Assignment of the hyperfine-shifted ^{1}H-NMR signals of the heme in the oxygen sensor FixL from *Rhizobium meliloti*

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Background: The Rhizobial oxygen sensor FixL is a hemoprotein with kinase activity. On binding of strong-field ligands, a change of the ferrous or ferric heme iron from high to low spin reversibly inactivates the kinase. This spin-state change and other information on the heme pocket have been inferred from enzymatic assays, absorption spectra and mutagenesis studies. We set out to investigate the spin-state of the FixL heme and to identify the hyperfine-shifted heme-proton signals by NMR spectroscopy.

Results: Using one-dimensional NMR we directly observed the high- and low-spin nature of the met- and cyanomet-FixL heme domain, respectively. We determined the hyperfine-shifted ^{1}H-NMR signals of the heme and the proximal histidine by one- and two-dimensional spectroscopy and note the absence of distal histidine signals.

Conclusions: These findings support the spin-state mechanism of FixL regulation. They establish that the site of heme coordination is a histidine residue and strongly suggest that a distal histidine is absent. With a majority of the heme resonances identified, one- and two-dimensional NMR techniques can be extended to provide structural and mechanistic information about the residues that line the heme pocket.

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Introduction

The oxygen sensor FixL is a modular protein consisting of a kinase domain and a heme domain on each polypeptide within a homodimer [1–3]. One of the physiological roles of FixL in Rhizobial nitrogen fixation is to restrict production of nitrogenase to anoxic conditions, thus preventing irreversible inactivation of the nitrogenase by oxygen. Deoxy-FixL phosphorylates the transcription factor FixJ, triggering gene expression from the promoters of nitrogen fixation genes (*nif*, *fix*) [4]. Oxy-FixL is inactive [2,5]. The FixL–FixJ–*nif* system thus provides for very simple and sensitive oxygen-controlled gene expression. Several other critical biological processes, such as red blood cell production in mammals, are known to be mediated by a hemoprotein acting as an oxygen sensor [6]. So far, FixL is the only one of these heme-based sensors to be identified and studied in purified form.

Various factors indicate that the heme environment in FixL is significantly different from that of oxygen carriers. The amino-acid sequence of FixL has no homology to that of known hemoproteins [7], and absorption spectra indicate that the ferric FixL does not coordinate water [3]. Thus, the distal amino acid is unlikely to be histidine or another hydrogen bond accepting residue. Despite this, the oxygen and carbon monoxide dissociation rates of FixLs from *Rhizobium meliloti* and *Bradyrhizobium japonicum* are comparable to those of myoglobins. It is their exceedingly slow association rates that account for their 30 to > 100-fold reduced affinities for oxygen and carbon monoxide compared to sperm-whale myoglobin [3]. These characteristics, which are undoubtedly related to the sensor function of the FixLs, support our classification of these proteins as heme-based sensors.

The unliganded deoxy- (Fe^{II}) and met-FixL (Fe^{III}) are equally active kinases. Binding of strong-field ligands such as CN^- or CO causes a transition of the FixL heme iron from high to low spin, which inactivates the kinase [5]. Thus, the binding of CN^- by met-FixL can be used to study the same sensing mechanism used for oxygen regulation of deoxy-FixL. Sequence comparisons, absorption spectra and mutagenesis studies suggest that His194 is probably the site of heme coordination in the *Rhizobium meliloti* FixL [3,5,8]. To understand the mechanism of oxygen sensing, it is crucial to elucidate the structural changes in the heme pocket on binding of ligands that lead to kinase inactivation.

