RE-INTERPRETATION OF THE ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF TRANSFERRINS

by

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SUMMARY: EPR spectra of transferrins from three sources have been recorded at a temperature near 5K. The spectra of hen's egg-white ovotransferrin (conalbumin) and human plasma transferrin are each interpreted as two partly overlapping Fe^{3+} spectra of the so-called rhombic type. Thus, the two iron-binding sites in these two transferrins are not identical but give distinctly different EPR spectra. The EPR spectrum of transferrin from the plasma of the atlantic hagfish (*Myxine glutinosa*) shows no signs of such an inhomogeneity.

INTRODUCTION: A special recurrent problem in transferrin chemistry has been the question of equivalence between the two metal binding sites. Equilibrium dialysis studies of human serum transferrin (1) have shown that the binding of Fe^{3+} to this protein may be described as a random process to two non-interacting sites. This has been corroborated by electrophoretic studies (2). Similar studies of hen's egg-white ovotransferrin, also known as conalbumin, seem to indicate that the first iron is slightly more strongly bound than the second iron (3). The equal or nearly equal binding strength of the two metal binding sites has been taken as an indication of equivalence between these sites.

There are, however, several observations which do not fit into this simple picture. The biological activity of human transferrin is not the same for molecules having one and two iron atoms, respectively (4). Lanthanide binding studies to transferrin have given indications of inequivalence between the two metal binding sites and furthermore there seems to exist only one specific binding site per molecule for some

lanthanides having large ionic radii (5).

Electron paramagnetic resonance (EPR) spectroscopy has revealed a distinct difference in the metal binding sites when Fe^{3+} is replaced by Cr^{3+} (6). It has also been difficult to interpret the EPR spectrum of native human transferrin in terms of a reasonably simple spin-Hamiltonian (1,7,8).

This paper presents evidence that the two iron binding sites in native human serum transferrin show up distinctly different EPR spectra. In ovotransferrin the difference between the sites is even larger as judged from their EPR spectra. Transferrin from the atlantic hagfish (Myxine glutinosa) has also been studied for comparative reason. The EPR spectrum of this transferrin has a more homogeneous appearance, which is interpreted as none or very small difference between the iron binding sites.

MATERIALS AND METHODS: Human transferrin was prepared as previously described (8).

Freeze-dried apo-ovotransferrin was obtained as a gift from Dr. Philip Aisen at Albert Einstein College of Medicine, New York. The protein was dissolved in Tris-HCl buffer, pH 8, and iron was added as the 1:2 Fe^{3+}-nitrilotriacetate complex. Excess amount of $NaHCO_3$ was added to ensure formation of the bicarbonate iron protein complex, called native ovotransferrin.

Transferrin from the atlantic hagfish (Myxine glutinosa) was prepared from about 18 ml blood drawn from 11 individual hagfishes. The erythrocytes were removed by centrifugation. Iron as the 1:2 Fe^{3+}-nitrilotriacetate complex was then added to the plasma in order to saturate all the transferrin. The plasma was concentrated about three times to a volume of 4 ml by dialysis against a 0.05 M Tris-HCl buffer, pH 7.6, also containing 0.1 M KCl. The transferrin was then fractionated by gel

Table 1. EPR parameters of Fe^{3+} in transferrins.

Source	Iron site I		Iron site II	
	D cm^{-1}	$E/D = \lambda$	D cm^{-1}	$E/D = \lambda$
Hen's egg-white	-	0.22-0.25	0.3	0.31
Human plasma	-	0.25-0.27	0.3	0.32
Hagfish plasma	0.3	0.315	0.3	0.315

filtration on a Sephadex G-150 column equilibrated with the same buffer (9). The fractions containing transferrin, which were detected by their characteristic EPR spectra, were then concentrated to a volume of about 0.4 ml. The final yield was estimated to be about 1 mg transferrin/ml plasma.

EPR spectra at 9.2 GHz were recorded with a Varian E-3 spectrometer. Spectra could be obtained at temperatures close to that of liquid helium by the use of a simple helium gas flow system (10).

Some spectra were also recorded at 3 GHz as described earlier (8).

