Iron Coordination Structures of Oxygen Sensor FixL Characterized by Fe K-edge Extended X-ray Absorption Fine Structure and Resonance Raman Spectroscopy

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Hideyuki Miyatake, Masahiro Mukai, Shin-ichi Adachi, Hiro Nakamura, Koji Tamura, Tetsutaro Iizuka, and Yoshitsugu Shiro

From the Institute of Physical and Chemical Research, RIKEN Harima Institute, Mikazuki-cho, Sayo, Hyogo 679-5143, Japan

Richard W. Strange and S. Samar Hasnain

From the Daresbury Laboratory, Warrington, Cheshire WA4 4AD, United Kingdom

FixL is a heme-based O₂ sensor protein involved in a two-component system of a symbiotic bacterium. In the present study, the iron coordination structure in the heme domain of Rhizobium meliloti FixLT (RmFixLT, a soluble truncated FixL) was examined using Fe K-edge extended x-ray absorption fine structure (EXAFS) and resonance Raman spectroscopic techniques. In the EXAFS analyses, the interatomic distances and angles of the Fe-ligand bond and the iron displacement from the heme plane were obtained for RmFixLT in the Fe₂⁺, Fe²⁺O₂, Fe²⁺CO, Fe²⁺F⁻, and Fe²⁺CN⁻ states. An apparent correlation was found between the heme-nitrogen (proximal His-184) distance in the heme domain and the phosphorylation activity of the histidine kinase domain. Comparison of the Fe-CO coordination geometry between RmFixLT and RmFixLH (heme domain of RmFixL), based on the EXAFS and Raman results, has suggested that the kinase domain directly or indirectly influences steric interaction between the iron-bound ligand and the heme pocket. Referring to the crystal structure of the heme domain of Bradyrhizobium japonicum FixL (Gong, W., Hao, B., Mansy, S. S., Gonzalez, G., Gilles-Gonzalez, M. A., and Chan, M. K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15177–15182), we discussed details of the iron coordination structure of RmFixLT and RmFixLH in relation to an intramolecular signal transduction mechanism in its O₂ sensing.

The soil bacterium Rhizobium meliloti establishes a symbiotic association with alfalfa, which facilitates nitrogen fixation by nitrogenase enzymes in plant root nodules (1–3). A requirement for nitrogen fixation is a low O₂ tension in the nodules, since the nitrogenase is readily deactivated by O₂ (4). In response to O₂ concentration, the transcription of nitrogen fixation (nif) genes (6, 7, 8) is stimulated in prokaryotic cells and even in some eukaryotic cells (9–13).

The O₂ sensor protein FixL, the transmitter molecule of the two-component system (14, 15), consists of two different functional domains (16–18), sensor and histidine kinase domains. Since the O₂ sensor domain of FixL contains a heme as a prosthetic group, FixL is a member of heme-containing kinases (19). The iron of the heme domain in FixL is in an equilibrium state between the O₂-bound (oxy) and the O₂-unbound (deoxy) states, in response to the O₂ concentration (19). However, compared with other O₂-binding hemoproteins, FixL is quite unique, since O₂ ligation to the heme iron is directly coupled with the switching of the kinase activity in itself. The kinase is active when the heme domain is in the deoxy state, whereas it is deactivated when O₂ is bound to the heme iron (6). As a result, the heme-based O₂ sensor FixL can be classified into a new class of hemoproteins (19).

For the case of FixL, the signaling for the O₂ association to/dissociation from the heme iron is transferred to its histidine kinase domain (20). Possibly, structural changes in the vicinity of the heme, caused by transition between the deoxy and oxy states, constitutes an initial event in O₂ sensing, which is then followed by the intramolecular signal transduction from the heme to the histidine kinase site, thus regulating kinase activity. In order to understand better the O₂-sensing mechanism in FixL, the structural changes of the heme environment induced by O₂ dissociation have been examined using bio- and physico-chemical techniques. Recently, resonance Raman (21, 22), NMR (23), and ESR (24, 25) spectroscopic studies have been applied to the characterization of the heme environmental structure of FixL, and kinetic measurements (19, 20, 24) have been used to determine its ligand binding properties as well. These studies have shown that the basic structure of the iron coordination is similar to that of hemoproteins such as myoglobin (Mb), but the environment around the iron sixth coordination site is different.