Over the past decade, multidimensional nuclear magnetic resonance (NMR) spectroscopy has permitted complete structural assignments for small, soluble, diamagnetic

proteins (<20 kDa), sometimes without the need for isotopic labeling [9,10]. In metalloproteins, proximity of protons to a paramagnetic center causes their signals to exhibit fast relaxation times, isotropic shifting and line broadening [11–13]. We and others have successfully exploited the isotropic shift that isolates the signals of the protons in the metal-binding site of metalloproteins from the crowded diamagnetic region (0–13 ppm) to determine structural features of hemoproteins [14–18]. Here, we examine the ferric forms of RmFixLH, a monomeric and quite soluble 17.6-kDa truncation of the *Rhizobium meliloti* FixL, containing only the heme domain. The paramagnetic met- (high-spin, S = 5/2) and cyanomet- (low-spin, S = 1/2) species correspond to the pertinent active and inactive conformations of FixL, respectively. For the low-spin paramagnetic form, isotropic shift and line broadening are sufficiently small that it becomes feasible to use two-dimensional NMR spectroscopy methods for signal assignment.

The theory behind the NMR studies of paramagnetic molecules is as follows. In environments where magnetic anisotropy is present, such as in low-spin Fe^{III} heme proteins, the dipolar shift becomes an important shift mechanism:

$$\frac{\Delta\nu^{dip}}{\nu} = -(1/3)N[(\chi_z - 1/2(\chi_x + \chi_y)]\left(\frac{3\cos^2\theta - 1}{r^3}\right) - (1/2N)(\chi_x - \chi_y)\left(\frac{\sin^2\theta\cos 2\Omega}{r^3}\right)$$

where r is the nucleus–metal distance, θ is the geometric angle of the **r** vector with the Z axis, and Ω is the angle of the X axis with the projection of the **r** vector on the XY plane. When the principal components of the magnetic susceptibility tensor (χ's) are known, one can obtain the configuration of the metal site. Other NMR techniques that help to determine molecular structures are estimation of proton–metal distances by the Solomon equation and Curie relaxation, detection of through-bond scalar coupling and observation of through-space nuclear Overhauser effects (NOEs) [11–14,19,20]. Because of better signal resolution, two-dimensional versions of these techniques are especially useful for the study of protein structures.

Coherence-transfer NMR techniques are helpful for signal assignment in paramagnetic species when it is possible to observe the crosspeaks due to scalarly coupled nuclei. The intensities of the cross-signals in a coherence-transfer correlation spectroscopy (COSY) experiment are functions of $\sin(\pi J_{ab}t_1)\exp(-t_1 / T_2)$, where J_{ab} is the scalar coupling constant, T_2 the spin–spin relaxation time, and t_1 the evolution time for the second dimension [21]. Because of the broadness (short T_2) of the hyperfine-shifted signals, coherence transfer between these signals may be so weak that it becomes undetectable. Since the scalar coupling between a vicinal pair of protons depends on their dihedral angle, however, the configuration of the moiety being studied, for example, the propionate groups of protoporphyrin IX, can also be revealed by the crosspeaks in a coherence-transfer spectrum.

The internuclear distance r_{ij} between a pair of nuclei i and j in a paramagnetic species can be estimated from the NOE:

$$NOE(i) = (\sigma_{ij} / \rho_i)[1-\exp(-\rho_i t)]$$

where $\sigma_{ij} = -\hbar^2\gamma^4\tau_c / 10r_{ij}^6$ is the cross relaxation, with γ the gyromagnetic ratio and τ_c the rotational correlation time, ρ_i is the intrinsic relaxation rate of *i*, and t is the saturation duration. The rotational correlation time τ_c also provides information about molecular dynamics and local motions. On the other hand, the large ρ_i of a hyperfine-shifted signal can result in very weak NOEs.

