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Mössbauer studies of electrophoretically purified monoferric and diferric human transferrin

Suzanne A. Kretchmar[1], Miguel Teixeira[2]*, Boi-Hanh Huynh[2], and Kenneth N. Raymond[1]

[1] Department of Chemistry, University of California, Berkeley, CA 94720, USA
[2] Physics Department, Emory University, Atlanta, GA 30322, USA

Summary. Electrophoretically purified ^{57}Fe-enriched monoferric and diferric human transferrins and selectively labeled complexes ([C-^{56}Fe,N-^{57}Fe]transferrin and [C-^{57}Fe,N-^{56}Fe]transferrin) were studied by Mössbauer spectroscopy. The data were recorded at 4.2 K over a wide range of applied magnetic fields (0.05-6 T) and were analyzed by a spin-Hamiltonian formalism. Characteristic hyperfine parameters were found and the obtained zero-field splitting parameters ($D = 0.25 \pm 0.05$ cm^{-1} and $E/D = 0.30 \pm 0.02$) agree with previous electron paramagnetic resonance (EPR) findings. The weak-field spectra of the [N-^{57}Fe]transferrin are slightly broader than those of the [C-^{57}Fe]transferrin, indicating that the N-terminal iron site may be more heterogeneous. However, the absorption line positions and the relative intensities of the subspectra originating from the three Kramers doublets of each Fe^{3+} site are identical. Thus the electronic structures of the two iron sites can be described by the same set of spin-Hamiltonian parameters, indicating that the ligand environments for the two sites are the same, as suggested by the recent X-ray crystallographic studies. This suggestion is further supported by the observation that the strong-field spectra of the two monoferric transferrins are indistinguishable. The selectively labeled mixed-isotope transferrins exhibit spectra that are identical to those of the corresponding monoferric ^{57}Fe-enriched transferrins, implying that the occupation of one iron site has little or no effect on the immediate envirnoment of the other site, a finding that is not surprising since the two sites are separated by approximately 4.2 nm.

* *Present address:* Centro de Quimica Estrutural, I.S.T., Universidade Nova de Lisboa, P-1000 Lisboa, Portugal

Offprint requests to: B. H. Huynh

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Introduction

As a class, the transferrins are mammalian glycoproteins which sequester and transport ferric ions. Human serum transferrin has been well characterized (Aisen and Listowsky 1980; Chasteen 1983). It has a relative molecular mass of approximately 80000 and is composed of a single polypeptide chain folded into two globular lobes, each of which binds one ferric ion. Although the amino-acid sequences of the two halves of the polypeptide chain exhibit nearly 40% homology, there are significant chemical differences (MacGillivray et al. 1982). The two metal-binding sites reversibly bind two Fe^{3+} ions along with two carbonate anions.

Although Fletcher and Huehns postulated 20 years ago that the two sites of transferrin behave differently (Fletcher and Huehns 1968), many of the studies which have been conducted to test the hypothesis, conflict. Some of the chemical differences between the two sites of serum transferrin are that the C-terminal site has three more disulfide bridges and is more acid-stable than the N-terminal site; irons is removed at different rates from the two sites by chelators; and high salt concentratitons can have opposite effects on the rates of iron release from the two sites (Baldwin 1980; Baldwin and de Sousa 1981; Chasteen 1983; Kretchmar and Raymond 1986). There has been disagreement as to whether the two iron coordination environments are identical, since the differences seen could be due to only protein folding.

In 1987, the X-ray crystallographic structures of lactoferrin and serum transferrin were resolved to 0.32 nm and 0.33 nm, respectively (Anderson et al. 1987; Baker et al. 1987; Bailey et al. 1987). The coordination environments of the iron atoms are identical and include two tyrosines, one histidine, one aspartate and a probable CO_3^{2-} (or HCO_3^-) and H_2O (or OH^-) molecule. The two iron atoms in the diferric protein are separated by about 4.2 nm.

