Beggs, J. D. (1978) *Nature 275*, 104-109.
Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., & Seed, B. (1989) *Science 243*, 1160-1165.
Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521-528.
Campbell, R. D., Law, S. K. A., Reid, K. B. M., & Sim, R. B. (1988) *Annu. Rev. Immunol. 6*, 161-195.
Dahlback, B., Smith, C. A., & Muller-Eberhard, H. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3461-3465.
Davis, D. G., & Bax, A. (1985) *J. Am. Chem. Soc. 107*, 2820-2821.
Day, A. J. (1990) in *Biochemistry and Molecular Biology of Complement* (Sim, R. B., Ed.) Kluwer Academic Publishers, Lancaster, U.K. (in press).
Day, A. J., Ripoche, J., Willis, A. C., & Sim, R. B. (1987) *Complement 4*, 147-148.
Day, A. J., Campbell, R. D., & Reid, K. B. M. (1989) in *Progress in Immunology* (Melchers, F., et al., Eds.) Vol. VII, pp 209-212, Springer-Verlag, Heidelberg.
Doolittle, R. F. (1985) *Trends Biochem. Sci. 114*, 233-237.
Driscoll, P. C., Clore, G. M., Beress, L., & Gronenburg, A. M. (1989) *Biochemistry 28*, 2178-2187.
Erdei, A., & Sim, R. B. (1987) *Biochem. J. 246*, 149-156.
Furie, B., & Furie, B. C. (1988) *Cell 53*, 505-518.
Hinnen, A., Hicks, J. B., & Fink, G. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1929-1933.
Janatova, J., Reid, K. B. M., & Willis, A. C. (1989) *Biochemistry 28*, 4754-4761.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Phys. Chem. 71*, 4546-4553.
Kingsman, A. J., & Kingsman, S. M. (1985) *Biotech. Genet. Eng. Rev. 3*, 377-416.
Klickstein, L. B., Bartow, T., Miletic, V., Rabson, L. D., Smith, J. A., & Fearon, D. T. (1988) *J. Exp. Med. 168*, 1699-1717.
Kumar, A., Ernst, R. R., & Wuthrich, K. (1981) *Biochem. Biophys. Res. Commun. 95*, 1-6.
Kurjan, J., & Herskowitz, I. (1982) *Cell 30*, 933-943.
Marion, D., & Bax, A. (1988) *J. Magn. Reson. 80*, 528-533.
Medved, L. V., Busby, T. F., & Ingham, K. C. (1989) *Biochemistry 28*, 5408-5414.
Mellor, J., Dobson, M. J., Roberts, N. A., Tuite, M. F., Emtage, J. S., White, S., Lowe, P. A., Patel, T., Kingsman, A. J., & Kingsman, S. M. (1983) *Gene 24*, 1-14.
Moore, M. D., DiScipio, R. G., Cooper, N. R., & Nemerow, G. R. (1989) *J. Biol. Chem. 264*, 20576-20582.
Pangburn, M. K., & Meri, S. (1989) *Complement Inflamm. 6*, 383.
Patthy, L. (1985) *Cell 41*, 657-663.
Perkins, S. J., Chung, L. P., & Reid, K. B. M. (1986) *Biochem. J. 233*, 799-807.
Perkins, S. J., Haris, P. I., Sim, R. B., & Chapman, D. (1988) *Biochemistry 27*, 4004-4012.
Piantini, U., Sorensen, O. W., & Ernst, R. R. (1988) *J. Am. Chem. Soc. 104*, 6800-6801.
Plateau, P., & Gueron, M. (1982) *J. Am. Chem. Soc. 104*, 7310-7311.
Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479-485.
Reid, K. B. M., & Day, A. J. (1989) *Immunol. Today 10*, 177-180.
Ripoche, J., Day, A. J., Harris, T. J. R., & Sim, R. B. (1988) *Biochem. J. 249*, 593-602.
Sim, R. B., & Perkins, S. J. (1989) *Curr. Top. Microbiol. Immunol. 153*, 99-122.
Springer, T. A. (1990) *Nature 346*, 425-434.
Tedder, T. F., Isaacs, C. M., Ernst, T. J., Demetri, G. D., Adler, D. A., & Disteche, C. M. (1989) *J. Exp. Med. 170*, 123-133.
Wharton, K. A., Johansen, K. M., Xu, T., & Artavansis-Tsakonas, S. (1985) *Cell 43*, 567-581.
Williams, A. F., & Barclay, A. N. (1988) *Annu. Rev. Immunol. 6*, 381-405.
Wüthrich, K., Wider, G., Wagner, G., & Braun, W. (1982) *J. Mol. Biol. 155*, 311-319.