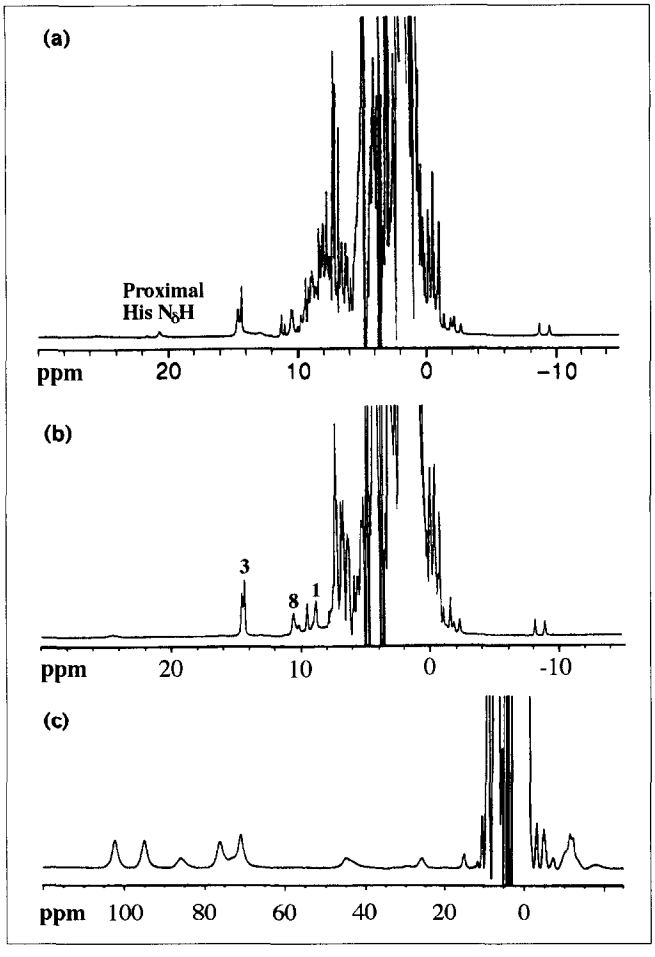
For paramagnetic hemoproteins such as the ferric RmFixLH, the heme–proton signals that are shifted outside of the diamagnetic region may be assigned by a combination of two-dimensional NMR techniques. Coherence transfer methods (COSY and total correlation spectroscopy (TOCSY)) can be used to identify spin systems, such as those of vinyl and propionate groups. Through-space interactions (determined by nuclear Overhauser enhancement spectroscopy (NOESY)) then establish the spatial arrangement of these sidechains and the methyl groups and *meso* protons with respect to each other around the porphyrin ring. The isotropically shifted proton signals of the amino acids lining the heme cavity can also be identified by their through-space interaction (NOEs) with the heme protons [12, 22]. Here, we describe the first ^{1}H-NMR study of FixL. We identify the isotropically shifted heme and proximal amino-acid signals of RmFixLH and compare our observations to data from selected myoglobins. This work also constitutes the first direct structural observation of the heme cavity of an oxygen sensor.

Results and discussion

The switch from high- to low-spin

The high-spin nature of the heme iron in met-RmFixLH at pH 8 is evident in the ^{1}H-NMR spectrum that spans a large spectral window from 100 to –20 ppm (Fig. 1c). The one-dimensional ^{1}H-NMR spectra in Figure 1a,c clearly show the switch from the high- to the low-spin Fe^{III} heme center that occurs on binding of cyanide. Consistent with FixL's low affinities for ligands, the low-spin cyanomet-heme is only evident after the addition of eight equivalents of cyanide. This species has a significantly different spectrum from those of cyanomet-myoglobin or the cytochromes [12]. For example, none of the heme-methyl signals of RmFixLH is isotropically shifted outside the

Figure 1



One-dimensional ^{1}H-NMR spectra of RmFixLH at pH 8 and 298 K show the switch from a high- to low-spin Fe^{III} heme center on binding of cyanide and features of the heme environment. **(a)** Met-RmFixLH in H_2O, **(b)** cyanomet-RmFixLH in D_2O and **(c)** cyanomet-RmFixLH in H_2O. A solvent exchangeable histidine ring-NH proton is indicated in (a); heme methyls 1, 3 and 8 are denoted in (b).

0–15 ppm region, whereas two heme-methyl signals are typically shifted outside of this range for the myoglobins (Table 1). Curiously, the relative shifts of the heme methyls in myoglobins are much less consistent than the common 8>3>5>1 shift pattern in the low-spin ferricytochromes *c* [11]. The small isotropic shifts and the shift pattern itself of cyanomet-RmFixLH suggest that the heme center has a unique magnetic environment and coordination chemistry. The differences between the heme environment of cyanomet-FixL and that of myoglobin, may relate to the fact that FixL functions as an oxygen sensor and not as an oxygen carrier.

