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Dynamic light-scattering and preliminary crystallographic studies of the sensor domain of the haem-based oxygen sensor FixL from *Rhizobium meliloti*

Hideyuki Miyatake,[a] Manabu Kanai,[b] Shin-ichi Adachi,[a] Hiro Nakamura,[a] Koji Tamura,[a] Hajime Tanida,[a] Terumasa Tsuchiya,[c] Tetsutaro Iizuka[a] and Yoshitsugu Shiro[a]*

[a]The Institute of Physical and Chemical Research (RIKEN), RIKEN Harima Institute, 323-3 Mikazuki-cho, Mihara, Sayo, Hyogo 679-5143, Japan, [b]Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka-shi, Kanagawa 259-1293, Japan, and [c]Department of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

Correspondence e-mail: yshiro@postman.riken.go.jp

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FixL is a transmitter protein in a two-component system which acts as an oxygen sensor when the symbiotic *Rhizobia* resides in root nodules of host plants. The oxygen-sensor domain of *Rhizobium meliloti* FixL (RmFixLH) was purified by His-tag affinity and isoelectronic focusing chromatographies, without the use of gel-filtration chromatography. Dynamic light-scattering measurements of RmFixLH thus obtained revealed it to be monodispersive and present as a homodimer in solution. A single crystal of RmFixLH in the met (Fe^{3+}) form was grown in 100 m*M* acetic acid/NaOH buffer at pH 4.6 in the presence of 200 m*M* ammonium acetate, using 40%(*w*/*v*) PEG 4000 as a precipitant. A crystal of the ferrous CO form of RmFixLH was also prepared by reduction of the met crystal with $Na_2S_2O_4$ in an atmosphere of CO. The crystals (0.2 × 0.05 × 0.01 mm) belong to the monoclinic system (*C*2) with unit-cell parameters $a = 60.94$, $b = 37.44$, $c = 54.14$ Å, $\beta = 115.29^\circ$ and diffract X-rays to 1.7 Å resolution at station BL44B2 of SPring-8, Japan. Bijvoet difference Patterson maps show a clear peak corresponding to the haem iron in RmFixLH.

1. Introduction

FixL is a haem-based oxygen sensor belonging to the two-component system of symbiotic *Rhizobia* in plant root nodules. The protein serves to regulate the gene expression of enzymes involved in nitrogen fixation (Stock *et al.*, 1989; Fisher & Long, 1992; Hill, 1988). Rhizobial FixL consists of three distinct functional domains: the membrane-binding, the haem-binding sensor and the histidine-kinase domains (Monson *et al.*, 1992). The haem iron in FixL is in an equilibrium state between the O_2-bound (oxy) and the O_2-unbound (deoxy) states, depending on the O_2 concentration (Gilles-Gonzalez *et al.*, 1995), and the ligation state of the haem domain (the sensor domain) is directly related to the regulation of the histidine kinase activity: in the oxy state the kinase is inactive, while the kinase is activated upon dissociation of O_2 from the haem iron (Lois *et al.*, 1993). Spectroscopic techniques, including Raman, NMR, ESR and EXAFS (Rodgers *et al.*, 1996; Lukat-Rodgers & Rodgers, 1997; Winkler *et al.*, 1996; Tamura *et al.*, 1996; Miyatake *et al.*, 1999), and mutagenesis techniques (Nakamura *et al.*, 1998) have been applied in order to characterize the oxygen-sensor domain of FixL. However, little is known concerning the mechanism of the intra-domain signal transduction.

Recently, Gong *et al.* (1998) have reported the crystal structure of the oxygen-sensor domain of *Bradyrhizobium japonicum* FixL (BjFixLH) and have proposed an oxygen-sensing mechanism for FixL on the basis of the crystal structure. Their report provides a good understanding of the overall topology of the haem domain of FixL and the structural characteristics of the haem pocket in BjFixLH. However, it is not very informative with regard to understanding the molecular mechanism of the intra-domain signal transduction, as the protein is monomeric in the crystalline state. Recently, it has been suggested that FixL shares folding topology with the PAS (Per–Arnt–Sim) domain superfamily (Zhulin *et al.*, 1997), which mediate homodimer/heterodimer formation with their basic helix–loop–helix (bHLH) N-terminal regions and transmit signals by protein–protein aggregation (Pellequer *et al.*, 1998). Thus, in order to discuss the signal-transduction mechanism in more detail, it is necessary to obtain structures of the dimeric form of FixL and its sensor domain. In the present study, we report a novel purification method for the haem domain of *Rhizobium meliloti* FixL (RmFixLH) and obtain its dimeric form in solution. The solution was then monitored by dynamic light-scattering measurements. In addition, we also report

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preliminary diffraction results from a single crystal of RmFixLH.