However, these studies have yielded little direct structural information on the heme site of FixL resulting from the association or dissociation of O₂. The relevance of the heme site coordination structure to the kinase activation is still not clear. Gilles-Gonzalez and co-workers (6, 20) have measured the ki-

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† To whom correspondence should be addressed.

‡ The abbreviations used are: Mb, myoglobin; EXAFS, extended x-ray absorption fine structure; BjFixLH, heme domain of B. japonicum FixL; RmFixLT, soluble truncated form of R. meliloti FixL, which contains both heme and kinase domains; RmFixLH, heme domain of R. meliloti FixL.
nase activity of FixL in several iron oxidation, ligation, or spin states and found that the kinase domain is inactive in the low spin state of the heme iron, whereas it is active in the high spin state. Based on these observations, they proposed the “spin state” hypothesis, where activation/deactivation of the kinase site could occur through changes in the spin state of the heme iron. Most recently, they reported the crystal structure of the heme domain of *Bradyrhizobium japonicum* FixL (BjFixLH) in the ferric (Fe3+) and ferric-cyanide (Fe3+CN-) forms at 2.4 and 2.7 Å resolution, respectively. Based on structural comparison between these two states, it was suggested that the heme doming/flattening upon the ligand dissociation/association results in rearrangement of the hydrogen bond network between the heme 6,7-propionates and the “regulatory loop” and is possibly relevant to the activation/deactivation of the kinase domain (26).

In the present study, we report Fe K-edge EXAFS (extended x-ray absorption fine structure) of FixL in several iron states, and we characterize its iron coordination structures in active and inactive forms of the kinase. The EXAFS provides structural information that is limited to the metal-ligand coordination site (bond distance and angle) but is more precise than crystallographic data. To support the EXAFS information, we also measured the resonance Raman spectra of the CO adduct of FixL. Based on these spectroscopic results, we examined the change in the iron coordination structure of FixL as this relates to the activation/deactivation of the kinase domain in FixL.

**MATERIALS AND METHODS**

**Sample Preparation**—A soluble truncated FixL (RmFixLT, monomer molecular mass of 43 kDa) and the heme-containing domain of FixL (RmFixLH, molecular mass of 17 kDa) of *E. coli* were overexpressed in *Escherichia coli* strain JM109. RmFixLH (monomer molecular mass of 23 kDa) was co-expressed with RmFixLT in the same system. Expression plasmid for RmFixLT was generated by subcloning of the BamHI-HindIII fragment of the *fixL* genes (27) with the EcoRI-BamHI adapter into pUC18 at the EcoRI and HindIII sites. The N-terminal amino acid sequence of the soluble FixL is MTMITSNSGV(28). RmFixLH is the heme-binding domain as defined for the Rhodobacter sphaeroides membrane and the kinase domains. We constructed the expression system of RmFixLT in *E. coli* using the *fixL* DNA as follows. The *XhoI* and *XbaI* sites were filled in with Klenow fragment and ligated to introduce the stop codon (L260STOP) after removal of the kinase domain. The filled-in *Rsr*II-*KpnI* DNA fragment encoding the heme domain and FixJ was joined to the filled-in *XhoI-*KpnI fragment of pRSET vector to generate the truncated RmFixLT (RmFixLT). The RmFixJ complex was obtained by expression of the *fixL* gene in the *E. coli* strain JM109. RmFixJ (monomer molecular mass of 43 kDa) and the heme-containing domain of FixL were converted into RmFixLT using a His-tagged N-terminal sequence, MRGSH6GMART128.