The proximal and distal residues

We establish by direct structural observation that a proximal histidine residue is present in FixL, which had been suggested by absorption spectra and mutagenesis studies [3,8]. The proximal histidine appears as a ring NδH shift at 21.0 ppm in the cyanomet-RmFixLH spectrum, which disappears in D_2O due to exchange with the solvent (Fig. 1a,b). We have confirmed this assignment with a one-dimensional WEFT-NOE experiment, where irradiation on the NδH signal revealed a cross relaxation on a $C_\beta H$ proton. We also assigned the shifts of the $C_\alpha H$ and $C_\beta H_2$ protons of the proximal histidine at 7.4, 9.1/9.9 ppm, respectively, by their connectivities in two-dimensional spectra (Table 1).

We did not find in the RmFixLH spectrum the second isotropically shifted, solvent-exchangeable signal that normally corresponds to a distal histidine. In sperm-whale and *Chironomus* myoglobins, one-dimensional NOE experiments have assigned the distal histidine NεH resonance to the downfield (14–24 ppm) region of the ^{1}H-NMR spectrum [23,24]. This ring proton signal is absent from the spectra of cyanomet-myoglobins having substitutions at the distal histidine, including genetically engineered sperm-whale myoglobins and the *Aplysia* and *Glycera* myoglobins that normally have a distal valine or leucine, respectively [25–28]. Interestingly, the proton signals of a distal valine are consistently not isotropically shifted to the paramagnetic region. Thus, cyanomet-RmFixLH most resembles the *Aplysia* and *Glycera* myoglobins in the shift pattern of the proximal histidine signals and in the absence of the distal histidine NεH resonance. These observations support the proposal that FixL has a distal residue other than histidine. This proposal was based on an 11-nm blue shift in the Soret band of the met-FixL absorption spectra, which is seen only in hemoproteins known to lack a hydrogen-bonding distal residue [3,29].

Assignment of the heme proton signals

Several well-resolved crosspeaks associated with the isotropically shifted heme proton signals of the cyanomet-RmFixLH were in the two-dimensional spectra at 314 K. Using bond-correlation techniques, the unique spin patterns of $-CH{=}CH_2$ and $-CH_2{-}CH_2-$ readily identified the vinyl and propionate groups, respectively. The TOCSY spectrum in Figure 2a displays the spin pattern for a propionate at 9.6 (T_1, 31 ms), 5.9, –1.2 (25 ms), and –1.8 ppm (52 ms). One vinyl spin system appears at 14.1, –7.0, and –7.6 ppm (T_1: 141, 111, and 84 ms, respectively) and the other at 8.0, –0.6 (T_1, 79 ms), and –0.1. From a COSY spectrum, the $C_\beta H_2$ signals of the vinyl groups could be further assigned to the proton either *cis* or *trans* to the $C_\alpha H$ proton (Fig. 2a, insets). The larger coupling constant of the *trans* pair (19.0 and 12.1 Hz, compared to 11.7 and 5.3 Hz for the *cis* pair in ethylene and dichloroethylene, respectively) results in a more intense crosspeak. Thus, the signal at –7.6 ppm, which gives a more intense crosspeak with the vinyl $C_\alpha H$ at 14.1 ppm, corresponds to the '*trans* proton'. Similarly, the signal at

Table 1

Chemical shifts of heme methyls and proximal and distal histidines in RmFixLH and selected low-spin myoglobins.

