First Spectroscopic Characterization of Fe^{II}-Fur, the Physiological Active Form of the Fur Protein

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Fur is a dimeric protein (2 × 17 kDa) involved in the iron uptake regulation in Gram negative bacteria. It is the key protein for the control of the intracellular iron concentration.[1] When the cellular iron concentration becomes too high, the Fur repressor binds the ferrous ion as a corepressor and then is able to complex specific DNA sequences.[2] *In vitro*, only a few dications such as Fe^{2+}, Co^{2+}, or Mn^{2+} are able to activate the protein whereas for unknown reasons Fur is not activated by Fe^{3+}.[3] Recent spectroscopic data on cobalt-substituted Fur indicate that the metal is hexacoordinated with only nitrogen and oxygen donor ligands including two (or three) histidines and one (or two) aspartate or glutamate.[4] In addition to this metallic site, Fur also possesses a structural tight-binding zinc site[5] that has been characterized by X-ray absorption studies[6] and shown to contain two sulfur ligands (cysteines 92 and 95[7]) and two N/O ligands including at least one histidine. Herein we present spectroscopic and magnetic data on the ferrous-substituted form of the Fur protein which is the active one *in vivo*.

The Fur apoprotein has been purified from *Escherichia coli* as previously described[4] and anaerobically reconstituted with 0.95 equiv of Fe^{2+} ions per Fur monomer.[8a] The X-band EPR spectrum recorded in parallel mode gives a g value around 9 which is in accordance with a high-spin state of the ferrous iron and shows the absence of ferric ions (data not shown). The samples have been analyzed by Mössbauer and X-ray absorption spectroscopies and saturation magnetization measurements.[8b]

Figure 1 shows the temperature dependence of the product of the molar magnetic susceptibility with temperature. The susceptibility data indicate the presence of an isolated high-spin ferrous center ($S = 2$). They were fitted with the Van Vleck equation derived by perturbation theory (valid for $g_i\beta H_i \ll |D|$ which is verified for $H < 1.5$ T) from the Hamiltonian appropriate for a spin $S = 2$ including an axial zero-field splitting. The fit of the data collected at 0.5 T leads to an estimate of the value of the axial ZFS parameter $|D|$ of 8.0(4) cm^{-1}.

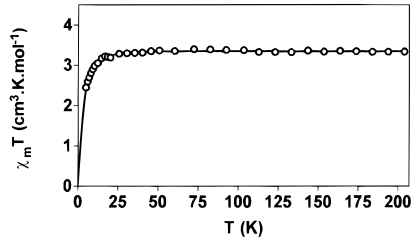

Figure 1. Temperature dependence of the product of the molar magnetic susceptibility with temperature at 0.5 T on an Fe(II)-Fur sample of concentration 4.14 mM in monomer in 0.1 M MOPS buffer at pH 8 containing 0.1 M potassium chloride and 10% v/v glycerol. The solid line is a theoretical curve corresponding to the best fit parameters.

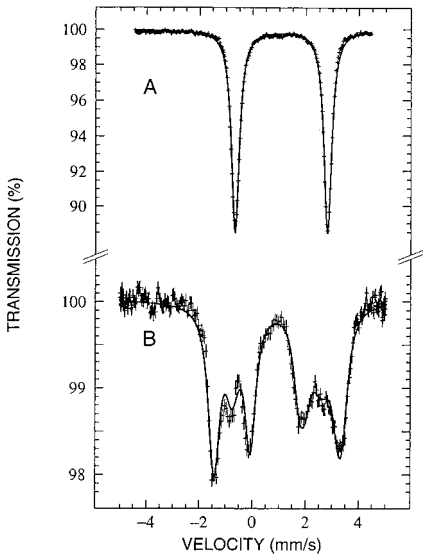

Figure 2. Mössbauer spectra of Fe(II)-Fur at 6 mM in monomer in 0.1 M Tris/HCl buffer at pH 8 containing 0.1 M potassium chloride and 10% v/v glycerol recorded at (A) 4 K without external field and (B) 200 K and 7 T external field. The experimental spectrum in part B was corrected for the contribution of iron impurities in the beryllium window. The following values of the **A** tensor have been used for the fit: $A_x = 0$ T, $A_y = -23$ T, and $A_z = -14$ T.