Mössbauer Spectroscopy of Iron–Ovotransferrin: A Crystal Field Interpretation†

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ABSTRACT: Mössbauer spectra from frozen solutions of ovotransferrin were recorded in a variety of applied external magnetic fields and at various temperatures in a small applied field. The results were fitted to a simple model for the electronic structure at the iron site. This model requires admixtures of the free ion ^6S and ^4P states, indicating a weak cubic crystal field. Possible implications of this model regarding the binding site are discussed.

The transferrins, a series of ca. 80-kDa glycoproteins found in the blood plasma and secretions of all vertebrates, serve a primary physiological role of sequestering and detoxifying ferric iron and, in the case of the plasma type, transporting iron to those tissues and cells of the body requiring iron (Aisen & Listowsky, 1980; Chasteen, 1983; Ponka et al., 1990). The X-ray crystal structures of two of these proteins reveal that each comprises two conformationally similar lobes connected by a short peptide sequence, each lobe being made up of two domains which define a deep cleft (Anderson et al., 1987, 1989; Baker et al., 1987; Bailey et al., 1988). Each cleft contains the amino acid side chains required to sequester one ferric ion and one synergistic anion, normally carbonate, essential for

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the binding of the iron. A wide variety of tri- and divalent metal ions can be accommodated by these binding sites (Tan & Woodworth, 1969; Pecoraro et al., 1981), and many small organic carboxylic acids with a second electron-donor group can substitute for carbonate in the ternary complex (Schlabach & Bates, 1975; Woodworth et al., 1975). Recent X-ray data from human apo-lactoferrin reveal that in the crystal the C-terminal site remains closed, as in the ternary complex, but that the N-terminal site is "open", the two domains having moved apart by some 15° relative to those in the ternary complex (Norris et al., 1989).

The nature of the two binding sites and their possible inequivalence have been the subject of several investigations since Fletcher and Huehns (1967) advanced their hypothesis that a difference in the sites possibly has physiological significance. Because they are direct probes of the iron's electronic and nuclear structure, electron paramagnetic resonance (EPR)[1] and Mössbauer spectroscopy have been applied to the study of site equivalence in the transferrins (Spartalian & Oosterhuis, 1973; Tsang et al., 1973, 1976; Kretchmar et al., 1988; Rottman et al., 1989). There is agreement among these studies that, at least as far as the electronic structure of the iron is concerned, the two sites are equivalent in both human serum transferrin and ovotransferrin. Of primary interest in the present work is not the site equivalence but the interpretation and significance of the EPR and Mössbauer results.

There is an abundance of interpretations of the EPR and Mössbauer results from human serum transferrin in terms of a spin Hamiltonian (Spartalian & Oosterhuis, 1973; Tsang et al., 1973, 1976; Kretchmar et al., 1988; Yang & Gaffney, 1987; Rottman et al., 1989). The authors generally agree that the spin Hamiltonian parameters fall in the range D = 0.15–0.3 cm^{-1} with asymmetry parameter λ near the "fully rhombic" value of 1/3. The interpretation of the EPR results, however, has resulted in consistently higher values for D than those obtained from Mössbauer spectroscopy. The early study of ovotransferrin by Aisen et al. (1973) did not present quantitative interpretations of the EPR and Mössbauer results. In this work we interpret Mössbauer spectra recorded in a variety of applied magnetic field values in terms of a crystal field model and discuss the implications of this model on the binding of iron by the transferrins.

Materials and Methods

Ovotransferrin was isolated and purified from hen's egg white as previously described (Williams & Woodworth, 1973) and saturated with ^{57}Fe in the presence of 20 mM $NaHCO_3$ as previously described (Aisen et al., 1973). Approximately 20 mM solutions of $^{57}Fe(III)$ were made by dissolving the requisite amount of $^{57}Fe_2O_3$ (AERE Harwell, U.K., or Oak Ridge National Laboratories) in a minimal volume of 6 N HCl and diluting this solution to the calculated volume. Actual concentrations of the stock ^{57}Fe were assessed by spectrophotometric titration of a known amount of ovotransferrin in 20 mM $NaHCO_3$ or by photometric evaluation of the color produced by reaction with sulfonated bathophenanthrolein with reference to a known iron standard (Schade, 1961). The iron was reduced to the ferrous form with $NaHSO_3$ in the former case and with ascorbate in the latter case. Similar preparations were made from $^{56}Fe_2O_3$ when a control giving no Mössbauer effect was required. N- and C-terminal half-molecules (OTF/2N, OTF/2C) were made from ^{57}Fe- or ^{56}Fe-saturated ovotransferrin as required. The Fe_2OTF preparations were purified by electrofocusing in a glass column (LKB, Bromma, Sweden) containing 0.4% Servalyt, pH 4–7 (Serva, Westbury, NY), in a 0–50% sucrose gradient. Each purified Fe_2OTF was cleaved into its half-molecules with TPCK-treated trypsin (Sigma, St. Louis, MO) in a mass ratio of OTF to trypsin of 30/1. The preparation was maintained at 37 °C for 2.5 h. The digest was then loaded onto an electrofocusing column as above and focused for 2–3 days to achieve resolution of the FeOTF/2N and FeOTF/2C. Protein preparations were concentrated and washed on Amicon PM-10 ultrafilters and Centricon 10s (Amicon, Danvers, MA). Preparations in water were finally lyophilized and dissolved in sufficient MilliQ-purified water to give the high concentrations required for the Mössbauer measurements.

