High-frequency EPR on high-spin transitions-metal sites

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Chapter 6

The two Fe^{3+} binding sites in human serum transferrin distinguished by high-frequency EPR

Continuous-wave electron-paramagnetic-resonance (EPR) spectra at 275.7 GHz of human serum transferrin allow to distinguish the signals of the high-spin Fe^{3+} ions bound by the two homologous lobes of the protein. This observation is confirmed by the 275.7 GHz spectra of two authentic monoferric human serum transferrins in which the iron binding by either the N-lobe or the C-lobe has been disabled by a site-directed mutation. The spectra of the monoferric mutants as well as the wild-type spectra are well reproduced by simulations and quantitatively analyzed in terms of their spin-Hamiltonian parameters.

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6.1 Introduction

The transferrin family of proteins plays a central role in the iron metabolism of vertebrates, and some invertebrates. [16] Human serum transferrin (hTF) transports iron to cells and assures that no free iron ions are present in the blood. The protein consists of two homologous lobes, termed the N- and C-lobe, each capable of binding strongly (binding constant in excess of 10^{20}) and reversibly an Fe^{3+} ion. Each lobe is comprised of two domains that open and close with a hinge motion. The iron is bound deeply in this cleft in a distorted octahedral coordination. Four ligands to the iron are provided by the conserved amino acid residues aspartic acid, histidine and two tyrosines. The two other ligands are constituted by a bound anion, the synergistic anion, which is naturally carbonate, CO_3^{2-}.

All modern, bilobal transferrins are thought to have resulted from the same early gene duplication of an ancestral, monolobal transferrin, [128] for which a candidate has been found in the ascidian *Ciona intestinalis*. [129] Evidence exists of differences in the iron-binding properties between the N- and C-lobe and of cooperativity between the lobes, [130–136] but no consensus in literature exists on what is the major driving force behind the evolution of bilobal transferrins.

Electron-paramagnetic-resonance (EPR) spectroscopy is a suitable and sensitive technique to investigate the electronic structure of paramagnetic transition-metal sites. The Fe^{3+} bound to transferrin is in a high-spin state with a spin angular momentum of $S = 5/2$. The first X-band (9.5 GHz) EPR spectrum of hTF was reported as early as 1963. [137] Since then X-band EPR spectra have been reported of other members of the transferrin family of proteins, [138–141] of transferrin in different environments, [142, 143], bound to the transferrin receptor, [133] or containing a synergistic anion other than carbonate. [144, 145] Moreover, spectra have been recorded at S-band (2.73 GHz), Q-band (34.05 GHz), W-band (94.1 GHz) and even at 285 GHz. [44, 55, 146] However, all of these spectra have withstood complete understanding, even if two or more components were assumed to contribute to the spectra. [86, 146]

Monoferric hTFs, which have iron bound only by the C-lobe or by the N-lobe, can be obtained by adding iron to apo-hTF in the form of ferric nitrilotriacetate (C-lobe) or ferric oxalate (N-lobe). [131, 147] Also, isolated recombinant C-lobe or N-lobe has been prepared, [133, 143] which made it possible to study the effects of site-directed mutations on the iron-binding through spectroscopic techniques. [148–151] The X-band EPR spectra acquired on the monoferric forms of transferrin have the same characteristics as the X-band spectra of the biferric forms. Subtle differences are observed between the N- and C-lobe, but the two lobes cannot be distinguished in the biferric spectra, nor can the differences be interpreted. The lack of an interpretation of the EPR spectra of transferrin constitutes a considerable barrier in the study of the iron binding by transferrin.

The higher the microwave frequency used, the more sensitive the EPR spectrum

of high-spin Fe^{3+} is to small variations in the electronic structure. The last decades have shown a strong increase in the possibilities of EPR at higher microwave frequencies. However, it remained a challenge to achieve the sensitivity to record spectra of frozen solutions of proteins that contain a transition-metal site in a high-spin state, like transferrin. [44, 146] The concentration and available amount of the protein are usually limited, and the spectra are hard to detect, because they cover large field ranges and the observed EPR signals are broad. Recently we have demonstrated that, using a single-mode cavity, it is possible to record high-quality EPR spectra in continuous-wave (cw) mode at 275.7 GHz (J band) of millimolar frozen solutions of the protein rubredoxin, whose active site in the oxidized state contains high-spin Fe^{3+}, see Chapter 2 of this thesis. [64]

Here we report the high-quality cw J-band spectra of hTF. In these spectra the signals due to the two iron-binding sites of hTF are clearly resolved. This observation is confirmed by the cw J-band spectra of two monoferric hTFs in which the iron binding by either the N-lobe or the C-lobe has been disabled by a site-directed mutation. [152] The spectra of the iron bound in the N- and C-lobe are well reproduced using the EPR simulation package EasySpin, [6] taking into account the effect of large conformational strain, and are quantitatively analyzed in terms of their spin-Hamiltonian parameters.