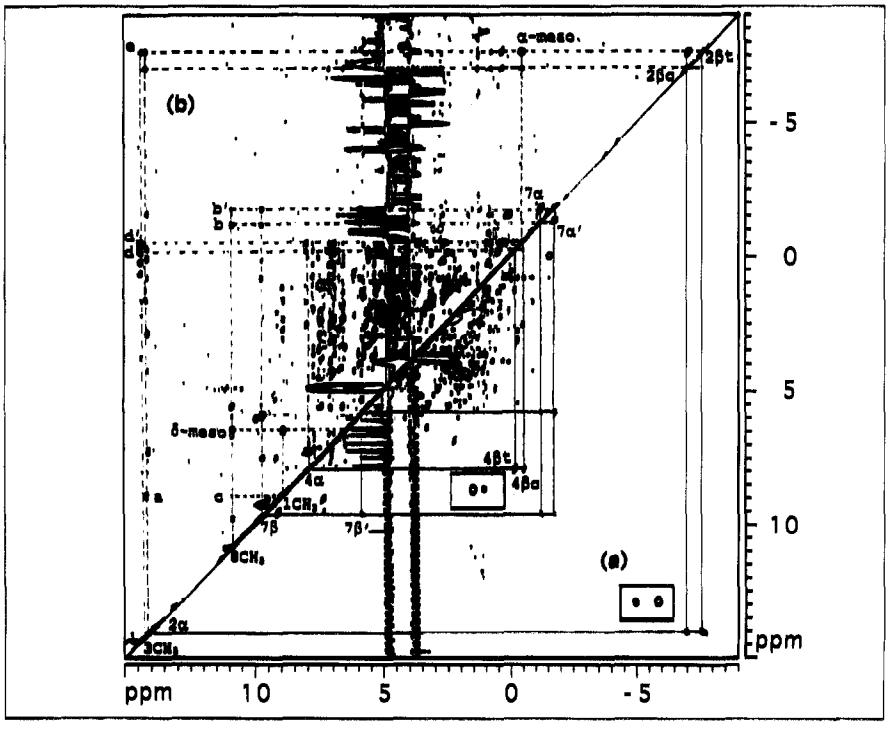
	Proximal His F8				Distal His E7	Heme methyls			
Protein	NδH	CαH	$C\beta_1H$	$C\beta_2H$	NεH	$1CH_3$	$3CH_3$	$5CH_3$	$8CH_3$
RmFixLH	21.0	7.4	9.9	9.1		8.8	14.3		10.8
Chironomus myoglobin	16.5		10.01	6.9	22.5				
	16.8				21.7				
Sperm whale myoglobin	20.1				23.7	18.6	4.8	27.0	12.9
Aplysia myoglobin	14.3	7.6	10.7	10.5	His → Val	11.8	17.8	15.6	9.9
Glycera myoglobin	23.7	8.6	11.7	7.1	His → Leu	1.0	18.3	6.7	19.3
SW Mb H(E7)V, T(E10)R	17.0	8.1	13.7	9.7	His → Val	17.8	5.8	26.0	12.6
SW Mb H(E7)V, T(E10)R, R(CD3)N	16.6	7.7	13.1	9.6	His → Val	16.7	5.9	25.2	12.8

Chironomus myoglobin was at pH 5.5–9.0, 298 K (the noted shift in ppm varied from 0.2 to 0.7); *Glycera* myoglobin was at pH 6.43, 288 K; *Aplysia* myoglobin was at pH 8.9, 298 K; sperm whale myoglobin (SW Mb) mutants were at pH 7.0, 298 K. SW Mb H(E7)V, T(E10)R is a double mutant with the distal histidine changed to valine and with threonine E10 changed to arginine; SW Mb H(E7)V, T(E10)R, R(CD3)N is a triple mutant with the additional change of arginine CD3 to asparagine. These multiply mutated sperm-whale myoglobins were designed to mimic *Aplysia* myoglobin.

–0.1 ppm corresponds to the proton *trans* to the vinyl $C_\alpha H$ found at 8.0 ppm.