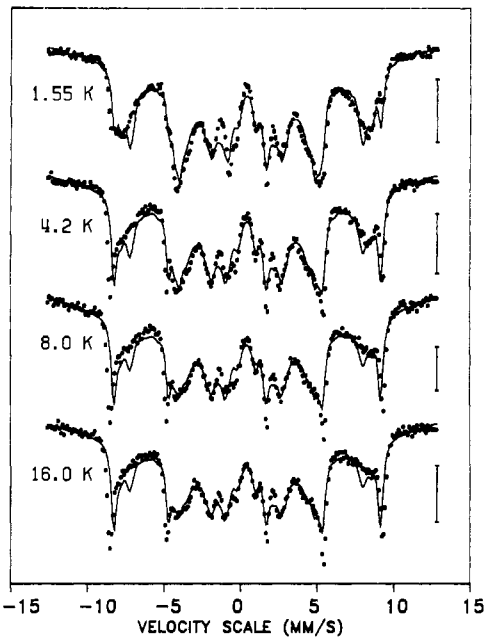

FIGURE 1: Mössbauer spectra of ovotransferrin in 55 mT at temperatures as indicated. Solid lines display simulated spectra based on the electronic model discussed in the text. Vertical bars denote 1% absorption.

Mössbauer spectra were recorded on a constant-acceleration spectrometer in horizontal transmission geometry, with a room-temperature ^{57}Co source diffused into a thin rhodium foil (Radiochemical Centre, Amersham). Calibrations were made with a thin room-temperature iron foil, and isomer shifts are relative to the center of its spectrum. The low applied field (55 mT) was obtained with a permanent magnet; higher fields were produced by a split-pair superconducting magnet, with the field perpendicular to the γ-beam direction. The 1.55 K and 4.2 K temperatures were determined from the vapor pressure of a helium bath in which the sample was immersed. Higher temperatures were obtained by placing the sample in contact with an electrically heated temperature-regulated copper plate within a counter-Dewar immersed in the helium bath. Computer simulations and least-squares fits were performed on a micro-VAX II with a descendant of the FORTRAN program described by Lang and Dale (1974).

Results and Analysis

Mössbauer spectra from frozen samples of ovotransferrin are shown in Figures 1 and 2. The spectra were recorded in a variety of applied magnetic fields as indicated. All spectra exhibit well-resolved paramagnetic hyperfine structure typical of the high-spin Fe(III) oxidation state in the slow paramagnetic relaxation regime. Comparison between the spectra

[1] Abbreviations: EPR, electron paramagnetic resonance; OTF, ovotransferrin; OTF/2N and OTF/2C, half-molecules of ovotransferrin from the N- and C-terminal lobes; zfs, zero-field splittings.

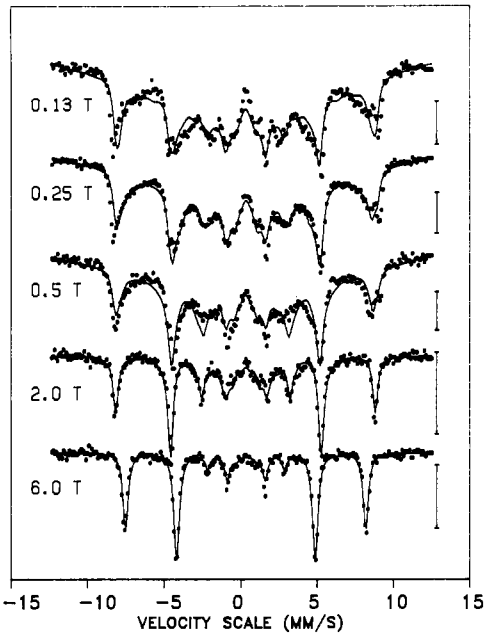

FIGURE 2: Mössbauer spectra of ovotransferrin at 4.2 K in applied fields as indicated. Solid lines display simulated spectra based on the electronic model discussed in the text. Vertical bars denote 1% absorption.

in a small applied field at 4.2 and 1.55 K shows that the relatively narrow, outermost peaks are associated with an excited state that originates in the top doublet. Thus, although the area under the ground-state subspectrum is greater, the excited-state subspectrum with its narrower lines significantly affects the profile of the composite spectrum. This means that all six contributions to the spectrum need to be determined with a good deal of accuracy for a successful simulation. Because of the unusually small zero-field splittings in ovotransferrin, our data are model-sensitive enough to reject the usual spin Hamiltonian analysis and require the use of a more comprehensive model. The data were fitted by computer, with the method of least squares, to model spectra that were generated with the crystal field model presented below.