In previous years, several electron paramagnetic resonance (EPR) studies (Aasa 1970 and 1972; Price and Gibson 1972; Cannon and Chasteen 1975; Chasteen et al. 1977; Folajtar and Chasteen 1982; O'Hara and Koenig 1986) have probed the iron sites, which could be distinguished. While EPR studies of vanadyl transferrin (Cannon and Chasteen 1975; Chasteen et al. 1977) further indicated the existence of two conformations for the metal-binding sites, the data could also be interpreted as due to a conformational difference among individual transferrin molecules. Previous Mössbauer studies of serum transferrin failed to detect any difference between the two iron sites (Tsang et al. 1973; Spartalian and Oosterhuis 1973; Oosterhuis and Spartalian 1976; Tsang et al. 1976). However, a Mössbauer study of a similar iron-transport protein, conalbumin, with selectively enriched ^{57}Fe sites indicated that the two sites were spectroscopically similar, but slightly different (Aisen et al. 1973a).

One intriguing problem to emerge from previous EPR and Mössbauer analyses of the Fe^{3+} electron structure is that the two spectroscopies yielded different spin-Hamiltonian parameters. In this paper, we report the first Mössbauer study of electrophoretically purified ^{57}Fe-enriched diferric and monoferric transferrins and of mixed isotope complexes: [C-^{56}Fe,N-^{57}Fe]- and [C-^{57}Fe,N-^{56}Fe]transferrin. The data have been analyzed in detail using a spin-Hamiltonian formalism. The parameters obtained are now in good agreement with the EPR results.

Materials and methods

Ferric chloride solutions. An unlabeled ferric chloride solution was prepared by dissolving $FeCl_3 \cdot 6H_2O$ in 12 M HCl, filtering the yellow solution through a 0.2-μm Gelman Acrodisc, and diluting with distilled deionized water to give a final concentration of 0.1 M at pH 1. A ^{57}Fe-enriched ferric chloride solution was prepared by heating the metal powder (95.45% enrichment, New England Nuclear) in 5 M HCl. A few drops of concentrated HNO_3 and HCl were added to speed up the process. The solution was filtered and diluted with water to give a final concentration of 7 mM at pH 1. The actual concentrations of the ferric chloride solutions were determined by titration with EDTA using Variamine Blue B indicator (Welcher 1958).

Transferrin preparations. Distilled deionized water was used at all times. Glassware was washed with phosphate-free RBS-pf (Pierce Chemical Company) and rinsed thoroughly with water. Dialysis tubing (Bethesda Research Laboratories) was boiled in water, placed in a solution containing 0.1% EDTA for short-time storage, and washed several times with water prior to use. Solutions were adjusted to pH 7.4 at 25° C. All ferric protein preparations were concentrated to 0.5–2.5 mM protein (1–5 mM total iron sites) with Centricon™ 30 microconcentrators, purified by electrophoresis (see below), transferred into 0.5-ml plastic Mössbauer sample holders, and frozen in liquid nitrogen.

Human serum apotransferrin (Calbiochem-Behring), C-terminal and N-terminal monoferric transferrins, and diferric transferrin were prepared as previously reported except ^{57}Fe-enriched ferric nitrilotriacetate, ^{57}Fe $(NTA)_2$, was used (Kretchmar and Raymond 1986 and 1988). Since some time-dependent hydrolysis of ferric nitrilotriacetate occurs, the Fe $(NTA)_2$ solutions were prepared just prior to use in the following manner: NTA (Eastman Chemical Company) was dissolved in water at pH 4.0, ferric chloride at pH 1.0 was added, and the pH was raised to ≈4.2 with dilute NaOH. The mixed ^{57}Fe-enriched diferric transferrins, [C-^{56}Fe,N-^{57}Fe]- and [C-^{57}Fe,N-^{56}Fe]transferrin, were prepared by adding enough unlabelled 2.00 mM Fe $(NTA)_2$ to solutions of ^{57}Fe-labelled C-terminal or N-terminal monoferric transferrins which had been purified by electrophoresis (see below), in a solution of 0.050 M Hepes buffer, 0.020 M $NAHCO_3$, and 0.10 M $NaClO_4$ to saturate 50% of the sites. After 2 h, the protein solutions were dialyzed at 4° C against several changes of 0.050 M Hepes buffer and 0.1 M $NaClO_4$, and finally against 0.050 M Hepes buffer.