6.2 Materials and methods

Human serum transferrin (hTF) was expressed in baby hamster kidney cells as described in reference 152 and the references therein. The recombinant hTF is non-glycosylated and contains an N-terminal hexa-histidine tag. It is functionally indistinguishable from hTF isolated from serum and containing two Asn-linked glycosylation sites. Monoferric hTF was realized by disabling one of the binding sites by local mutations: monoferric C (Fe_C): N-His Y95F/Y188F hTF-NG, and monoferric N (Fe_N): N-His Y426F/Y517F hTF-NG. The proteins were kept in 100 mM HEPES buffer at pH 7.4.

The cw J-band EPR spectra were recorded on an in-house developed spectrometer, [9] using a probe head specialized for operation in cw mode as described in Chapter 2. [64] The effective sample volume, limited by the microwave cavity, is approximately 20 nl.

6.3 Results

The two upper spectra in Figure 6.1 are the cw J-band EPR spectra of frozen solutions of two hTF mutants, in which either only the C-lobe (Fe_C) or only the N-lobe (Fe_N) is able to bind iron. The Fe_C spectrum shows a broad, positive EPR signal at 8.68 T, and another, less broad, positive signal at 9.24 T, which occurs roughly

halfway between the 8.68 T signal and the signals around $g = 2$ (at 9.838 T). The spectrum also shows a broad, negative signal at 11.11 T, and another, less broad, negative signal at 10.48 T, which again occurs roughly halfway between the 11.11 T signal and the signals around $g = 2$. The same pattern is found in the Fe_N spectrum, with positive signals at 8.47 and 9.12 T and negative signals at 10.66 and around 11.5 T.

The lowest spectrum in Figure 6.1 is the cw J-band EPR spectrum of a frozen solution of wild-type hTF, which has an Fe^{3+} ion bound in both lobes. The Fe_2 spectrum clearly shows both the two positive signals observed in the Fe_N spectrum and the two positive signals observed in the Fe_C spectrum. Both negative signals of the Fe_C spectrum and the negative signal observed at 10.66 T in the Fe_N spectrum are present in the Fe_2 spectrum. The shallow 11.5 T signal in the Fe_N spectrum cannot be detected with certainty in the Fe_2 spectrum.

Figure 6.2 shows the $g = 2$ region of the Fe_N, Fe_C and Fe_2 hTF spectra in detail. The positive signals below $g = 2$ and negative signals above $g = 2$ form a complex pattern, which is of much stronger intensity in the Fe_C spectrum than in the Fe_N spectrum. The Fe_2 hTF spectrum is dominated by the signals that also show up in the Fe_C spectrum, but a contribution from the Fe_N spectrum is clear from the weak signals at 9.779 T and around 9.930 T, and a change in shape of the central signal around 9.820 T compared to the Fe_C spectrum.

Apart from the signals already discussed, all spectra in Figure 6.1 show a positive signal just below the $g = 2$ region. The shape of this signal could not be accurately determined, because it is distorted by a negative signal due to an unknown impurity at 9.6 T.

6.4 Analysis

The EPR spectra of high-spin Fe^{3+}, $S = 5/2$, are interpreted using the following spin Hamiltonian [3]

$$H = \mu_B \vec{B_0} \cdot \vec{\vec{g}} \cdot \vec{S} + \vec{S} \cdot \vec{\vec{D}} \cdot \vec{S} \tag{6.1}$$

The first term describes the electron Zeeman interaction. The g tensor gives the anisotropy of the Zeeman splitting, which is small for high-spin Fe^{3+}. The second term describes the zero-field splitting (ZFS) of the six magnetic sublevels into three Kramers doublets, $|m_s \pm 1/2\rangle$, $|\pm 3/2\rangle$, and $|\pm 5/2\rangle$. The ZFS tensor, $\vec{\vec{D}}$, is symmetric, can be taken traceless, and is characterized by two parameters, D and E.

$$D = 3/2 D_z,\ E = 1/2(D_x - D_y) \tag{6.2}$$

The rhombicity of $\vec{\vec{D}}$ is given by the ratio $\lambda = E/D$. The principal axes are chosen such that $|D_z| > |D_y| > |D_x|$ and $0 < \lambda < 1/3$.