**Iron Coordination Structure of Oxygen Sensor FixL**

**EXAFS Measurements and Analyses**—For the Fe K-edge EXAFS measurement, we prepared the ferrous deoxy, ferrous CO, ferrous O2 (oxy), ferric (met), ferric fluoride (metF−), and ferric cyanide (metCN−) forms of the (RmFixLT)/(RmFixLH) complex. The iron concentration of the samples was less than 1 mM for RmFixLT, because it precipitated when RmFixLT was concentrated over 1 mM. In addition, the ferrous CO complexes of RmFixLH (2 mM) and the O2, deoxy, and CO complexes of Mb (8 mM) were also prepared. The samples were frozen in liquid nitrogen in Perspex EXAFS cells with Mylar windows. The EXAFS measurements were carried out using synchrotron radiation at Station 8.1 of the synchrotron radiation source of Daresbury Laboratory (UK) and at BL12C of Photon Factory of KEK (Japan) (29, 30). All measurements were carried out at liquid nitrogen temperature in the fluorescence mode using multi-element germanium detector. Each EXAFS scan took approximately 1 h. Due to the low concentration of iron in the samples, it took 20–24 h to obtain good quality EXAFS data. The sample chamber was kept at 4 °C. The calibration of monochromator position to energy, the EXAFS was normalized to a unit iron atom and extracted from the background absorption using the Daresbury Laboratory program EXBACK (31). The EXAFS data were converted into a space using h = (2mIE - Er - ph )1/2, where E and Er are the energy of the incident x-ray radiation and the absorption edge of the iron atom, respectively, and h is the photoelectron wave vector. The counting of the data was performed using the non-linear least squares program EXCURV92 (32), which calculates the theoretical EXAFS function using fast curved wave theory and incorporates multiple scattering up to 3rd order (33, 34). Phase shifts were calculated using the Hedin-Lundqvist approximation (35). Curve fitting was carried out in a space on raw EXAFS data weighted by K2 to compensate for the diminishing amplitude of the EXAFS at high K.

Constrained and restrained refinement procedures were used to minimize the number of free parameters in the least squares analysis (36, 37). The quality of the simulations was assessed by the least squares fit index and R factor (37). The most important information obtained by the fitting procedures were the radial distances of the inner metal ligands in the iron coordination sphere (Fe-Npyr, Fe-NHα, Fe-ligand, and Fe-Ct), where the (Fe-Ct) represents the distance of the iron from the center of the pyrrole plane (iron displacement). The errors in the structural parameters deduced from EXAFS analysis arise from the data collection (e.g. photon counting statistics or sample inhomogeneity) and the subsequent data analysis (e.g. uncertainties in the EXAFS theory used or the quality of the background subtraction). For the FixL and Mb spectra, estimates of the errors are ±0.02 Å for metal-ligand distances of the inner metal ligands, and ±0.03 Å for the histidine ligand. The Debye-Waller factors for atoms belonging to the same ligand were found to increase systematically with their distance from the iron atom.
ments). The excitation sources were Kr⁺ laser (Coherent) at 406.7 and 413.1 nm. The Raman cell was spinning and kept below 10 °C by flushing with cold N₂ gas.

RESULTS AND DISCUSSION

Solution States of FixL and FixL-FixJ Complex

Prior to Fe K-edge EXAFS measurements, the solution states of RmFixLT were checked using the dynamic light scattering technique. Fig. 1 shows the results for RmFixLT in the met (Fe³⁺) state in the presence or absence of RmFixJ, which show the distribution of $R_H$ (hydrodynamic radius) of the protein in the solution. This measurement indicated that $R_H$ of RmFixLT (RmFixJ-free form; bold line in Fig. 1) is widely distributed and that the average molecular mass estimated from the $R_H$ value is about 300 kDa, suggesting that RmFixLT is highly and randomly aggregated in the solution, since the molecular mass of monomeric RmFixLT is 43 kDa. On the other hand, the RmFixLT-RmFixJ complex gives the result shown in Fig. 1 (thin line), in which the distribution of the $R_H$ is narrow and the average molecular mass is 130 kDa. The same results were obtained for the metF²⁻, deoxy, oxy (Fe²⁺O₂), and CO (Fe²⁺CO) forms of RmFixLT (data not shown). These results suggest that the RmFixLT-RmFixJ complex is mono-dispersive in the solution and is most probably in the heterotetrameric form, $(RmFixLT)₂(RmFixJ)₂$, since the monomer molecular mass of RmFixJ is 23 kDa.

The $(RmFixLT)₂(RmFixJ)₂$ in the met, metF²⁻, oxy, and CO states are stable at 10 °C, since their dynamic light scattering patterns were unchanged for several hours after the purification. The deoxy form was stable in the tetrameric form for 30 min after the fresh preparation under anaerobic condition but gradually aggregated afterward. Therefore, we prepared the EXAFS samples immediately after the preparations.