Electrophoresis. Analytical urea/polyacrylamide gel electrophoresis (PAGE) was carried out as described by previous workers (Makey and Seal 1976; Chasteen and Williams 1981). The acrylamide, bisacrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylethylenediamine (TEMED), and Coomassie blue R250 were purchased from Bio-Rad. A vertical gel apparatus (Bethesda Research Laboratory) was used with 4-mm spacers. Preparative urea/PAGE (Kretchmar 1988) was performed with gels in which the proportions of the components were exactly as those for the analytical gels. However, the pH of the run buffer was adjusted to 8.4 at 4° C since the gels were run at 4° C to help dissipate the heat which arose from the high voltages applied. At 4° C, TEMED was added to degassed solutions of acrylamide, bisacrylamide, and ammonium persulfate, and the gels were poured into glass columns (14 × 2 cm) (Shuster 1971). About 1 ml of a very dilute sodium dodecyl sulfate solution (Matheson, Coleman, and Bell) was carefully layered over the gel to give a smooth gel surface. This was removed just prior to use by washing with distilled water. The electrophoresis apparatus (see Fig. 1) used included a 15 × 7 cm glass column (the upper reservoir) fitted with a clean rubber stopper, in which a hole was bored to fit the glass columns tightly, and a large evaporating dish (the lower reservoir). Platinum wire was twisted and attached with electrical tape to long strips of insulated copper wire connected to the positive and negative terminals of a power supply box (either Bio-Rad model 500/200 or model 3000/300). The samples (50–100 mg concentrated transferrin, 0.020 ml bromophenol blue, and two drops glycerol) were applied to the top of the

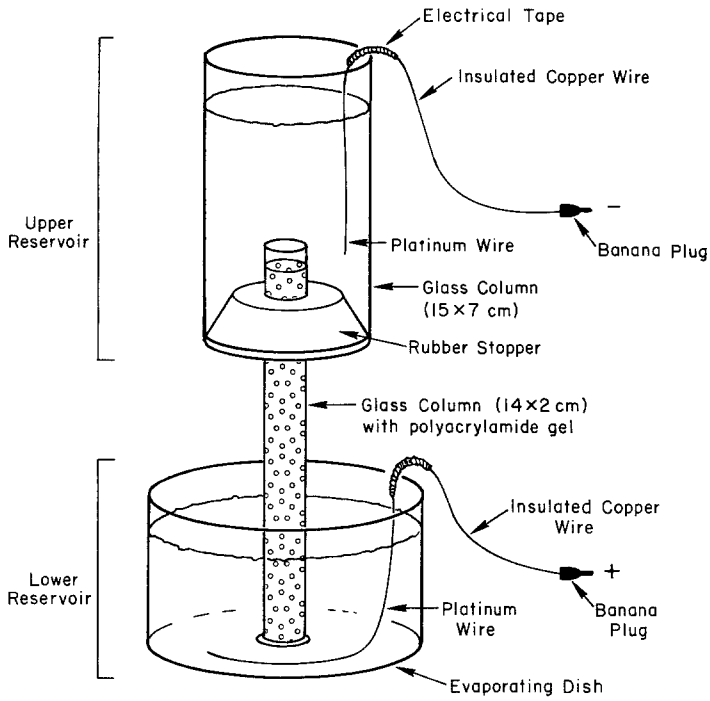

Fig. 1. Apparatus for preparative urea/polyacrylamide gel electrophoresis

gels. A constant voltage was applied to give currents of 40 mA. The gels were run for 15–20 h, and the voltages were periodically adjusted to keep currents of 40 mA. Upon separation of the desired protein bands, the bands were cut with a clean razor blade and the proteins extracted by individually grinding each slice in a 7-ml or 15-ml dounce homogenizer (Wheaton). Analytical urea/PAGE showed one band for each purified sample.

Mössbauer spectroscopy. Standard transmission measurements were made with a 50-mCi ^{57}Co(Rh) source driven by a Doppler velocity transducer operating at constant-acceleration mode. The weak-field Mössbauer spectrometer was equipped with a Ranger VT-700 velocity transducer and a top-loading Janis 8DT SuperVaritemp cryostat. The strong-field spectrometer was equipped with a top-loading Janis 12 CNDT/SC SuperVaritemp cryostat completed with an American Magnetics 8-T superconducting magnet and a home-built Doppler velocity transducer. Absorber temperatures (1.5–250 K) were controlled by a Lake Shore 520 cryogenic temperature controller. The velocity scale was calibrated using room-temperature Mössbauer spectra of a metallic iron foil. The zero velocity was referred to the centroid of these spectra.