RESULTS AND DISCUSSION: EPR spectra of the three kinds of investigated transferrins are shown in Fig. 1. All spectra are recorded at temperatures near 5 K but at two different microwave powers. Spectra obtained at 77 K are identical to those shown in Fig. 1A.

There are some main features which are quite similar for all spectra. They consist of an absorption around $g' = 4.2$ and a much weaker absorption near $g' = 9$. This type of EPR apectra is very common for organic Fe^{3+} compounds and often called rhombic type of spectra. The spin-Hamiltonian commonly used to interpret EPR spectra of high spin ferric ions is

$$\underline{H} = g\beta \underset{\sim}{B} \cdot \underset{\sim}{S} + \underline{D}\left[\underline{S}_z^2 - \tfrac{1}{3}\underline{S}(\underline{S}+1)\right] + \underline{E}\left[\underline{S}_x^2 - \underline{S}_y^2\right]$$

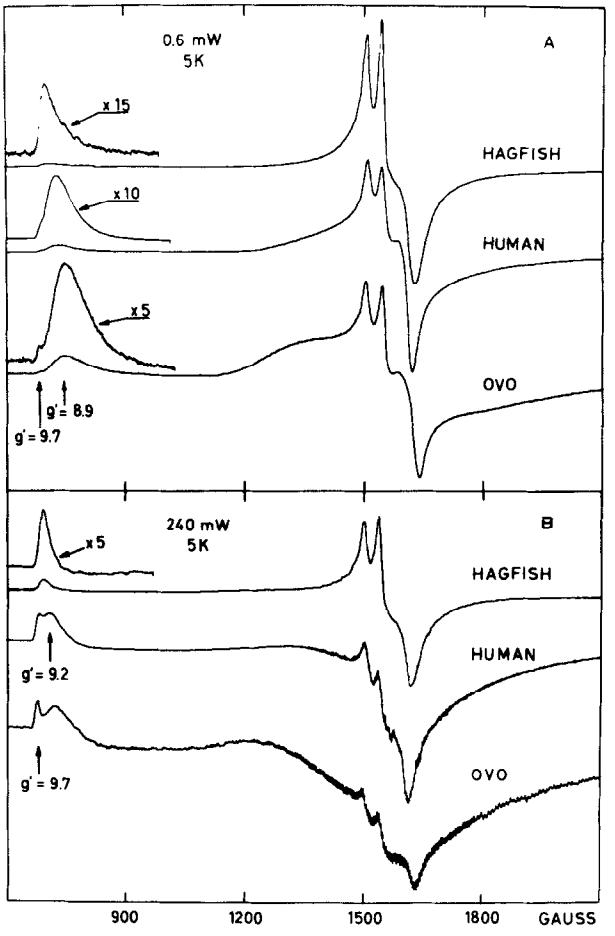

Figure 1. EPR spectra of transferrins recorded at a temperature close to 5K and microwave frequency near 9180 MHz. The microwave power is for A, 0.6 mW and for B, 240 mW.

with $S = 5/2$ and isotropic $g \sim 2.00$. The rhombic type is obtained if the Zeeman term is small compared to the crystal field terms, i.e. $g\beta B < E, D$, and in addition $\lambda = E/D$ lies approximately in the range 0.2 - 1/3. Then, the middle Kramer's doublet gives rise to a line cluster at an apparent g-value (or g') near 4. The other two Kramer's doublets give lines at $g' \sim 9$ and also lines, which are difficult to observe, at $g' < 2$ (8). The number of powder lines at $g' \sim 4$ can vary from one to six depending on the size of the spin-Hamiltonian parameters. The number of lines near $g' \sim 9$

can be either one (for λ exactly equal to 1/3) or two. There must, of course, exist a direct relation between the positions of the individual lines in the $g' \sim 4$ cluster and the positions of the lines near $g' = 9$. In addition, there is a relation between the intensities of the $g' \sim 4$ lines and the $g' \sim 9$ lines. Due to changes in transition probabilities and anisotropies one can rather generally state that the more λ deviates from 1/3 the more intense will the $g' \sim 9$ lines appear compared to the lines at $g' \sim 4$ (7,8).