2. Materials and methods

2.1. Purification and crystallization

A plasmid expression vector with an N-terminal polyhistidine tag, pET-14b (Novagen), was used to express RmFixLH in *Escherichia coli* strain JM109(DE3). We constructed the expression system of RmFixLH with a His_6-tag at the N-terminal (M_w = 17 kDa) in *E. coli* using the truncated version of *R. meliloti* FixL (RmFixLT) cDNA as follows. The *Xho*I and *Xba*I sites were filled in with a Klenow fragment and ligated to introduce the stop codon (L260-D-stop). The filled-in *Rsr*II–*Bam*HI DNA fragment encoding the haem domain was joined to the filled-in *Xho*I–*Bam*HI fragment of the pET-14b vector. The His-tagged RmFixLH was subjected to nickel–nitrilotriacetic acid (Ni–NTA) metal-affinity chromatography (Porath *et al.*, 1975). To remove the His-tags, the eluted protein was treated with thrombin (1 unit per milligram of protein) at 298 K for 5 h. After removal of the His-tags, serious isoelectric and dispersive heterogeneity of RmFixLH was observed. Owing to this heterogeneity, it was difficult to obtain a single crystal. Therefore, anion-exchange chromatography using MONO-Q (Amersham Pharmacia Biotech) was carried out to confirm the isoelectrical and dispersive purity, or an isoelectronic focusing technique was employed using IsoPrime (Amersham Pharmacia Biotech) to purify the sample on a larger scale. The major fraction of the sample was focused in the chamber between pH 4.19 and pH 4.24 membranes. The chemical purity of the sample was monitored during the purification by SDS–PAGE and isoelectronic focusing using PhastSystem (Amersham Pharmacia Biotech). The sample was concentrated to 1 m*M* in a 20%(*v*/*v*) glycerol solution and stored at 193 K. The preparation is stable for several months under these conditions.

A single crystal of RmFixLH in the met (Fe^{3+}) state was obtained by the vapour-diffusion method using the hanging-drop technique. Crystals were grown at 293 K in 100 m*M* sodium acetate pH 4.6, 200 m*M* ammonium acetate using 40%(*w*/*v*) PEG 4000 as a precipitant. The initial droplets contained 3 µl protein solution (1 m*M*) and 3 µl precipitant solution, and were equilibrated against a 500 µl precipitant solution reservoir. We also prepared crystals of RmFixLH in the ferrous CO form using procedures which were similar to those reported for haemoglobins (Park *et al.*, 1996); the met crystals were soaked in a 50 m*M* sodium dithionite ($Na_2S_2O_4$) solution under anaerobic conditions and exposed to CO gas.

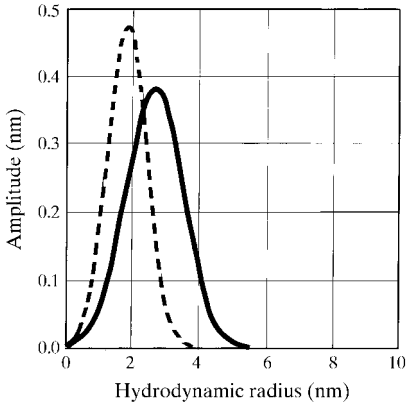

Figure 1
Distribution of the hydrodynamic radius of RmFixLH (solid line) and myoglobin (dashed line) at 293 K in 5 m*M* potassium phosphate pH 7.0 containing 20%(*v*/*v*) glycerol. Values of the diffusion coefficient D_T (10^{-13} m^2 s^{-1}) and the hydrodynamic radius R_H (nm) of RmFixLH obtained were 505 and 2.8, respectively, whereas those of Mb were 607 and 2.0, respectively.