Results and discussion

Mössbauer spectra of diferric and monoferric transferrins were recorded at 4.2 K over a wide range of applied magnetic fields. We begin by discussing the spectra of the monoferric [C-^{57}Fe]transferrin, since it contains only one occupied iron site and exhibits the sharpest spectra.

Analysis of the monoferric [C-^{57}Fe]transferrin spectra

Figure 2 shows the Mössbauer spectra of monoferric [C-^{57}Fe]transferrin recorded at 4.2 K with applied magnetic fields of 50 mT (A and B), 0.5 T (C), 2 T (D), and 6 T (E). For biological molecules, the spectra shown in Fig. 2 are relatively sharp and show well-resolved hyperfine absorption peaks. These spectra can be interpreted by the following spin-Hamiltonian:

$$\hat{H}=D\left[S_z^2-S(S+1)/3+\frac{E}{D}(S_x^2-S_y^2)\right]$$
$$+\beta\vec{S}\cdot\tilde{g}\cdot\vec{H}+\vec{S}\cdot\tilde{A}\cdot\vec{I}+\frac{eQV_{zz}}{4}$$
$$\left[I_z^2-I(I+1)/3+\frac{\eta}{3}(I_x^2-I_y^2)\right]-g_n\beta_n\vec{H}\cdot\vec{I}, \qquad (1)$$

where $\eta=(V_{xx}-V_{yy})/V_{zz}$.

For the high-spin Fe^{3+} ion in transferrin, S equals 5/2. In a weak magnetic field or in the absence of an applied field, the sextet spin multiplet of the ferric ion splits into three Kramers doublets with energy separations described by the first three terms of Eq. (1). Each doublet, in general, can yield a paramagnetic six-line spectrum. The spectra shown in Fig. 2A and B, therefore, are superpositions of three distinct subspectra weighted with appropriate Boltzmann populations. Stick diagrams indicating the absorption-line positions of the subspectra generated from the three Kramers doublets are shown on top of Fig. 2. Since the subspectra from the ground and the highest excited doublets exhibit comparable intensity, the zero-field splitting parameter D must be small in comparison with 4.2 K ($\approx 3\ cm^{-1}$), a general result that has been concluded by previous investigators. With all three doublets populated, the resulting spectra are inevitably complex. But, at the same time, they contain enough information for an accurate determination of the spin-Hamiltonian parameters.

From the EPR studies the iron sites in transferrin were found to have low symmetry ($E/D\approx 1/3$) (Aasa 1972). In this situation, the effective g values are very anisotropic for the ground and the highest Kramers doublets, ($g_x\approx 0.9$, $g_y\approx 9.7$, $g_z\approx 0.6$) and ($g_x\approx 0.9$, $g_y\approx 0.6$, $g_z\approx 9.7$), respectively. The Mössbauer spectra resulting from the lowest and highest doublets are therefore very sensitive to the hyperfine parameters along the y and z directions, respectively, allowing