The crystal field model that we used to obtain the electronic structure of ovotransferrin allows a ground sextet to interact with excited quartet states via spin–orbit coupling but is otherwise a departure from the usual description in which the electrons in a many-electron ion are assumed to occupy one-electron states described by e_g and t_{2g} orbitals. This latter approach has been successful with other "$g = 4.3$" high-spin ferric complexes but could not account for the small zero-field splittings (zfs) exhibited by the transferrins[2] (Spartalian & Carrano, 1983).

Our starting point is the recognition that the relatively small zero-field splittings indeed indicate a weak crystal field. Instead, however, of modeling the zero-field splittings by requiring quartet states at unreasonably high energies, we resolve the difficulty by selecting electronic basis states that imply no crystal field, namely, the free ion states. These are the states for which L and S are good quantum numbers, $| L\ M_L\ S\ M_S >$. Clearly, the choice of basis states is immaterial to the solution of the problem if a complete set, 252 states in d^5, is considered. The necessity, however, to cast the problem in a mathematically tractable form demands the selection of a small subset of the complete set. The smallest and most obvious subset of basis states consists of the ground sextet (^{6}S), which we know is present, and the excited spin-quartet orbital-triplet (^{4}P), which is the only multiplet for which the spin–orbit coupling matrix has off-diagonal elements with the ground sextet. Electron–electron repulsion results in a separation between ^{6}S and ^{4}P of $E_0 = 7B$, where B is the standard Racah parameter (Griffith, 1961). In zero applied field, spin–orbit coupling splits the quartet into three multiplets with $J = 1/2,3/2,5/2$ but admixes ^{4}P character to the ground sextet in such a way as merely to shift but not split the sextet energy levels. A weak crystal field of strength Δ will by itself perturb the quartet but not the spherically symmetric sextet. However, the *combined* effect of crystal field and spin–orbit coupling admixes ^{4}P character to the sextet in a way that splits it into three doublets, the overall separation of which is roughly $\zeta^2\Delta/E_0^2$. This last result enables us to generate arbitrarily small zero-field splittings by adjusting the strength of the crystal field.

We write the electronic part of our Hamiltonian as an 18 × 18 matrix in the form

$$H = H_{e\text{-}e} + \zeta\sum_i \vec{l}_i\cdot\vec{s}_i + H_{CF} + \beta\sum_i \vec{H}_{app}\cdot(\vec{l}_i + 2\vec{s}_i) \quad (1)$$

The first term represents electron–electron repulsion and is diagonal with zero value for the 6 ground-state elements and E_0 for the 12 excited-state elements, the second term is the spin–orbit interaction, the third term is the crystal field Hamiltonian, and the last term is the Zeeman interaction with the applied field H_{app} (β is the Bohr magneton). The summation extends over the five d electrons.

The crystal field Hamiltonian is basically an electrostatic potential term that can be represented as a sum of even-order spherical harmonics:

$$H_{CF} = AY_{2,0} + B(Y_{2,2} + Y_{2,\bar{2}}) \quad (2)$$

Matrix elements of higher order spherical harmonics vanish for the ^{4}P basis states. We can exploit operator equivalence and rewrite the crystal field Hamiltonian more suggestively as

$$H_{CF} = \sum\left\{V\left[l_z^2 - \frac{1}{3}l(l+1)\right] + \Delta(l_x^2 - l_y^2)\right\} \quad (3)$$

where the summation, with indices omitted for clarity, extends over the five d electrons.

As is customary with this type of calculation, the electronic problem was solved first by diagonalizing the 18 × 18 Hamiltonian in eq 1 to obtain the eigenvalues and eigenfunctions. These were used to obtain expectation values of the components of the paramagnetic hyperfine field (often called the internal field) for a given direction of the applied magnetic field. We formally wrote the internal field as the sum of the orbital, dipolar, and Fermi-contact contributions even though the first two are expected to be vanishingly small for a high-spin ferric ion:

$$\vec{H}_{int} = \vec{H}_{orb} + \vec{H}_{dip} + \vec{H}_{cont} \quad (4)$$

where

$$\vec{H}_{orb} = H_0\sum_i \vec{l}_i$$

$$\vec{H}_{dip} = H_0\sum_i\left[\frac{3(\vec{r}_i\cdot\vec{s}_i)\vec{r}_i}{r_i^2} - \vec{s}_i\right] \qquad \vec{H}_{cont} = -H_0\sum_i \kappa\vec{s}_i$$

[2] We can estimate the sextet–quartet separation E_0 by noting that, in the e_g–t_{2g} description, the zero-field splittings are of the order ζ^2/E_0, where ζ is the spin–orbit coupling constant. Considering that the overall zero-field splittings in the transferrins is about 1 cm^{-1} or less (as evidenced by the reported spin Hamiltonian parameters), we see that E_0 must be of the order of 100 000 cm^{-1}. Computer calculations support this rough estimate.