2.2. Dynamic light-scattering measurements

The dynamic light scattering (DLS) of RmFixLH in the met form was measured at 293 K using DynaPro-MS (Protein Solutions Inc., http://www.protein-solutions.com/), in which the sample was filtered with a disposable 0.02 µm pore size Anotop-10 inorganic membrane filter (Whatman) just prior to the DLS measurement. The protein concentration for this measurement was 0.5 m*M*, which is the same as that for the crystallization experiment. For comparison, the DLS of 0.5 m*M* myoglobin (Sigma) in 20%(*v*/*v*) glycerol solution was also measured. In the DLS measurement, a beam of monochromatic light was directed through the sample to monitor fluctuations in the light intensity scattered by the protein molecules. From analysis of the data, the translational diffusion coefficient, D_T, of the protein particles in solution was obtained. Assuming Brownian motion, this coefficient was converted to the hydrodynamic radius, R_H, of the protein particles using the Stokes–Einstein equation ($R_H = k_bT/6\pi\eta D_T$), where k_b represents Boltzman's constant, T is the absolute temperature in Kelvin and η is the solvent viscosity. The hydrodynamic molecular weight of the protein particles can be estimated from the measured value of D_T using a calibration curve obtained from proteins of known mass. All DLS analysis was carried out using the *DYNAMICS* version 3.0 program (DynaPro control software, Protein Solutions Inc.). The program provided us with values of the refractive index (1.33) and viscosity (1.734) of the 20%(*v*/*v*) glycerol solution. All other DLS parameters were used without any modification.

2.3. Diffraction data collection from native and CO forms of RmFixLH

Diffraction data of the RmFixLH crystals in the met and the ferrous CO forms were obtained using synchrotron radiation of wavelength 1.0 Å at the BL-44B2 station at SPring-8, Japan (Adachi *et al.*, 1996). Intensity data were collected with an R-AXIS IV image-plate detector, which was mounted on a Huber alignment table. The cryogenic head of the Oxford Cryosystems Cryostream was mounted close to the goniometer head. Data were collected at 100 K. The collected intensities were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski, 1988); all other calculations were carried out using the program *PHASES*95 (Furey & Swaminathan, 1998).

2.4. Multiwavelength anomalous diffraction (MAD) data collection

Since RmFixLH contains one haem iron per protein molecule, we collected additional diffraction data using the multiwavelength anomalous diffraction (MAD) method in order to obtain initial phase information for structural determination. The crystal was mounted so that its *b* axis was approximately coincident with the rotational φ axis, and data were collected at 100 K. Prior to the collection of diffraction data, the fluorescence spectrum of the RmFixLH crystal was measured in order to determine the absorption edge of the haem iron using the single-element SSD detector (ORTEC SLP-φ6165-S). Three data sets for MAD calculation were collected, with wavelengths of 1.7396 (peak), 1.7417 (edge) and 1.6500 Å (remote), using one crystal at BL44B2 of SPring-8. The distance between the crystal and detector was set to be 140 mm. The data were processed as described above.

3. Results and discussion

3.1. Solution state of RmFixLH characterized by dynamic light scattering (DLS)

Fig. 1 shows the distribution pattern of the hydrodynamic radii of RmFixLH and

Table 1
Crystallographic data statistics of RmFixLN.

Values in parentheses are for the highest resolution shells.