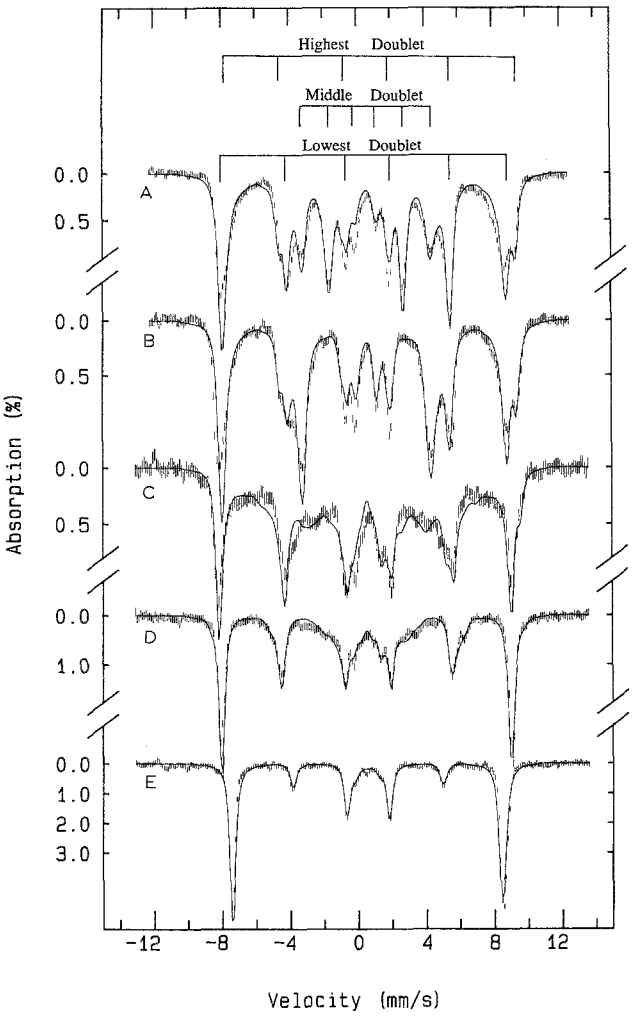

Fig. 2. Mössbauer spectra of monoferric [C-^{57}Fe]transferrin. The data were recorded at 4.2 K with a magnetic field of 50 mT applied perpendicular to the γ beam (*A*), and with magnetic fields of 50 mT (*B*), 0.5 T (*C*), 2 T (*D*), and 6 T (*E*) applied parallel to the γ beam. The stick diagrams shown on top of the figure indicate the line positions of the subspectral components originating from the three Kramers doublets at a magnetic field of 50 mT. The *solid lines* plotted over the experimental data are theoretical simulations using the parameters listed in Table 1

accurate determination of the magnetic hyperfine coupling tensor components, A_{yy} and A_{zz}, and the electric field gradient tensor components, V_{yy} and V_{zz}. The components of $\tilde{A}$ can be estimated from the total splitting of the subspectra, and the components of $\tilde{V}$ are evaluated from the line positions. The third component of the electric field gradient tensor V_{xx} can be calculated from the relation $V_{xx}+V_{yy}+V_{zz}=0$, and the remaining hyperfine component, A_{xx}, can then be estimated by fitting the spectra using the spin-Hamiltonian formalism given by Eq. (1).

In principle, the zero-field splitting parameter, D, can also be estimated from the weak-field spectra by the relative intensities of the three subspectra. However, due to the possible heterogeneous distribution of the crystal field, it is difficult to simulate the accurate shapes of the weak-field spectra (Huynh et al. 1978). Consequently, intensity estimation is not reliable. Instead, the parameter D is determined from the strong-field spectra. In strong applied fields, the three Kramers doublets are split into six singlet states, and at low temperature only the ground state is appreciably populated. In the situation where the Zeeman interaction is comparable to the zero-field splitting ($g\beta H \approx D$), an effect of the applied field is to mix the excited states into the ground state, resulting in an internal field at the iron nucleus that is strongly dependent on the ratio $g\beta H/D$ (Huynh et al. 1979). For the iron site in transferrin, this situation occurs at $H \approx 0.3$–1.0 T. Consequently, the parameter D can be estimated precisely from the 0.5-T spectrum (Fig. 1C).

With the understandings outlined above, a complete set of spin-Hamiltonian parameters for the C-terminal Fe site was determined through a series of theoretical simulations and visual comparisons of the simulations with experimental data. The parameters thus obtained are listed in Table 1; the theoretical spectra are plotted in Fig. 2. The agreement is excellent for the strong-field spectra and acceptable for the others. In comparison with the weak-field spectra, the theoretical simulations appear to be sharper at the outer regions and broader in the central region (Fig. 2A, B). These discrepancies are most likely caused by the intrinsic heterogeneity of the molecule, which was not taken into consideration in our simulations. The effect of this heterogeneity is to cause distribution of the fine and hyperfine tensors. This effect has been discussed in detail and successfully applied by Dwivedi et al. to simulate the

Table 1. Spin-Hamiltonian parameters for the ferric ion in transferrin

D (cm^{-1})	0.25 ± 0.05
E/D	0.30 ± 0.02
$A_{xx}/g_n\beta_n$ (T)[a]	-22.3 ± 0.5
$A_{yy}/g_n\beta_n$ (T)	-21.9 ± 0.2
$A_{zz}/g_n\beta_n$ (T)	-22.3 ± 0.2
ΔE_Q (mm/s)	0.30 ± 0.10
η	1.0 ± 0.5
δ (mm/s)	0.54 ± 0.02