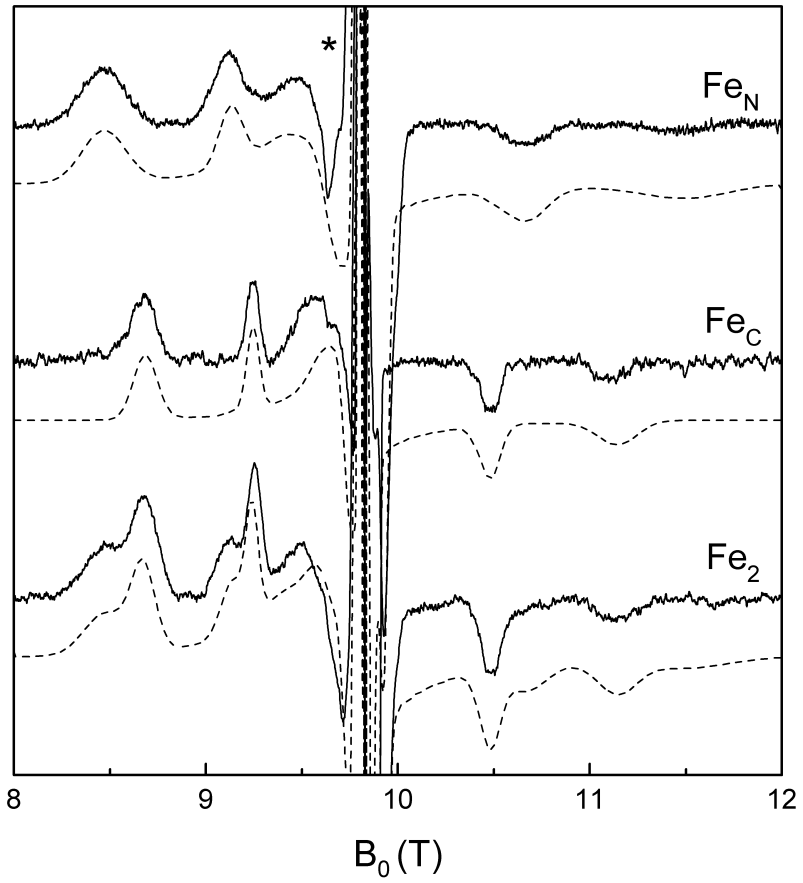

Figure 6.1: J-band cw EPR spectra of 1 mM frozen solutions of Fe_N, Fe_C and Fe_2 human serum transferrin at 10 K. Experimental conditions: modulation amplitude: 3 mT, microwave power: 1 μW, microwave frequency: 275.7 GHz. Solid lines: experimental spectra, dashed lines: spectra calculated using EasySpin and the spin-Hamiltonian parameters given in the text. The scale of the graph is set to optimally show the broad signals outside the $g = 2$ (at 9.838 T) region. The $g = 2$ region is shown in detail in Figure 6.2. The experimental spectra are baseline corrected. An unknown impurity gives a negative signal at 9.6 T, marked with $\star$.

In the high-field limit, $\vec{S} \cdot \vec{\vec{D}} \cdot \vec{S} \ll \mu_B \vec{B_0} \cdot \vec{\vec{g}} \cdot \vec{S}$, the ZFS term may be treated as a perturbation. [45] [64] As a result the magnetic sublevels split linearly with the applied magnetic field and only allowed transitions ($\Delta m_s = \pm 1$) occur. In a frozen solution spectrum this leads to equidistant transitions with their spacing determined by D_i, the principal values of the ZFS tensor, according to