	Native	CO–RmFixLN	MAD data		
			Remote	Peak	Edge
Space group	Monoclinic *C*2	Monoclinic *C*2	Monoclinic *C*2		
Unit-cell parameters (Å, °)	a = 59.26, b = 36.99, c = 52.72, β = 115.44	a = 60.50, b = 37.24, c = 53.49, β = 115.66	a = 60.94, b = 37.44, c = 54.14, β = 115.29		
Wavelength (Å)	1.000	1.000	1.6500	1.7396	1.7417
Crystal-to-detector distance (mm)	230	230	140	140	140
Resolution (Å)	100–1.7	100–2.0	100–2.8	100–2.8	100–2.8
Oscillation angle (°)	3.0	5.0	10.0	10.0	10.0
σ cut-off	0.0	0.0	0.0	0.0	0.0
$R_{\rm merge}$†	0.042 (0.115)	0.058 (0.211)	0.066 (0.132)	0.075 (0.15)	0.080 (0.163)
$R_{\rm anom}$	—	—	—	0.038	0.033
Number of measurements	26389	21913	9333	9451	9273
Number of independent reflections	10498	6829	2746	2770	2751
Completeness (%)	90.3 (81.8)	92.4 (84.4)	97.8 (96.0)	98.6 (96.8)	98.2 (96.8)
Multiplicity	2.52	3.20	3.40	3.41	3.37
Mean $\langle I/\sigma(I)\rangle$	20.6	12.7	12.6	10.7	11.0

† $R_{\rm merge} = \sum\sum_i |I(h) - I(h)i| / \sum\sum_i I(h)$, where $I(h)$ is the mean intensity after rejections.

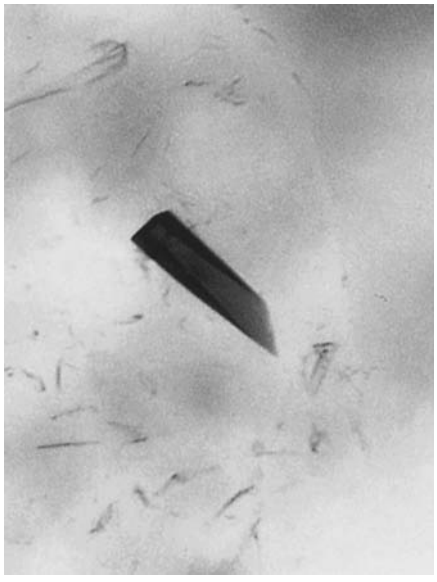

Figure 2
A single crystal of RmFixLH grown from 40%(*w*/*v*) PEG 4000, 100 m*M* acetic acid/NaOH buffer at pH 4.6 containing 200 m*M* ammonium acetate. Approximate dimensions of the crystal are 0.2 × 0.05 × 0.01 mm.

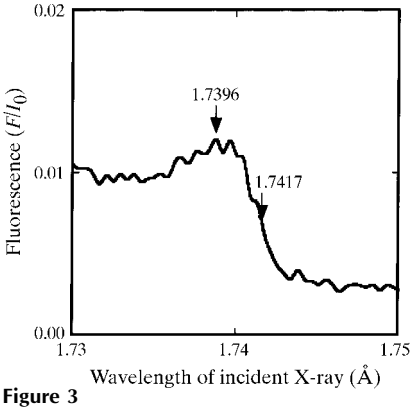

Figure 3
EXAFS spectrum of Fe absorption in RmFixLH. The peak (1.7396 Å) and edge (1.7417 Å) are indicated by arrows.

myoglobin (Mb), as obtained from the DLS measurements at 293 K. Polydispersities for RmFixLH (0.6 nm) and Mb (0.7 nm) are below 1.0 nm, suggesting that both proteins are in the monodispersive state in 20%(*v*/*v*) glycerol solution (Amare & Burley, 1997). From the hydrodynamic radii, the molecular weight of RmFixLH was estimated to be 35 kDa, while that of Mb was estimated to be 16 kDa. The estimated molecular weight of Mb was nearly the same as that of the monomeric Mb (17 kDa), showing that the DLS measurement was sufficiently reliable to compare the dispersive states of RmFixLH and Mb. The DLS results suggest that the 0.5 m*M* solution of RmFixLH was appropriate for the initial crystallization condition, since systematic observations show that macromolecules existing in the unimodal state are likely to be crystallized (D'Arcy, 1994).