[a] The symbol β_n represents the nuclear magneton, and g_n has the value 0.1806 and -0.1033 for the ground and the excited states of the ^{57}Fe nucleus

spectra of cytochrome c_{551} (Dwivedi et al. 1979). In a strong applied field, however, the effect of molecular heterogeneity diminishes. This is so because at a strong applied field, regardless of the distributions, the sextet is well separated and the ground state is always the $S_z = -5/2$ state which yields a well-defined spectrum. The fact that the strong-field spectra agree well with the simulations strongly suggests that the less-than-perfect agreement for the weak-field spectra is indeed caused by molecular heterogeneity.

The parameter set listed in Table 1 contains certain characteristics that are unique for transferrin. The values for the zero-field parameter D and the quadrupole splitting ΔE_Q, 0.25 cm^{-1} and 0.30 mm/s, respectively, are relatively small for high-spin ferric ions in biological molecules. The anisotropy found for the $\tilde{A}$ tensor is also small; the components are within 1% of their average value. The more commonly observed anisotropy for biological compounds is 10–15% (Huynh and Kent 1984). These characteristic values suggest that the ground electronic state of the ferric ions in transferrin must have a near isotropic charge distribution and zero angular momentum. In other words, the ground state is almost a pure ^6S state ($^6A_1$ in cubic symmetry). Mixing of other orbital excited states is minimal, indicating a strong ligand field for the iron site in transferrin.

Spin-Hamiltonian parameter sets for the iron site in transferrin have been deduced from previous Mössbauer measurements (Spartalian and Oosterhuis 1973; Tsang et al. 1976). However, there remained some unresolved problems. The zero-field parameters, D and E/D, obtained from the earlier Mössbauer studies did not agree with the EPR results, which yielded $D = 0.30$–0.32 cm^{-1} and $E/D = 0.31$ (Aasa 1972; Aisen et al. 1973b). From the zero-field and weak-field Mössbauer spectra, Spartalian and Oosterhuis have derived a value of 0.15 cm^{-1} for D (Spartalian and Oosterhuis 1973). Later, by analyzing the strong-field spectra, Tsang et al. redetermined D as 0.3 cm^{-1} (Tsang et al. 1976). In the analysis of Tsang et al., however, the magnetic hyperfine tensor $\tilde{A}$ was assumed to be isotropic, resulting in E/D value of 0.26 which disagreed with the EPR result. Inclusion of fourth-order terms in the spin-Hamiltonian was suggested as a possible explanation for the discrepancy. In the present analysis, we included both the weak-field and strong-field spectra, and did not restrict the tensor $\tilde{A}$ to being isotropic. The values found for the parameters D and E/D, 0.25 cm^{-1} and 0.30, respectively, are in