Fe K-edge EXAFS of RmFixLT

In view of the results of the light scattering measurements, EXAFS studies were carried out on the $(RmFixLT)₂(RmFixJ)₂$ complex to investigate the iron coordination structure of RmFixLT, since the $(RmFixLT)₂(RmFixJ)₂$ complex is apparently more intact than the aggregated RmFixLT. Fig. 2a shows the EXAFS data and their Fourier transforms for RmFixLTs in the oxy, CO, and deoxy forms. Also shown are the corresponding data for horse heart myoglobin. The clear similarity between these spectra for each pair of samples implies a general structural equivalence between their iron coordination sites. From this observation it was anticipated, for example, that the iron atom in deoxy RmFixLT is displaced from the porphyrin plane, as is the case for deoxy Mb (38). The detailed curve-fitting of the deoxy RmFixLT EXAFS confirmed this observation.

We have also examined the Fe K-edge EXAFS for the met, metF⁻, and metCN⁻ complexes (Fig. 2b). The strategy used in
the data analysis was to adopt the relevant model for the iron coordination site for the RmFixLT samples that is known, by crystallography (39), to hold true for Mb. The three-dimensional crystallographic information on Mb was simplified, and the application of restraints guaranteed the structural integrity of these ligands throughout the fitting procedure and also ensured that the refinements remained overdetermined. These features of the data analysis method are described in detail elsewhere (37) for the EXAFS of fetyal deoxy Mb. The results obtained by this approach are compiled in Table I, top and middle parts.

**Fifth Ligand His-194 Imidazole Coordination**—In our EXAFS analyses of RmFixLT, the best fit of the theoretical curves to the raw data was obtained in all cases when the imidazole ring was placed at the iron axial position in all states, indicating coordination of the histidyl imidazole to the heme iron of RmFixLT as the fifth axial ligand. The result agrees with previous conclusions based on optical absorption and the resonance Raman measurements (19, 22). Support for the 5-coordination of the met iron in RmFixLT was also provided in the iron near-edge spectra (41), in which the magnitude of the pre-edge for RmFixLT was comparably larger than that for six-coordinated metMb (data not shown).

| RmFixLT          | Deoxy                       | Oxy (O2)                  | CO  |
|------------------|-----------------------------|----------------------------|-----|
|                  | \( R \) 2\( r^2 \)        | \( R \) 2\( r^2 \)       |     |
| Fe-Ct            | \( \AA \) \( \AA^2 \)      | \( \AA \) \( \AA^2 \)    |     |
| Fe-Npyr          | 2.07 (0.005)                | 2.01 (0.002)              | 1.99 (0.003) |
| Fe-Nim           | 2.11 (0.002)                | 2.01 (0.006)              | 2.08 (0.002) |
| Fe-L1            | 1.91 (0.003)                | 1.81 (0.003)              | 1.81 (0.003) |
| Fe-L1-L2 (*)     | 142                         | 157                       |     |

| RmFixLT          | Met                         | Met F                     | Met CN |
|------------------|-----------------------------|----------------------------|--------|
|                  | \( R \) 2\( r^2 \)        | \( R \) 2\( r^2 \)       |     |
| Fe-Ct            | \( \AA \) \( \AA^2 \)      | \( \AA \) \( \AA^2 \)    |     |
| Fe-Npyr          | 2.04 (0.005)                | 2.01 (0.005)              | 1.98 (0.004) |
| Fe-Nim           | 2.11 (0.001)                | 2.11 (0.002)              | 2.06 (0.003) |
| Fe-L1            | 1.95 (0.005)                | 1.85 (0.005)              | 1.85 (0.005) |
| Fe-L1-L2 (*)     | 163                         |                           |       |

| RmFixLT          | MbCO                        | Deoxy-Mb                  |
|------------------|-----------------------------|----------------------------|--------|
|                  | \( R \) 2\( r^2 \)        | \( R \) 2\( r^2 \)       |     |
| Fe-Ct            | 0.00                        | 0.02                       | 0.48   |
| Fe-Npyr          | 2.00 (0.003)                | 2.00 (0.004)              | 2.05 (0.004) |
| Fe-Nim           | 2.00 (0.004)                | 2.07 (0.004)              | 2.11 (0.003) |
| Fe-L1            | 1.77 (0.003)                | 1.82 (0.003)              |       |
| Fe-L1-L2 (*)     | 171                         | 149                       |       |

**Sixth Ligand Coordination**—The EXAFS analysis clearly showed that the iron sixth coordination site in the met form of RmFixLT was vacant (5-coordination), which is in good agreement with previous conclusions based on optical absorption and the resonance Raman measurements (19, 22). Support for the 5-coordinate iron in RmFixLT was also provided in the iron near-edge spectra (41), in which the magnitude of the pre-edge for RmFixLT was comparably larger than that for six-coordinated metMb (data not shown).