Furthermore, the estimated M_r of RmFixLH (35 kDa) showed that the molecule was dimeric in solution, since the molecular weight of monomeric RmFixLH calculated from its DNA sequence is 17 kDa. This result may be biologically meaningful as the PAS domain superfamily, to which RmFixLH belongs, are thought to mediate signal transduction through dimerization processes (Huang *et al.*, 1993; Zhulin *et al.*, 1997). In addition, it is widely known that the transmitter proteins in the large two-component system family act as homodimers, as first characterized in CheA (Surette *et al.*, 1996), EnvZ (Yang & Inoue, 1985) and NRII (Ninfa *et al.*, 1993). The result, however, is inconsistent with that reported by Gilles-Gonzalez *et al.*, who suggested that RmFixLH was monomeric with a molecular mass of 21 kDa based upon estimation by gel filtration (Gilles-Gonzalez *et al.*, 1994, 1995; Gonzalez *et al.*, 1998). They also maintain that BjFixLH is a monomer in solution state. In their preparation, a dimer of RmFixLH may become dissociated or denatured during the gel filtration. Indeed, we found a denaturation of the RmFixLH sample when we applied it to a HiLoad 26/60 Superdex75 gel-filtration column (Amersham Pharmacia Biotech). In any case, the dimerization mechanism of RmFixLH related to PAS signal transduction could be discussed after the crystallographic analysis of RmFixLH by a comparison between the crystal structures of RmFixLH and BjFixLH.

3.2. Characterization of crystals and analyses of data quality

RmFixLH crystals in the met form were obtained within a few days at 293 K. Typically, the size of the crystals obtained was 0.2 × 0.05 × 0.03 mm (Fig. 2). The crystals belong to the monoclinic space group *C*2, with unit-cell parameters a = 60.94, b = 37.44, c = 54.14 Å, β = 115.29°. Assuming that each asymmetric unit of the crystal contains one molecule of the protein, the partial specific volume (V_m) is estimated to be 1.54 Å^3 Da^{-1}. This value corresponds to a solvent content of approximately 22%. The solvent content in the crystal is lower than those of other protein crystals studied (Matthews, 1968). The reflection data for the native crystal of RmFixLH in the met form at a wavelength 1.0 Å have an $R_{\rm merge}$ of 4.2% for 10498 independent reflections derived from 26285 total observations. The crystal-to-film distance was set to 230 mm. The completeness of the data set is 90.3% in the 100–1.7 Å range.

The crystallographic parameters of the ferrous CO form of RmFixLH were basically

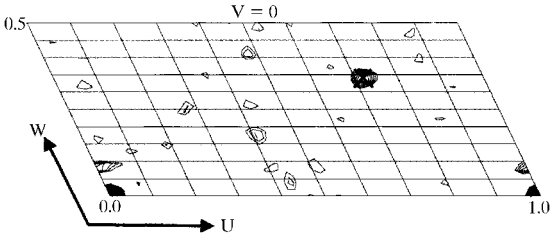

Figure 4
Bijvoet difference Patterson map using the data collected at 1.7396 Å (peak data). Diffraction data between 6 and 3 Å resolution ($<3\sigma$) were used for calculation. The cross symbol corresponds to haem iron–iron self-vectors. The position of the haem iron is refined as (0.362, 0.000, 0.168) by maximum-likelihood phase refinement.

the same as those of the met form. As no serious damage was observed on binding CO to the crystal, the crystal was suitable for ligand formation. The crystal-to-film distance was set to 230 mm. We were able to collect data from the CO–RmFixLH crystal to 2.0 Å resolution, with an $R_{\rm merge}$ of 5.8% for 6829 independent reflections derived from a total of 21913 reflections. The completeness of the data set is 92.4% in the 100–2.0 Å range. Crystallographic statistics are summarized in Table 1.

3.3. Position of haem Fe atom from anomalous dispersion

The fluorescence spectrum of the RmFixLH crystal (Fig. 3) was measured in order to select three wavelengths for the MAD measurements which maximize anomalous dispersion effects. The MAD data were collected under cryogenic conditions using only one crystal. No serious radiation damage was observed during the data collection. The results of data reduction are summarized in Table 1.

A Harker section at $V = 0$ of the Bijvoet anomalous difference Patterson map between the remote (1.6500 Å) and peak (1.7396 Å) wavelengths clearly shows the position of the Fe atom in RmFixLH (Fig. 4). The position of the haem iron was refined as (0.362, 0.00, 0.168) by maximum-likelihood phase refinement. The initial electron-density map was calculated using MAD data and model building is now under way.

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