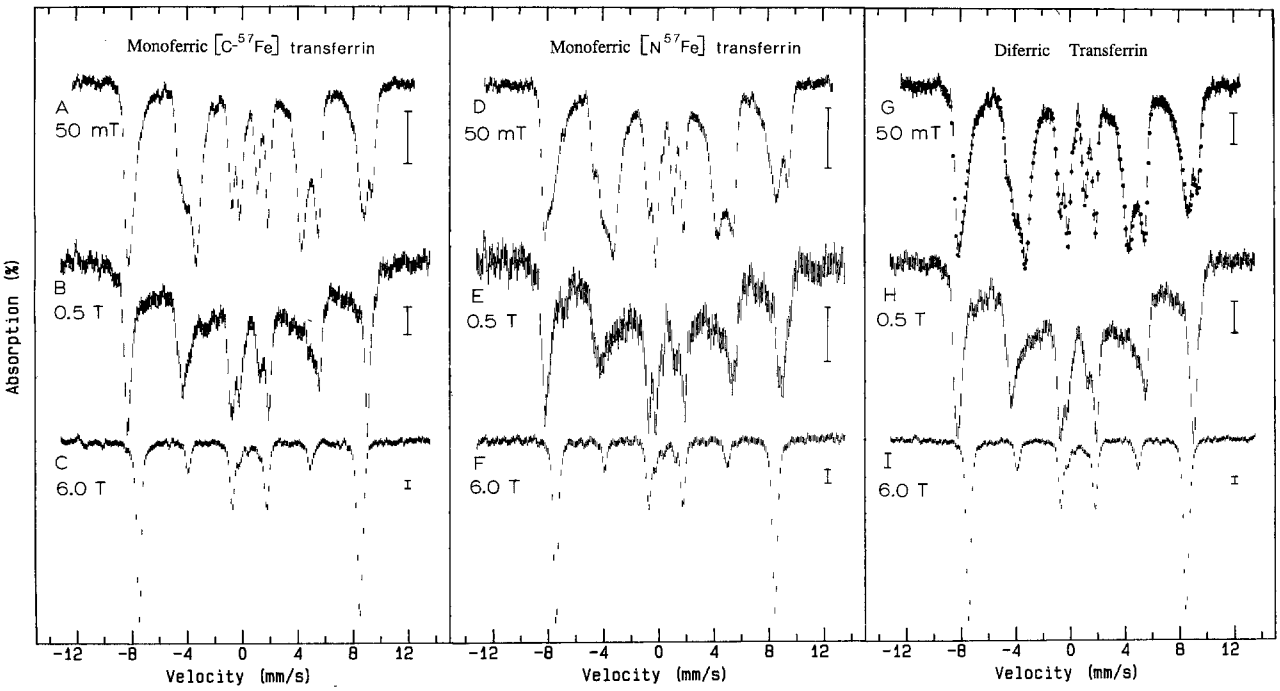

Fig. 3. Mössbauer spectra of the monoferric and diferric transferrins. The data were recorded at 4.2 K with the indicated magnetic fields applied parallel to the γ beam. For comparison, the sum of the two monoferric spectra recorded at 50 mT (*A, D*) is plotted as dots on top of the diferric spectrum (*G*, dashed lines). The *vertical bars* indicate absorption of 0.2%

good agreement with the EPR results. Inclusion of higher order terms appears to be unnecessary.

Comparison of the monoferric and diferric spectra

In Fig. 3, the Mössbauer spectra of the monoferric [C-^{57}Fe]- and [N-^{57}Fe]transferrins and diferric transferrin are compared. The data were recorded at 4.2 K with magnetic fields of 50 mT, 0.5 T, and 6 T applied parallel to the γ beam. Under the same experimental conditions, the spectra of the different transferrins are very similar. In strong applied fields (e.g., 0.5 T and 6 T), the spectra for the two monoferric and the diferric transferrins are identical. In a weak applied field, the spectrum of the [N-^{57}Fe]transferrin is broader than that of the [C-^{57}Fe]transferrin at the outer regions, indicating that the N-terminal iron site may be more heterogeneous. However, the absorption line positions and the relative intensities of the subspectra corresponding to the three Kramers doublets are identical for the two monoferric transferrins. This observation indicates that the electronic structures of the two iron sites can be described by the same set of spin-Hamiltonian parameters, suggesting that the ligands and their geometrical arrangements for the two iron sites are the same. This is supported by the recent X-ray crystallographic studies (Anderson et al. 1987; Baker et al. 1987; Bailey et al. 1987).

We have also recorded the spectra of diferric transferrins in which the iron sites were selectively labelled with ^{57}Fe ([C-^{56}Fe,N-^{57}Fe]transferrin and [C-^{57}Fe,N-^{56}Fe]transferrin); these exhibit spectra that are identical to those of the corresponding monoferric [^{57}Fe]transferrins, implying that the occpuation of one iron site has little or no effect on the immediate environment of the other site due to the 4.2-nm separation of the two iron centers. Such an implication can also be seen by comparing the spectrum of the ^{57}Fe-saturated diferric transferrin with the sum of the spectra of the two monoferric [^{57}Fe]transferrins. In Fig. 3 the additive spectrum (dots) is overlayed onto the diferric spectrum (dashed lines) and, within experimental error, the two spectra are indistinguishable.

In summary, electrophoretically purified ^{57}Fe-enriched diferric and monoferric transferrins and selectively labelled mixed-isotope transferrins have been prepared. Mösbauer studies of these samples showed slight differences between the two iron sites. The data suggest that these differences reflect the different degrees of inhomogeneities of the two iron sites, although the ligands and their geometrical arrangements are very similar. Detailed analysis of the spetra using a spin-Hamiltonian formalism has determined the characteristic parameters for the iron sites, which now agree with previous EPR findings.

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