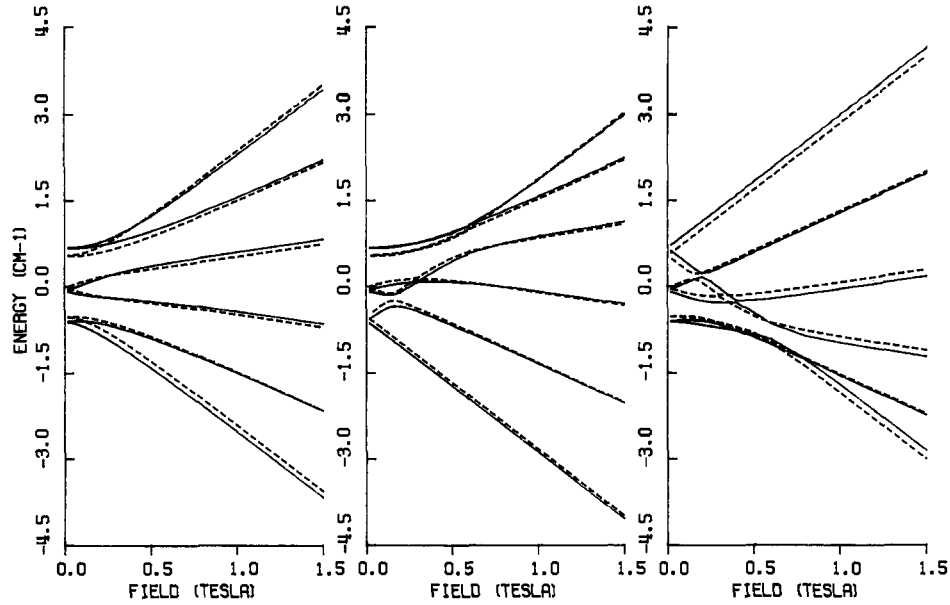

FIGURE 3: Splitting of the ground sextet in an external field applied along the principal axes, x (left), y (middle), and z (right). Solid lines represent the predictions of the crystal field model and dashed lines the spin Hamiltonian predictions.

Here, H_0 measures the strength of the magnetic hyperfine interaction and is equal to $2\beta\langle r^{-3}\rangle_{\text{eff}}$; κ measures the strength of the Fermi-contact term.

The nuclear problem was then solved by assuming that each member of the sextet generates an effective field $\vec{H}_{\text{eff}} = \vec{H}_{\text{app}} + \vec{H}_{\text{int}}$ at the nucleus. The nuclear Hamiltonian was written as

$$H_n = -g_n\beta_n\vec{H}_{\text{eff}}\cdot\vec{I} + \frac{QV_{zz}}{4}\left[I_z^2 - \frac{1}{3}I(I+1) + \frac{\eta}{3}(I_x^2 - I_y^2)\right] \quad (5)$$

in which the first term is the Zeeman interaction with the effective field and the second term is the quadrupole interaction. For each orientation of the applied field, six subspectra were calculated (one associated with each member of the sextet), and in the slow relaxation regime, these were weighted by the appropriate Boltzmann factor and added to yield the final calculated spectrum.

In our least-squares fitting procedure, the only adjustable parameters in the calculation, apart from the isomer shift and quadrupole splitting, were the crystal field parameters Δ and V. The Racah parameter B was chosen at the typical value of 7000 cm^{-1} (Griffith, 1961), and the spin–orbit coupling constant was set at $\zeta = 400$ cm^{-1}. Parameters H_0 and κ have well-established values of −61.5 T and 0.355, respectively (Lang, 1970). In order to simulate the random orientation of the external field with respect to the crystal axes in our frozen solution samples, the spectra were generated with 100 different orientations of the external field, distributed over an octant of a sphere in equal solid-angle increments. A consistent set of parameters Δ and V was found by fitting four selected spectra (1.55 K – 50 mT, 4.2 K – 50 mT, 4.2 K – 0.25 T, 4.2 K – 6 T) *simultaneously* and then simply generating spectra for the remaining combinations of temperature and applied field with all parameters fixed. Table I shows the set of parameters that provided the best fits to the spectra. Note that the crystal field parameters Δ and V are given in units of the spin–orbit coupling constant ζ, which is a measure of the overall splitting of the energy levels.