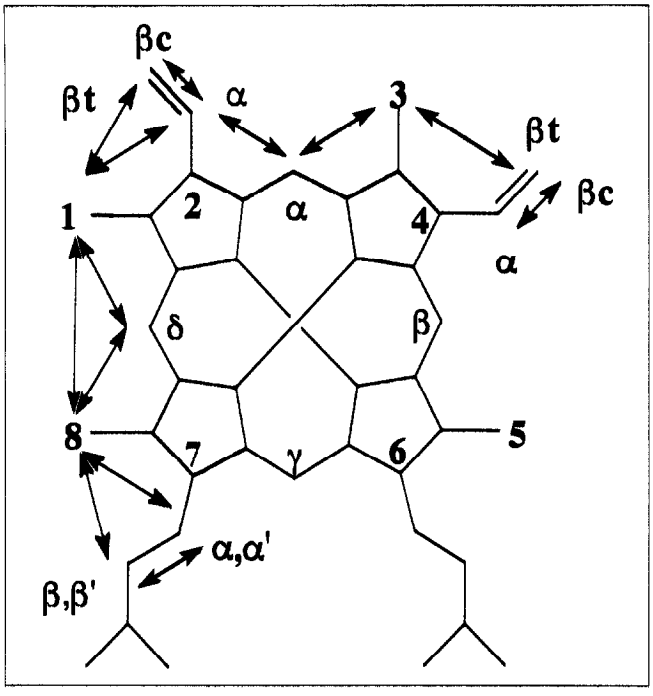
We have confirmed the above assignments and identified other heme signals by their through-space NOE interactions. In the one-dimensional ^{1}H-NMR spectrum, we easily identified three shifted heme methyls at 8.8, 14.3, and 10.8 ppm by simple integration (Fig. 1b). A fourth signal at 15.0 ppm may also correspond to a heme methyl, but this assignment has not yet been verified. Given the structure of protoporphyrin IX, methyls 1 and 3 should interact with vinyl spin systems, whereas methyls 8 and 5

Figure 2



The heme-proton signals can be assigned in the two-dimensional ^{1}H-NMR spectra of cyanomet-RmFixLH at 314 K and pH 8 (without calibration against temperature). **(a)** TOCSY with a mixing time of 20.5 ms. The insets in (a) are COSY crosspeaks that clearly show high intensities for *trans* pairs. **(b)** NOESY with a mixing time of 100 ms. The 100-ms mixing time is used because it reveals the methyl 1–methyl 8 fingerprint. No losses of the other heme signals were detectable at shorter mixing times (50 and 60 ms).

Figure 3



Structure of a protoporphyrin IX ring with observable NOEs indicated by arrows. Of particular interest is the 'fingerprint' NOE interaction between the methyl 1 and methyl 8 protons.

should sense propionate systems (Fig. 3). Furthermore, a fingerprint NOE should exist between methyls 1 and 8. From the NOESY spectrum, crosspeak *a* shows the interaction of the vinyl *a* signal at 14.1 ppm with the methyl signal at 8.8 ppm, permitting the assignment of methyl 1 or 3 to the latter signal (Fig. 2a). Crosspeaks *b* and *b'* show the interaction of a propionate CH_2 pair at −1.2 and −1.8 ppm with the methyl signal at 10.8 ppm. Thus, we assigned the signal at 10.8 ppm to methyl 5 or 8. Crosspeak *c*, associated with the methyl signals at 8.8 and 10.8 ppm, is identifiable as the fingerprint NOE between methyls 1 and 8. These NOE connectivities thus indicate that the signals at 8.8 and 10.8 ppm correspond to methyls 1 and 8 respectively.

Based on these methyl assignments, we assigned the corresponding vinyl 2 and propionate 7. This further allowed the assignment of the other vinyl spin system as vinyl 4. The spin system for propionate 6 is buried in the diamagnetic region, and its identification will require isotopic labeling. Crosspeaks *d* and *d'* show the interaction of the vinyl 4 $C_\beta H_2$ protons with the methyl signal at 14.3 ppm. Either methyl 3 or 5 could show this interaction. However, the existence of another crosspeak, *e*, between the *trans*-$C_\beta H$ proton of vinyl 2 and this methyl signal allows unambiguous assignment of the signal at 14.3 ppm to methyl 3. Unambiguous identification of the heme methyl 5 and of the β and γ *meso* protons awaits the recognition of the buried propionate 6 signals. The signal at −0.5 ppm, which shows crosspeaks with the *trans* vinyl 2 $C_\beta H$ proton and methyl 3, is assigned to the α-*meso* proton. The δ-*meso* proton (6.5 ppm) is recognizable from its crosspeaks with methyls 1 and 8.