^{57}Fe(II)-Fur protein has been studied by ^{57}Fe Mössbauer spectroscopy from 4.2 to 200 K in applied magnetic fields parallel to the γ-rays up to 7.0 T. At 4.2 K in zero magnetic field, the obtained spectrum consists of a quadrupole doublet, characterized by a quadrupole splitting, $\Delta E_Q = 3.47$ (2) mm/s, and an isomer shift relative to iron metal at room temperature, $\delta_{Fe} = 1.19$ (1) mm/s (Figure 2A). These values characterize a high-spin ferrous ion in a tetragonal environment, i.e. t_{2g} as the fundamental state.[9] The slight temperature variation of ΔE_Q (3.31(2) mm/s at 200 K) indicates that the first excited state of the orbital system lies much higher than the ground state (in the range of 400−600 cm^{-1}). The high-field high-temperature spectrum is shown in Figure 2B. It indicates a positive value for the largest component of the electric field gradient tensor and a value of the asymmetry

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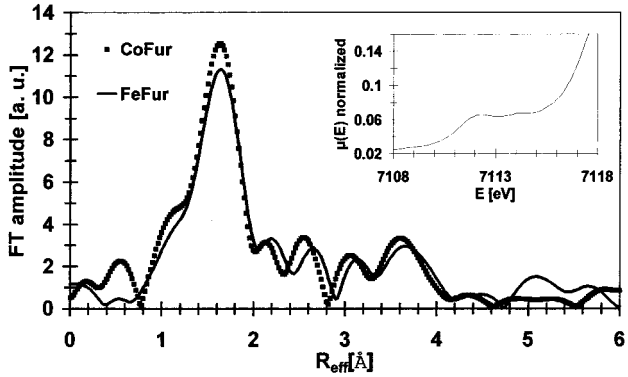

Figure 3. Fourier transforms of EXAFS spectra of Fe(II)-Fur and Co(II)-Fur. The concentrations in monomer were 5.5 and 11 mM for Fe(II)-Fur and Co(II)-Fur, respectively. Both samples were in 0.1 M Tris/HCl buffer at pH 8 containing 0.1 M potassium chloride and 10% v/v glycerol. The insert represents the preedge features of the X-ray absorption spectrum of Fe(II)-Fur.

parameter η in the range of 0.3−0.7. This suggests that the d_{xy} orbital is the lowest in energy and points to a low symmetry at the iron site. At 4.2 K in various applied magnetic fields, a complex set of spectra of combined electric and magnetic hyperfine interactions is obtained. A preliminary analysis[10] within the spin Hamiltonian formalism[11] allows the determination of the zero-field splitting parameters $D = +8(2)$ cm^{-1} and $E/D = 0.20(5)$. A very anisotropic magnetic hyperfine tensor was obtained; a precise determination of its components is in progress.

X-ray absorption spectroscopy[12] was used to investigate further the electronic structure and the chemical environment of the iron site. Figure 3 illustrates the Fourier transform of the EXAFS spectra of iron- and cobalt-substituted Fur. The two FT curves are very similar with a major peak at 1.6 Å (uncorrected for phase shifts) and the three-peak pattern characteristic of the multiple scattering of histidine imidazoles[13] with the same intensity, which suggests that the two metals occupy the same site. Simulation of the first shell could be done with five nitrogen/oxygen atoms at 2.13 Å and inclusion of sulfur did not give physically meaningful simulations. A 25% improvement of the fit was obtained by splitting the first shell in two independent subshells of nitrogens and oxygens. Analogous results were obtained with two subshells comprising two oxygens at 2.05 Å and three nitrogens at 2.17 Å or two subshells including three oxygens at 2.08 Å and two nitrogens at 2.19 Å. Clearly these values are consistent with the binding of oxygens from carboxylate residues, aspartate or glutamate, and histidine nitrogens, respectively. On the other hand, inclusion of a sixth donor at longer distance did not significantly improve the fit. The insert of Figure 3 shows Fe(II)-Fur normalized preedge domain. It comprises two main transitions: a broad one centered at ca. 7112.6 eV and a weaker one at higher energy ca. 7114.7 eV. These preedge transitions are more intense than those observed in systems of pure octahedral symmetry indicating a distortion of the iron coordination sphere.[14]