**Fluoride Coordination to Ferric Iron**—We also compared the iron displacement relative to the heme plane, \( d(\text{Fe-Ct}) \), is also dependent on the iron spin states; in the oxy, CO, and metCN \(^-\) forms (low spin state), the iron is located in the heme plane (in-plane configuration), whereas it is displaced by 0.44 ± 0.06 and 0.55 ± 0.06 Å from the heme plane (out-of-plane configuration) in the met and deoxy forms (high spin state), respectively. The results are consistent with the general properties of hemoproteins in that the high spin iron is of considerably larger covalent radius than the low spin iron (40), thus making it difficult to force the high spin iron into the rigid porphyrin plane.

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respectively, on the basis of the Fe K-edge EXAFS data; these values are shorter than the corresponding ones obtained by crystallographic analysis mentioned above. Compared with the EXAFS data of sperm whale Mb, the (Fe-N_pyr), d(Fe-N_His), and d(Fe-F) of the metF\textsuperscript{−} complex of RmFixLT (Table I, middle part) are slightly short. Although the issue of whether the slight differences have arisen from structural differences in the iron coordination structure between RmFixLT and Mb, or from differences of the EXAFS analytical methods is unclear, the values obtained for RmFixLT appear to fall into the allowable range for the iron coordination in hemoproteins. In the iron coordination of the metF\textsuperscript{−} complex of RmFixLT (Table I, middle part), it is notable that the d(Fe-N_pyr) and d(Fe-N_His) are longer than the corresponding ones in the metCN\textsuperscript{−} complex and that the iron is displaced by 0.20 ± 0.06 Å from the heme plane.

Changes of Iron Coordination Structure of FixL upon Ligand Binding

The present EXAFS study clearly indicates that the iron coordination structure of the O\textsubscript{2}-bound heme in RmFixLT, in which the kinase domain is inactive, is significantly different from that of the deoxy form, which contains the active kinase domain. This structural difference is comparable to the pattern observed for oxy and deoxy Mb (38) and for oxy (R state) and deoxy (T state) hemoglobin (Hb). In the case of Hb, the iron moves up to 0.3 Å in the R-T conversion (46, 47). Thus, comparing the EXAFS data (Table I, top part), we found two changes of the iron coordination structures between the oxy and the deoxy states. One is an iron displacement (Fe-Ct) from the heme plane by 0.55 Å, which accompanies an increase in the Fe-N_pyr length from 2.01 to 2.07 Å. The similarity between deoxy Mb and deoxy RmFixLT EXAFS spectra already suggested this similarity in structure. Another similarity is an increase in the Fe-N_His bond length from 2.01 to 2.11 Å. As a consequence, the distance between the heme plane and the nitrogen atom of the proximal His-194 imidazole, d(Fe-Ct) + d(Fe-N_His), is increased by 0.63 Å upon the dissociation of O\textsubscript{2} from the ferrous heme iron.

It has been reported that the kinase of FixL is inactive in the metCN\textsuperscript{−} state of its heme domain, although active in the met and metF\textsuperscript{−} states (20). It was also shown, without any raw data, that CO inhibits the kinase activity of ferrous FixL. Therefore, we also examined the d(Fe-Ct) of these complexes of RmFixLT. In the met form, the d(Fe-Ct) is 2.55 Å, comparable to that of the kinase-active deoxy form (2.66 Å). The d(Fe-Ct) of the FixL in the metF\textsuperscript{−} state is 2.31 Å. On the other hand, the d(Fe-Ct) of the metCN\textsuperscript{−} and CO complexes are 2.06 and 2.08 Å, respectively, which are similar to that of the kinase-inactive oxy (2.03 Å) forms. In Fig. 3, the kinase activities of the RmFixLT complexes are plotted against the d(Fe-Ct) estimated in the present study. Inspection of this figure clearly shows that a threshold is present in the region of 2.1–2.3 Å of the d(Fe-Ct); the RmFixLT complexes having a d(Fe-Ct) longer than the threshold contains active kinase, whereas the kinase domain of the RmFixLT complex with the shorter d(Fe-Ct) is inactive. The result indicates that the iron displacement relative to the heme plane is apparently correlated to the kinase activation/deactivation.