Mössbauer spectra of $^{57}Fe_2$OTF, ^{57}FeOTF/2C, and an equimolar mixture of ^{57}FeOTF/2C with ^{54}FeOTF/2N were indistinguishable, suggesting no discernible differences between the irons in the two sites in either the intact protein or its half-molecules.

Table I: Mössbauer and Electronic Parameters for Ovotransferrin

isomer shift δ (mm/s) with respect to metallic Fe	0.405
quadrupole splitting ΔE (mm/s)	0.1
asymmetry parameter η	0
hyperfine coupling constants	
H_0 (T)	61.5
κ	0.355
spin-orbit coupling constant ζ (cm^{-1})	400
crystal field parameters	
Δ/ζ	7.82
V/ζ	1.68

Discussion

As is seen from Figures 1 and 2, the calculation based on our model provides good agreement with experiment considering that this is essentially a two-parameter fit of nine spectra. In view of the wide use of the spin Hamiltonian in the interpretation of the Mössbauer and EPR spectra of human serum transferrin and of the $g = 4.3$ complexes in general, we believe that it is important first to examine how and why the spin Hamiltonian fails as a theoretical basis for the interpretation of the ovotransferrin Mössbauer spectra. We will do this by comparing the predictions of our crystal field model for ovotransferrin with the corresponding predictions of a quadratic spin Hamiltonian with $\lambda = 0.31$ and $D = 0.153$ cm^{-1}. This particular choice of λ was made in order to match human serum transferrin while D was scaled to match the zfs predicted by our model.

The splitting of the sextet state as a function of an external magnetic field applied along the three principal axes is shown in Figure 3. Solid lines are predictions based on our crystal field model for ovotransferrin and dashed lines the spin Hamiltonian predictions. Figure 4 shows the spin expectation values associated with each member of the ground sextet. These were computed by using the eigenfunctions corresponding to the eigenvalues displayed in Figure 3. Solid lines represent the predictions of the crystal field model and dashed lines the spin Hamiltonian predictions. The members of the sextet are numbered 1–6 in ascending order of energy. For clarity, we have limited the abscissa values to the low-field region ($H_{\text{app}} < 0.3$ T) in which the magnetic energy is less than

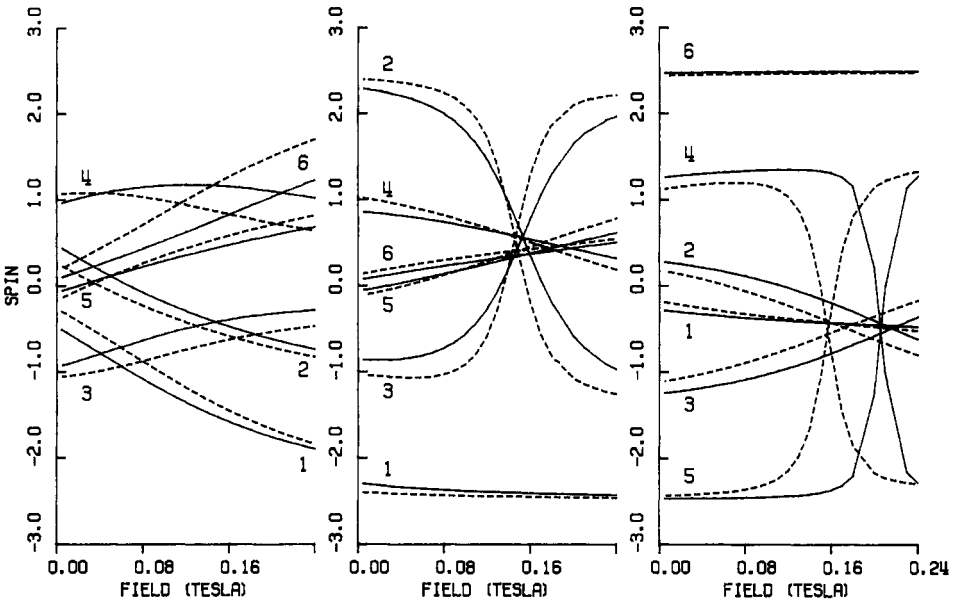

FIGURE 4: Spin expectation values in an external field applied along the principal axes, x (left), y (middle), and z (right). Only the low-field region is shown for clarity of presentation. The members of the sextet are labeled 1–6 in ascending order of energy. Solid lines represent the predictions of the crystal field model and dashed lines the spin Hamiltonian predictions.

or comparable to the zfs. This is the region where the largest discrepancy between the two theories occurs and also the region of interest for X-band EPR. Note that in Figure 4 labels "3" and "4" denote the states that are associated with the $g = 4.3$ EPR resonance.