All spectroscopic and magnetic measurements concur to show that the iron ion in Fe-Fur is in the high-spin ferrous state. Magnetization and Mössbauer spectroscopic data have provided an evaluation of the zero-field splitting parameters of the ferrous center to $D \sim +8.0$ cm^{-1} and $E \sim 1.6$ cm^{-1}. Using perturbation theory this value allows us to estimate to ca. 800 cm^{-1} the energy splitting (Δ) of the d_{xy} and $d_{xz,yz}$ orbitals.[15] Moreover the energy splitting between d_{xz} and d_{yz} can be estimated to ca. $V = 350$ cm^{-1} from the published graphical relationships[15] between E and $V/|\Delta|$ pointing to a high rhombicity of the ferrous center. This places the energy difference between the ground and the first excited states at ca. $\Delta - V/2 \sim 625$ cm^{-1}. This value compares favorably with that derived from the analysis of the slight temperature dependence of the quadrupole splitting which amounts to 500(100) cm^{-1}. In addition, the observation of the more intense transition at lower energy in the preedge region of the X-ray absorption spectrum indicates that the d_{z^2} orbital is close to those originating from the t_{2g} level, suggesting that the ferrous center is significantly elongated.[14]

EXAFS spectroscopy favors a pentacoordinate ferrous site although the presence of a sixth ligand at a longer distance cannot be strictly excluded. Indeed the X-ray structure of lipoxygenase[16] has shown the presence of iron bonds so weak that they cannot be detected by EXAFS experiments.[17] It is therefore possible that the coordination belongs to the 5+1 type in agreement with the elongation apparent in the preedge and edge spectra. NMR and EXAFS studies of Co^{II}-Fur[4] have shown the presence of at least two histidines and at least one carboxylate residue and the spectroscopic similarity of the iron and cobalt derivatives strongly suggests that these residues contribute to the iron ligation as well. Therefore it appears that the iron center in Fur is likely to possess the 2-his-1-carboxylate triad which has recently emerged[18] as a structural motif common to mononuclear non-heme iron(II) enzymes such as isopenicillin N synthase, lipoxygenase, superoxide dismutase, and protocatechuate 3,4-dioxygenase. Indeed the Mössbauer parameters of Fe(II)-Fur are very close to those of these enzymes (δ_{Fe} = ca. 1.10 mm/s and $\Delta E_Q = 3.0-3.5$ mm/s)[19,20] in agreement with an environment constituted of oxygen and nitrogen donors.

Nevertheless, Fe(II)-Fur differs from these enzymes in several respects, a major one being that so far it has not been oxidized aerobically with hydrogen peroxide at pH 8, suggesting a high potential value for the Fe^{2+}/Fe^{3+} couple in Fur. This property may be related to the fact that Fur is not activated by Fe^{3+} ions.[3] In addition, optical, EPR and X-ray absorption data concur to show that the Fur metal site provides Mn^{2+}, Co^{2+}, and Fe^{2+} ions with the same basic environment and geometry,[4,21] therefore not adapting to the intrinsic structural preference of every metal. This property may be linked to the similarity of the dissociation constants of these metals which all fall in the 10−100 μM range.[3,22] This apparent rigidity of the metal environment and its moderate affinity for divalent ions such as Fe^{2+} may be a key factor in obtaining the easy metal uptake and release processes essential for Fur to function as a cellular iron sensor. The redox properties of Fe(II)-Fur in this respect, its similarities with non-heme iron enzymes, and the mechanism of iron release are currently under scrutiny in our laboratory.

Supporting Information Available: Full experimental details and data analysis of the XAS experiments together with a table and a graph of the EXAFS fits (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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