In hemoproteins, it is a general feature that the iron moves out of the heme plane in conversion from the low to the high spin state, due to the increment of the iron covalent radius. Perutz (46, 47) focused on this iron movement induced by the O\textsubscript{2} dissociation from hemoproteins and proposed a well known mechanism in the R-T transition of Hb, i.e. the “trigger” model, where the movement of the proximal histidine (fifth ligand) relative to the heme plane upon the dissociation of O\textsubscript{2} from one Hb subunit acts as a trigger to induce the conformational change of the Hb tertiary and quaternary structure, eventually controlling the O\textsubscript{2} affinity of the other subunits. The present EXAFS results on deoxy and oxy RmFixLT suggest that the proximal trigger model might be applicable to FixL; the movement of the imidazole group of His-194 relative to the heme plane upon the association/dissociation of O\textsubscript{2} is related to the deactivation/activation of its histidine kinase domain.

However, recent crystallographic study of BjFixLH (26) suggested that the Hb-type mechanism is not operative in the O\textsubscript{2} sensor FixL, because of no structural change in the heme proximal side upon the CN\textsuperscript{−} association to the ferric iron. Instead, another mechanism was proposed, in which rearrangement of the hydrogen bond pattern of the heme 7-propionate with its surroundings, caused by the heme flattening upon the ligand (CN\textsuperscript{−}) association, is possibly important in the signal transduction to the kinase domain. The present EXAFS data of RmFixLT appears to be consistent with the proposal based on the crystal structures of BjFixLH, since the heme flattening/doming is closely associated with the iron movement relative to the heme plane. These results support the idea that ligand binding could act as a trigger in the intramolecular signal transition in FixL through the heme flattening/doming.

**FIG.3.** Plot of heme-N_His distances against kinase activity of (RmFixLT)\textsuperscript{−} (RmFixJ)\textsuperscript{−}. The kinase activities were calculated from the results reported by Gilles-Gonzalez et al. (19). The activity value of the CO complex has not been reported.
In the heme-based O₂ sensor FixL, the O₂ binding to the heme sixth site is a signal that induces kinase deactivation (6). Therefore, the ligand (O₂, CO, NO, CN⁻, and F⁻) binding properties of FixL and the property of the autoxidation of its oxy complex have been extensively studied via kinetic and spectroscopic techniques and have been discussed in relation to the structure around the iron sixth coordination side (the distal heme pocket) (6, 7, 19–24). Through these extensive studies, it has also been suggested that steric and/or electrostatic properties of the distal heme pocket are different for RmFixLT vis-à-vis RmFixLH (heme domain of FixL), and consequently interaction of the iron-bound ligand with the heme pocket is also different between these forms. In the present study, we attempted to characterize the structure in the distal heme pocket and to reveal the effect of the kinase domain, by virtue of the Fe K-edge EXAFS and the resonance Raman spectroscopy of the CO complexes of RmFixLT, RmFixLH, and Mb. The simulation of the EXAFS of RmFixLT is shown in Fig. 4 together with the data for the CO complexes of RmFixL and Mb, and their resonance Raman spectra are also illustrated in Fig. 5. The main reason for the choice of the CO complex is that the coordination of CO to the ferrous heme iron has been the most comprehensively investigated using many biochemical and physicochemical techniques and has been discussed in detail with relevance to the heme pocket structure and its chemical environments (48, 49 and references therein). The CO coordination is a sensitive marker to evaluate characters around the Fe-CO moiety in hemoproteins.