It is immediately obvious from Figures 3 and 4 that the two theoretical approaches agree on the energies but not on the spin expectation values. This can be understood by noting that, as a second-order perturbation treatment (Griffith 1961), the spin Hamiltonian is expected to match the "exact" energies to second order but the "exact" eigenfunctions only to first order. Expectation values are computed from eigenfunctions; therefore, the difference between spin Hamiltonian and crystal field model predictions will be more conspicuous when spin expectation values are compared than when energy splittings in an applied field are compared.

For Mössbauer spectroscopy this means that the Boltzmann populations, and therefore the fractional contribution of each subspectrum to the total, are not critically sensitive to the choice of model. What is indeed sensitive to the choice of model is the position of each peak that depends on the magnetic hyperfine field, which itself is proportional to the spin expectation value for contact-dominated $S = {}^5/_2$ Fe(III). A change in spin expectation value by as little an amount as 0.2 translates to a shift in peak position that is a large as 1.4 mm/s. Thus, the spectral profile will, in principle, depend on the choice of model. In practice, however, discrepancies between the predictions of a crystal field model and a spin Hamiltonian will be experimentally noticeable when the magnetic energy βH_{app} is comparable to the zfs and all six members of the sextet are appreciably populated at a given temperature. These two criteria appear to be fulfilled in the case of ovotransferrin and human serum transferrin. In cases where the zfs is larger, such as Fe(EHPG), there are no discrepancies and the spin Hamiltonian predictions closely match those of the crystal field model (Spartalian & Carrano, 1983).

It may be possible to minimize the discrepancy between the crystal field model and the spin Hamiltonian by adjusting the latter's parameters; however, parameter λ by itself is not sufficient to provide the needed eigenfunctions. Thus, if we were to analyze our results on the basis of a spin Hamiltonian, we would have to augment the number of adjustable parameters by invoking anisotropic A values, perhaps quartic terms (a and F), and even anisotropic g values in the Hamiltonian itself. Even if this proliferation of spin Hamiltonian parameters succeeded in putting the solid line through the data points, it would certainly not be simpler than our ^6S–^4P model, nor would it provide the extra physical insight that, we believe, our model provides. It appears, in this case at least, that the "exact" situation is simpler than the approximation.

At this point it is appropriate to discuss our model vis-á-vis the EPR experiments on the transferrins. Nearly all transferrins exhibit the same characteristic EPR profile, yet attempts to simulate this EPR signal with a spin Hamiltonian have met with limited success. Scullane et al. (1982) could not reproduce the EPR profile. Yang and Gaffney (1987) came close to a simulation at the price of invoking a composite signal from three components, each having its own spin Hamiltonian D value and a distribution of λ values.

Unlike the position of the resonance lines in the Mössbauer spectra, the position of the EPR resonances depends entirely on the energy splitting of the sextet for a given value and orientation of the applied magnetic field with respect to the crystal axes. Resonances occur at field values for which the quantum of microwave energy matches the energy separation between members of the sextet. Therefore, as Figure 3 shows, a spin Hamiltonian and a crystal field model ought to account for the positions of the EPR resonances equally well. However, the two theories are expected to diverge more significantly in their predictions of that which depends directly on the eigenfunctions, the EPR transition probabilities. Our finding that a two- or three-parameter spin Hamiltonian is inadequate to provide the correct hyperfine fields implies that a spin Hamiltonian is inappropriate as a model for simulations of EPR spectra from ovotransferrin and, by extension, from the rest of the transferrins. Simulations of EPR spectra using as basis states the set in which our electronic Hamiltonian is diagonal may elucidate this point.

A study of the zfs exhibited by the so–called $g = 4.3$ complexes shows that the transferrins form a group of their own within these complexes. The members of this group have relatively small zfs, between 1 and 2 cm^{-1}. Relatively small zfs mean relatively weak crystal fields. If one wishes to provide a quantum mechanical description of a crystal field as weak

as the one in the transferrins, one can in principle formulate the problem using a complete set of basis states and adjust the appropriate crystal field parameters to small values, but this is computationally intractable. We chose the reasonable alternative of using an incomplete set of *free ion* basis states. This last result is not a claim that the iron bound by ovotransferrin is a free ion; it is a mathematical description of a weak crystal field, an acknowledgment that the electronic structure of iron in ovotransferrin is closer to the spherical symmetry of a free ion than to the cubic symmetry of an ion in a formally octahedral complex. After all, we do have to invoke non-zero crystal field parameters in order to simulate our spectra, and furthermore, the characteristic optical absorption peak at 470 nm is attributed to a ligand to metal charge transfer.