We have assigned the majority of the proton signals of the heme moiety in FixL and directly demonstrated the presence of a proximal histidine. Some interesting features of the NMR spectra, such as the smaller isotropic shifts of the heme signals and the absence of an NH ring signal from a distal histidine, already indicate that the FixL heme environment is unique among hemoproteins. This work will facilitate assignment of the amino acids lining the heme pocket and identification of the changes that occur when the heme iron switches to the low-spin form, inactivating the kinase. We will detect the amino acids around the heme first by their interaction with the heme protons; we will then apply sequence-specific methods for a better assignment. Because of the relatively slow nuclear-relaxation rates and large magnetic anisotropy of the paramagnetic low-spin Fe^{III} heme center, we can investigate these changes in detail by two-dimensional NMR experiments such as magnitude COSY, NOESY and TOCSY, as discussed here. These studies will be aided by the availability of *fixL* point mutants and the reconstitution of FixL with alternative hemes. These experiments are in progress in our laboratories.

Significance

This report presents the first structural information on a new class of hemoproteins, the heme-based sensors. FixL is a key regulator of the expression of nitrogenase, the main enzyme of the nitrogen fixation cycle. A kinase activity in the FixL protein is reversibly inhibited by heme ligands. Ferrous FixL responds to oxygen, which is thought to be the physiological ligand. However, met-FixL can also function as a sensor, responding to cyanide and other strong-field ligands of ferric heme. NMR provides an excellent means for studying the changes in the heme pocket that inactivate the kinase. The advantage of NMR methods for the study of paramagnetic proteins is that they emphasize the resonances of protons that are near the heme. We have identified hyperfine-shifted ^{1}H-NMR signals of the heme and proximal histidine. These assignments will be useful for the determination of solution three-dimensional structures of the activating and inactivating conformations of the heme pocket.

Materials and methods

Sample preparation

The *R. meliloti* FixL heme domain, RmFixLH, was produced at high levels in *Escherichia coli* strain TG1 (harboring plasmid pRH61) and purified as described previously [3]. The met-RmFixLH (1.4 mM) was initially in H_2O or D_2O buffered with deuterated 10 mM Tris (pH 8.0). The cyano form was prepared as follows. A solution of 70 mM KCN

(1 eq./10 μl) was made in 99.5 % pure D_2O. The pH was adjusted to 7.6 (pD = pH + 0.4) by the addition of concentrated DCl. The pH was measured with a Fischer Scientific Accumet 910 pH meter. Titration of the ferric heme with KCN was monitored by the disappearance of signals in the 75–105 ppm region of the one-dimensional spectrum (Fig. 1). After addition of eight equivalents of cyanide, the sample was saturated.

^{1}H-NMR analysis

All measurements were performed on a Bruker AMX360 NMR spectrometer at 360.13 MHz using a 5-mm inverse-detection probe. For all spectra, a 90-degree pulse width of 8.5 μs was used with a presaturation pulse for water suppression, and they were all referenced to water signal at 4.65 ppm. Nonselective T_1 relaxation time measurements were taken using the inversion-recovery pulse sequence, i.e., 180°–τ–90°-acquisition. The signal intensity with respect to each delay time τ was fitted using a three-parameter fitting routine on the spectrometer. Phase-sensitive (TPPI) NOESY and TOCSY spectra (1024 x 512 data points) were recorded with presaturation of the residual HDO signal during the relaxation delay and the mixing time. To resolve overlapping peaks, experimental temperatures ranged from 288 to 314 K. A 90-degree pulse of 13 μs was used for the MLEV-17 spin-lock mixing period in the TOCSY experiments. This strong pulse caused significant increases in the sample temperature. Exact temperatures in the TOCSY experiments were determined by comparing the chemical shifts of the hyperfine-shifted signals in the two-dimensional NMR experiments with those of one-dimensional spectra obtained at temperatures ranging from 288 to 314 K. A 45-degree shifted sine-bell squared window function was applied to both dimensions prior to Fourier transformation using a Bruker UXNMR software.

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