**Structural Characterization of Heme Pocket in Sixth Ligand (Distal) Side**

In the heme-based O₂ sensor FixL, the O₂ binding to the heme sixth site is a signal that induces kinase deactivation (6). Therefore, the ligand (O₂, CO, NO, CN⁻, and F⁻) binding properties of FixL and the property of the autoxidation of its oxy complex have been extensively studied via kinetic and spectroscopic techniques and have been discussed in relation to the structure around the iron sixth coordination side (the distal heme pocket) (6, 7, 19–24). Through these extensive studies, it has also been suggested that steric and/or electrostatic properties of the distal heme pocket are different for RmFixLT vis-à-vis RmFixLH (heme domain of FixL), and consequently interaction of the iron-bound ligand with the heme pocket is also different between these forms. In the present study, we attempted to characterize the structure in the distal heme pocket and to reveal the effect of the kinase domain, by virtue of the Fe K-edge EXAFS and the resonance Raman spectroscopy of the CO complexes of RmFixLT, RmFixLH, and Mb. The simulation of the EXAFS of RmFixLT is shown in Fig. 4 together with the data for the CO complexes of RmFixL and Mb, and their resonance Raman spectra are also illustrated in Fig. 5. The main reason for the choice of the CO complex is that the coordination of CO to the ferrous heme iron has been the most comprehensively investigated using many biochemical and physicochemical techniques and has been discussed in detail with relevance to the heme pocket structure and its chemical environments (48, 49 and references therein). The CO coordination is a sensitive marker to evaluate characters around the Fe-CO moiety in hemoproteins.

**Structural Characterization of FixL in Heme Distal Side by Comparison with Myoglobin—** To characterize the heme pocket structure, we compared the CO binding geometry between RmFixLT and Mb (see Table I, top and bottom parts), and in particular, we focused on the bond angle of the CO coordination, 208 Fe—C–O, which is different between RmFixLT (157°) and Mb (149°). A relative difference of 8° is significant in terms of the maximum fitting errors, but a note of caution is required for the absolute errors. The Fe-CO coordination in RmFixLT is less bent than that of Mb, although other parameters, including the Fe-CO distance (1.81 Å) and the Fe-Nim distance (2.08–2.09 Å), are basically identical.

Because the linear coordination is favorable for the CO coordination in the model system, the bent CO binding in the proteins can be explained in terms of effects of protein parts in the distal heme pocket. To examine the protein effect on the Fe-CO bond, we also measured the resonance Raman spectrum of the CO-bound form of RmFixLT (Fig. 5), in which the Fe-CO stretching ($\nu_{\text{Fe-CO}}$) and the C-O stretching ($\nu_{\text{CO}}$) frequency bands were observed at 498 and 1962 cm⁻¹, respectively. These bands were shifted to 496 and 1921 cm⁻¹ upon the $^{13}$CO replacement and to 490 and 1873 cm⁻¹ upon the $^{13}$C$^{18}$O replacement, respectively. Compared with the spectrum of MbCO ($\nu_{\text{Fe-CO}} = 507$ cm⁻¹, $\nu_{\text{CO}}$ (main band) = 1945 cm⁻¹), the $\nu_{\text{Fe-CO}}$ locates at lower positions, whereas the $\nu_{\text{CO}}$ locates at higher positions, indicating that the Fe-CO bond character in RmFixLT is different from that in Mb. The back donation from the iron $d^{π}$ orbital to the ligand CO $p^{π}$ orbital is less in RmFixLT than in Mb, decreasing the Fe-CO bond order, while increasing the C-O bond order.

According to the comprehensive studies of vibrational spectroscopies of the CO adduct of hemoproteins (49–52), two factors that significantly affect CO coordination have been considered as follows: the steric hindrance to the iron-bound CO by the distal residues and electrostatic interactions of the bound CO with a hydrophobic heme pocket. The less bent CO character in RmFixLT is different from that in Mb. The back donation from the iron $d^{π}$ orbital to the ligand CO $p^{π}$ orbital is less in RmFixLT than in Mb, decreasing the Fe-CO bond order, while increasing the C-O bond order.

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2 The fitting errors on these angles is $±2°$, but absolute error is likely to be $±5°$ in view of approximation in theory, limited data range, and quality.
$d\pi$ orbital and the CO $p\pi^*$ orbital, resulting in increase in the $\pi$ back donation. Therefore, the Fe-CO bond order must be increased, but this is not the case for RmFixLT and Mb.