Unlike the inorganic $g = 4.3$ complexes or even siderophores such as enterobactin in which the ligand wraps itself around the iron, it appears that the bound iron in the transferrins is presented with a rather rigid environment. In view of the ability of the transferrins to bind a wide diversity of tri- and divalent metal ions regardless of their preferred coordination geometries (Tan & Woodworth, 1969; Pecoraro et al., 1981) and various carboxylic synergistic anions (Schlabach & Bates, 1975; Woodworth et al., 1975), as well as the present interpretation of the Mössbauer data, we advance the notion that, rather than acting as a metal chelator, the transferrin active site acts as a sequestering agent for metals with the synergistic anion acting as a latch to keep the "sequestration box" closed. The X-ray crystallographic data on apo- and differic human lactoferrin support this notion with the two domains of the N-terminal lobe being open by 15° relative to the ternary complex (Norris et al., 1989). The X-ray data also reveal that the active site cleft is rather spacious and accommodates a few to several water molecules in addition to the ferric ion and synergistic anion in the ternary complex (Bailey et al., 1988; Evans et al., 1989). The synergistic anion carbonate appears to be doubly coordinated to the iron and to be bound to the protein electrostatically by ^{124}Arg and via hydrogen bonding to a few peptide nitrogens (Lindley et al., 1988; Evans et al., 1989).

In summary we propose that ovotransferrin (and the transferrins in general) ought to be regarded not as metal chelating agents but rather as metal sequestering agents. The mechanistic details of the sequestration reaction have yet to be worked out.

Registry No. Fe, 7439-89-6.

References

Aisen, P., & Listowsky, I. (1980) *Annu. Rev. Biochem. 49*, 357.

Aisen, P., Lang, G., & Woodworth, R. C. (1973) *J. Biol. Chem. 248*, 649.

Anderson, B. F., Baker, H. M., Dodson, E. J., Norris, G. E., Rumball, S. V., Waters, J. M., & Baker, E. N. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1769.

Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W., & Baker, E. N. (1989) *J. Mol. Biol. 209*, 711.

Bailey, S., Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnaint, S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R., & Watson, J. L. (1988) *Biochemistry 27*, 5804.

Baker, E. N., Rumball, S. V., & Anderson, B. F. (1987) *Trends Biochem. Sci.*, 350.

Chasteen, N. D. (1983) *Adv. Inorg. Biochem. 5*, 201.

Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnain, S., Jhoti, H., Lindley, P. F., Sarra, R., & Walton, A. (1989) *IXth International Conference on Proteins of Iron Transport & Storage Abstracts*, p 8 (Abstract).

Fletcher, J., & Huehns, E. R. (1967) *Nature 215*, 584.

Griffith, J. S. (1961) *Theory of Transition Metal Ions*, University Press, Cambridge.

Kretchmar, S. A., Texeira, M., Hyunh, B. H., & Raymond, K. N. (1988) *Biol. Met. 1*, 26.

Lang, G. (1970) *Q. Rev. Biophys. 3*, 1.

Lang, G., & Dale B. (1974) *Nucl. Instrum. Methods 116*, 567.

Lindley, P. F., Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnain, S. S., Jhoti, H., Sarra, R., & Walton, A. R. (1988) *Biochem. Soc. Trans. 16*, 825.

Norris, G. E., Baker, H. M., & Baker, E. N. (1989) *J. Mol. Biol. 209*, 329.

Pecoraro, V. L., Harris, W. R., Carrano, C. J., & Raymond, K. N. (1981) *Biochemistry 20*, 7033.

Ponka, P., Schulman, H. M., Woodworth, R. C., & Richter, G. W. (1990) *Iron Transport and Storage*, CRC Press, Boca Raton, FL.

Rottman, G. A., Doi, K., Zak, O., Aasa, R., & Aisen, P. (1989) *J. Am. Chem. Soc. 111*, 8613.

Schade, A. L. (1961) *Behringwerk-Mitt. 39*, 3.

Schlabach, M. R., & Bates, G. W. (1975) *J. Biol. Chem. 250*, 2182.

Scullane, M. L., White, L. K., & Chasteen, N. D. (1982) *J. Magn. Reson. 47*, 383.

Spartalian, K., & Oosterhuis, W. T. (1973) *J. Chem. Phys. 59*, 617.

Spartalian, K., & Carrano, C. J. (1983) *J. Chem. Phys. 78*, 4811.

Tan, A. T., & Woodworth, R. C. (1969) *Biochemistry 8*, 3711.

Tsang, C. P., Boyle, A. J. F., & Morgan, E. H. (1973) *Biochim. Biophys. Acta 328*, 84.

Tsang, C. P., Bogner, L., & Boyle, A. J. F. (1976) *J. Chem. Phys. 65*, 4584.

Williams, S. C., & Woodworth, R. C. (1973) *J. Biol. Chem. 248*, 5848.

Woodworth, R. C., Virkaitis, L. M., Woodbury, R. G., & Fava, R. A. (1975) in *Proteins of Iron Storage and Transport in Biochemistry and Medicine* (Crichton, R. R., Ed.) p 39, North-Holland Publishing Co., Amsterdam.

Yang, A.-S., & Gaffney, B. J. (1987) *Biophys. J. 51*, 55.