Now let us turn to an examination of the experimental spectra of the transferrins, starting with the ovotransferrin spectrum at the bottom of Fig. 1A. The three narrow lines at 1500, 1550, and 1640 Gauss, having g' between 4.4 and 4.0, can fairly well be accounted for by taking $\lambda \sim 0.31$. However, there are broad lines on both the high and low field side of these narrow lines. It is possible to get lines near $g' = 4$ from transitions other than the middle Kramer's doublet. This, however, demands a rather narrow range of $h\nu / D$ (8). A spectrum recorded at 3 GHz, i.e. $h\nu$ decreased by a factor of three, clearly shows both the narrow and the broad components around $g' = 4$. Thus, one is left with the remaining possibility, namely that the broad lines around $g' = 4$ do not arise from the same Fe^{3+} species as the narrow lines. These two species are naturally interpreted as the two iron sites in the ovotransferri molecule which accordingly yield clearly dissimilar EPR spectra. Further evidence for this conclusion is provided by the shape of the lines near $g' = 9$. An Fe^{3+} spectrum with $\lambda = 0.31$ should give two lines at $g' = 9.60$ and 9.70 while the experimental spectrum shows a weak line at $g' = 9.7$ and a comparatively strong line at about $g' = 8.9$. These lines are better resolved in Fig. 1B, which shows spectra recorded at 240 mW. At that microwave power, all lines are saturated but not to the same extent. Thus, the line at $g' = 8.9$ saturates more easily than the line at $g' = 9.7$ and the same is true for the narrow components of $g' = 4$ compared to the broad

parts. The most natural interpretation of the observed lines is then that the broad parts around $\underline{g}' = 4$ and the $\underline{g}' = 8.9$ line belong to one Fe^{3+} site, while the narrow three-component part near $\underline{g}' = 4$ and the $\underline{g}' = 9.7$ line belong to the other Fe^{3+} site in the ovotransferrin molecule. Indeed, spectra recorded of partly Fe^{3+} saturated ovotransferrin support this designation and also indicate that the iron which is first bound (3) gives the broad type of spectrum.

Similar analyses of the spectra of hagfish and human plasma transferrins can also be made. The hagfish transferrin spectra show no sign of inhomogeneties neither at low microwave power (Fig. 1A) nor at high power (Fig. 1B). The human transferrin spectrum at low power has a weak but visible shoulder down-field the narrow $\underline{g}' = 4$ lines and also two low field lines with $\underline{g}' = 9.7$ and $\underline{g}' = 9.2$ essentially like the ovotransferrin spectrum. These features are much more clearly seen in Fig. 1B due to the different saturation behavior of the various components. Accordingly, the two iron binding sites in human plasma transferrin are not identical with respect to their EPR spectra. In the hagfish transferrin, however, the sites seem to be identical or the difference is too small to be manifested in the EPR spectra. Of course, one cannot with certainty exclude the possibility that the difference between the sites is instead very large in hagfish transferrin. The EPR spectra of this transferrin in Fig. 1 could then be due to only one iron site, while the other site gives a spectrum which is very broad and too weak to be seen, e.g. like the spectrum of a ternary complex between iron, oxalate and human transferrin (11).

One should observe the marked difference in the intensity of the strongest peak at $\underline{g}' \sim 9$. This line increases in the order hagfish, human, and ovotransferrin compared to the corresponding intensity of the $\underline{g}' \sim 4$ lines. As the narrow lines at $\underline{g}' \sim 4$ in all these transferrins can be interpreted within a small range of $\underline{\lambda}$ (0.31-0.32) there should not

be such a large difference in intensity of the $g' \sim 9$ lines if only one species was present. Thus, not only the positions of the lines at low fields but also an intensity comparison speaks for the presence of two different iron species in human transferrin and ovotransferrin.

Due to lack of any detailed structure it is rather difficult to give any accurate spin-Hamiltonian parameters for the broad type of spectrum in human transferrin and ovotransferrin. Also, the positions of the lines near $g' = 4$ and $g' = 9$ are rather insensitive to the value of D (8). Those values of D and λ which can be given with reasonable certainty are collected in Table 1. This table also summarizes the conclusions drawn in this paper, i.e. the two iron binding sites in native ovotransferrin and human plasma transferrin are not identical and give distinctly different EPR spectra.

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