On the other hand, since the polar and cationic His-64 (distal His) is present in the distal heme pocket of Mb, the $\pi$ back donation is facilitated to increase in the Fe-CO bond order. Therefore, we can suggest that the Fe-CO environment of Rm-FixLT should be apolar, retarding the $\pi$ back donation and decreasing the Fe-CO bond order (53). In the case of RmFixLT, the hydrophobic effect is predominant to determine its Fe-CO bond character. This suggestion is in good agreement with that reported on the basis of the NMR, ESR, resonance Raman, and kinetic studies (21–25). In addition the recent crystallographic study showed that its distal heme pocket is constructed by some hydrophobic residues such as Ile-215, Leu-236, and Ile-238 of BjFixLH.

Considering the van der Waals radii of side chains of the distal residues, the heme sixth sites of FixL are packed. Despite such crowded space in the distal heme pocket, some spectroscopic results including EXAFS have suggested the less bent CO coordination in FixL than in Mb. These observations could suggest that steric environment in the distal heme pocket of FixL allows the less bent CO coordination, but highly bent coordination might be unfavorable. Indeed, the CN$^-$ ligand is linearly bound to the ferric iron, as was found in the crystal structure of BjFixLH (26), whereas the triatomic pseudohalides, SCN$^-$ and N$_3^-$, which generally bind the heme ferric iron in the highly bent fashion (54), do not bind to FixL (21, 23). On the other hand, the bulky imidazole can bind to RmFixLT (55). Therefore, the relationship between the ligand binding and the steric effect in RmFixLT still seems controversial. Further studies to elucidate the ligand selection mechanism of FixL should be carried out.

Interaction of Heme Distal Side with Kinase Domain—As shown in Table I, top and bottom parts, the linear CO coordination is more favorable in RmFixLH (171°) than in RmFixLT (157°). The change of the CO coordination from bent to linear increases in the overlap of the porphyrin and CO $\pi^*$ orbitals, eventually increasing the $\pi$ back donation from the iron/porphyrin moiety to the CO ligand. Indeed, in the resonance Raman spectra of the CO complex of RmFixLH (Fig. 5), the $\nu_{Fe-CO}$ and $\nu_{C-O}$ frequency bands are located at 503 and 1955 cm$^{-1}$ respectively, which were shifted to 499 and 1912 cm$^{-1}$ upon the $^{13}$CO substitution and to 492 and 1868 cm$^{-1}$ upon the $^{13}$C$^{15}$O substitution, respectively. The Raman spectral results show that the Fe-CO bond order in RmFixLH is increased, whereas the C-O bond order is decreased, compared with those of RmFixLT ($\nu_{Fe-CO} = 498$ cm$^{-1}$, $\nu_{C-O} = 1955$ cm$^{-1}$). The results are quite consistent with the previous Raman result (21), in which the porphyrin $\pi$-electron density marker, $\nu_{\pi}$, is shifted to higher frequency (up-shifted) by 2.7 cm$^{-1}$ from the ferrous-CO complex of RmFixLT to that of RmFixLH. The
up-shifted \( \nu_3 \) line in the spectrum of RmFixLH suggests diminished \( \pi^* \) electron density resulting from the increased \( \pi \) back donation from the iron to the ligand CO, in good consistency with the Fe-CO bond of RmFixLH stronger than that of RmFixLT.

According to the suggestion discussed above, the Fe-CO bond is affected by steric hindrance and/or hydrophobicity in the distal heme pocket. Since it is not expected that the hydrophobicity around the iron-bound ligand (CO) is drastically altered from RmFixLT to RmFixLH, the change in the CO coordination geometry is possibly caused by change in steric factors. In RmFixLH, the steric constraint to the iron-bound CO is less than in RmFixLT. The steric interaction between the iron-bound ligand (CO) and its surrounding is present, and its magnitude is different between RmFixLT and RmFixLH.

Recently, we have reported that RmFixLH which we purified is stable in a dimeric form like RmFixLT (28). Therefore, the structural difference in the heme distal pocket between RmFixLH and RmFixLT is caused by removal of the kinase domain, rather than by the monomer/dimer formation. It is also true that the heme distal pocket indirectly communicates to the kinase domain. These indications allow us to suggest that the heme distal pocket indirectly communicates to the distal heme pocket. Since it is not expected that the hydrophobicity and/or hydrophilicity in the distal heme pocket is stable in a dimeric form like RmFixLT (28). Therefore, the magnitude is different between RmFixLT and RmFixLH.

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