$$\Delta B_0 = \frac{3D_i}{\mu_B g_i} \tag{6.3}$$

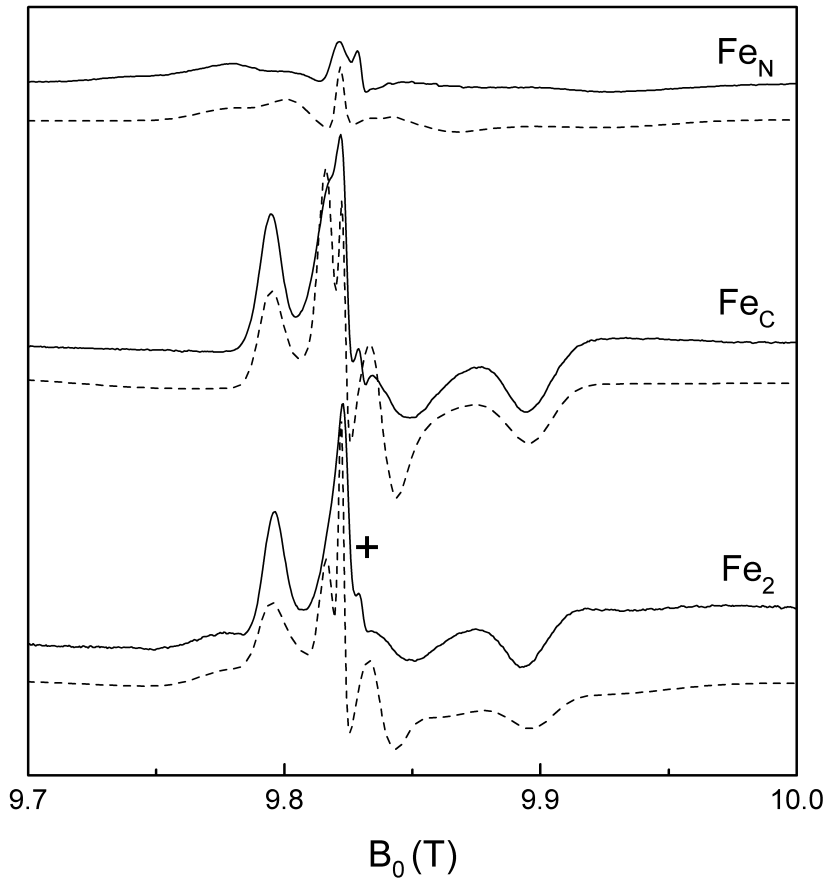

Figure 6.2: The $g = 2$ region of the J-band cw EPR spectra of 1 mM frozen solutions of Fe_N, Fe_C and Fe_2 human serum transferrin at 10 K. Experimental conditions were the same as for the spectra shown in Figure 6.1, except for the modulation amplitude, which was set to 0.8 mT. Solid lines: experimental spectra, dashed lines: spectra calculated using EasySpin and the spin-Hamiltonian parameters given in the text. The signal observed in all spectra at 9.830 T, marked with +, is due to an impurity in the frozen solution.

At the lowest temperatures transitions will show up above or below $g = 2$ depending on whether D_i is positive or negative, respectively, because the magnetic sublevels are populated according to Boltzmann.

From Equation 6.3 we estimate the values of D_z and D_y of the iron bound in the N- and the C-lobe from the two upper spectra in Figure 6.1. For both lobes the distance between the negative signals above $g = 2$ is larger than the distance between the positive signals below $g = 2$, which means that the negative signals yield a positive D_z and the positive signals yield a negative D_y. D_x follows from

$D_x + D_y + D_z = 0$. We estimate for the N-lobe $D = 11.7$ GHz and $\lambda = 0.21$ and for the C-lobe $D = 9.0$ GHz and $\lambda = 0.27$.

Fine tuning of the spin-Hamiltonian parameters was done by comparing the experimental spectra to spectra calculated by numerical diagonalization of the spin Hamiltonian using the EPR simulation package EasySpin. [6] The ZFS parameters that describe the electronic structure of the bound high-spin Fe^{3+} ion best are

$$\text{N-lobe: } D = 12.0 \text{ GHz}, \ \lambda = 0.20$$

$$\text{C-lobe: } D = 9.3 \text{ GHz}, \ \lambda = 0.25$$

We estimate the uncertainties to be $\pm$ 0.3 GHz for D and $\pm$ 0.01 for λ from the changes the J-band spectra induced by a change in these parameters as calculated using EasySpin. An increase of the g values from the free-electron value to $g_x = 2.004 \pm 0.0005$, $g_y = 2.0042 \pm 0.0002$, $g_z = 2.0042 \pm 0.0002$ was found to improve the match with the experimental spectra, particularly in the $g = 2$ region. These spin-Hamiltonian parameters were used for the simulated spectra shown in Figure 6.1 and Figure 6.2. In order to reproduce the width and shape of the resonances, the spectra were simulated taking into account a strain in D and E, using a first-order approximation.[1] For the Fe_C spectra a strain of 20 % was used in both D and E, for the Fe_C spectra a strain of 20 % was used in E and of 30 % in D.

The Fe_2 spectrum is clearly a sum of the Fe_C spectrum and the Fe_N spectrum. For the simulation of the Fe_2 spectrum, shown in Figure 6.1 and 6.2, equal contributions of the N- and C-lobe are assumed.

6.5 Discussion

The cw J-band spectra of wild-type hTF show the signals of the high-spin Fe^{3+} ions bound to the two lobes of hTF clearly resolved. This allows in principle quantitative analysis of the electronic structure of the iron bound in the individual lobes, although, particularly concerning the shallow negative peaks, doubts may arise as to which signals belong together. The spectra of the monoferric mutants of hTF erase those doubts and, moreover, show that the Fe_2 spectrum is a sum of the Fe_C spectrum and the Fe_N spectrum. We conclude that, as far as we can resolve with cw EPR at 275.7 GHz, the presence of an Fe^{3+} ion in one lobe does not alter the electronic structure of the iron-binding site in the other lobe.

The ZFS parameters of the bound Fe^{3+} ion differ considerably for the two lobes. To interpret these differences these parameters have to be translated into electronic structure using advanced quantum-chemical methods. Considerable progress has

[1] See the online documentation on EasySpin. A Gaussian is added to the spectrum of a width proportional to the derivative of the resonance field with respect to, for instance, D of a given transition.

been made with such calculations in recent years, in particular based on density-functional theory. [8] The calculation of the ZFS for transition-metal ions is, however, still in an exploratory stage and interpretation of the spin-Hamiltonian parameters of the high-spin Fe^{3+} bound to transferrin in terms of the electronic structure and the relation between geometry and function is not yet within reach.

In 1972 Aasa *et al.* concluded that the characteristic spectrum of hTF at X band had to derive from at least two components, one with an almost rhombic ZFS tensor, $\lambda = 0.325$, and one with a ZFS tensor of lower rhombicity, between 0.2 and 0.27. [140] Their logical guess was that these two components correspond to the two lobes of hTF. In their paper from 1987 Yang and Gaffney even invoke three components to explain the X-band hTF spectrum. [86] The remarkable fact is that the X-band spectra of the monoferric forms of transferrin show the same characteristics, [131, 133, 143, 147] which removes the basis for an interpretation of these spectra in terms of multiple components.

Neither our J-band spectra of the two monoferric hTFs nor our J-band spectra of wild-type hTF show a sign of the presence of high-spin Fe^{3+} sites with another electronic structure than those described by the parameter sets we obtained for the N-lobe and the C-lobe. Moreover, the rhombicity of the ZFS-tensors we found for both iron-binding sites, $\lambda = 0.20$ and 0.25 for the N- and the C-lobe respectively, is not compatible with the rhombicity found from X-band spectra by Aasa *et al.* We have attempted to simulate the X-band spectra of hTF with the parameter sets established from the J-band spectra using EasySpin, but were not successful. Thus, the cw J-band spectra of hTF have provided us with essential, new information on the electronic structure of the two iron-binding sites of hTF, but more information is still hidden in the X-band spectra.

6.6 Conclusion

The high frequency and the high sensitivity with which the EPR spectra of hTF are recorded make it possible to distinguish in the wild-type spectrum the high-spin Fe^{3+} ions bound in the N-lobe and in the C-lobe. The EPR signals of the iron bound by the N-lobe or the C-lobe are quantitatively analyzed in terms of their spin-Hamiltonian parameters. The quantitative understanding of the high-frequency transferrin EPR spectra has brought us in a good starting position to finally understand the X-band spectrum of transferrin. Moreover, the sensitivity of the high-frequency spectra to small changes in the electronic structure of the high-spin Fe^{3+} binding sites opens up the way to deepen our understanding of the iron binding and the iron-binding mechanism by transferrin through a combination of biochemistry and EPR spectroscopy.