Magnetization and Electron Paramagnetic Resonance Studies of Reduced Uteroferrin and Its “EPR-silent” Phosphate Complex*

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The exchange coupling of reduced uteroferrin has been measured (19.8(5) cm\(^{-1}\) \(S_1 \cdot S_2\)) using recently developed techniques for studying metalloprotein magnetization. A spin Hamiltonian describing the coupled binuclear Fe(II)--Fe(III) center has been used to fit the low and high field magnetization data, the EPR \(g\) values, and the highly anisotropic effective hyperfine tensor of the ferric site. The exchange coupling of the phosphate complex of reduced uteroferrin has also been measured (6.0(5) cm\(^{-1}\) \(S_1 \cdot S_2\)) using the same techniques. The smaller exchange coupling of the phosphate complex is comparable with the zero field splittings of the iron sites. This results in increased sensitivity of the system \(g\) values (found by calculation from the spin Hamiltonian) to variations of the zero field splitting parameters arising from heterogeneities in the protein microenvironment. Consequently, there is a very significant (9-fold) increase in the "effective \(g\) strain" of the system compared to the situation in the absence of phosphate. This, together with the larger \(g\) anisotropy (\(g = (1.06, 1.51, 2.27)\)), gives rise to an EPR signal for the phosphate complex of reduced uteroferrin which is extremely broad and difficult to detect but which has now been identified for the first time.

The best characterized of the purple acid phosphatases (1) are those from porcine uterus (also called uteroferrin) (2, 3) and bovine spleen (4, 5). These are glycoproteins of molecular weight 35,000 with significant primary sequence homology (6). Each enzyme contains an antiferromagnetically coupled binuclear iron site which can be obtained in two oxidation states. The oxidized form is purple (\(\lambda_m = 550\) nm), catalytically inactive, and EPR-silent (2-5); the metal site consists of the two high spin ferric centers (4, 7) coupled antiferromagnetically (\(H = JS_1 \cdot S_2\)) with lower limits on \(J_m\) ranging from 80 to 300 cm\(^{-1}\) (2, 4, 5, 8, 9). The reduced form is pink (\(\lambda_m = 510\) nm), catalytically active, and exhibits an EPR signal with \(g_{\text{ex}} = 1.75\); this signal arises from the spin \(S = 1/2\) ground state resulting from the coupling of a high spin ferric center with a high spin ferrous center (2, 4, 7). The antiferromagnetic coupling in the reduced form has been estimated to be large from Faraday balance measurements (\(J_{\text{ed}} > 35\) cm\(^{-1}\)) (3) and from SQUID\(^1\) susceptibility measurements (\(J_{\text{ed}} > 100\) cm\(^{-1}\)) (6), but these values are contradicted by estimates from variable temperature NMR studies (\(J_{\text{ed}} = 20\) cm\(^{-1}\)) (9) and EPR studies (\(J_{\text{ed}} = 11, 14\) cm\(^{-1}\)) (2, 4).

The addition of phosphate to reduced uteroferrin at pH 4.9 causes an immediate red shift in the visible spectrum and immediate apparent loss of its characteristic EPR signal but a slow (\(\tau_m = 1\) h) loss of enzyme activity (10-13). The red shift of the visible spectrum and the apparent loss of EPR signal have been interpreted by Aisen and co-workers (11, 13) as indicative of the immediate formation of the oxidized phosphate complex. However, this conclusion is inconsistent with the slow loss of enzyme activity, leading Zerner and co-workers (10) and Que and co-workers (12) to propose instead the formation of a reduced uteroferrin phosphate complex.

The Mössbauer spectra of this complex provide clear evidence for a mixed valence paramagnetic complex after phosphate addition, despite its apparent EPR silence (12). Upon standing in air, the reduced uteroferrin phosphate complex converts slowly to the oxidized phosphate complex at a rate parallel to the loss of enzyme activity (10, 12).

In this paper, we report the results of SQUID magnetization measurements on reduced uteroferrin and its phosphate complex at several fixed fields using new techniques developed for metalloprotein studies (14). We also present the first report of the EPR signal of the phosphate complex of reduced uteroferrin. We have obtained a \(J\) value for reduced uteroferrin that is consistent with, but more precise than, the values estimated from NMR and EPR studies (2, 4, 9). Fits to the data for reduced uteroferrin yield a single set of spin Hamiltonian parameters describing the coupled binuclear site which simulates the entire range of magnetization data. The EPR \(g\) values, and the highly anisotropic effective \(A\) tensor of the ferric site derived from Mössbauer spectroscopy. The magnetization data on the reduced uteroferrin phosphate complex verifies the presence of a paramagnetic complex and provides a basis for understanding the previous difficulty in observing its EPR signal.

**MATERIALS AND METHODS**

**Protein Purification**—Uteroferrin was purified by a modification of previously published procedures (12, 15). The resulting protein (\(A_{280}/A_{400} < 1.5\)) was obtained in 0.1 M NaO\(_2\)C\(_2\)H\(_4\) buffer, pH 4.9, 0.2 M in NaCl. Activities were measured at 25 °C using the reaction with p-nitrophenylphosphate. The specific activity of samples varied with preparation, but treatment with 100 mM HO\(_2\)C\(_2\)H\(_2\)SH and catalytic amounts of Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\) resulted in specific activities of 350-500.

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†The abbreviations used are: SQUID, superconducting quantum interference device; EXAFS, extended x-ray absorption fine structure.
units (1 unit = 1 μmol of p-nitrophenol produced per min per mg).

Excess reagents were removed by gel filtration on Sephadex G-25.

Spectroscopy—Electronic absorption spectra were obtained on either a Cary 219 or a Hewlett-Packard HP8451 spectrophotometer. EPR spectra at X-band were recorded on one of two Varian E-Line Century spectrometers equipped either with a commercial (2.3–15 K) or a homemade (6–15 K) continuous flow cryostat operating in the indicated temperature range.

Sample Preparation—Magnetization samples were prepared by exchange into 0.1 M NaO$_4$CD$_4$/DO$_4$CD$_2$ and 0.1 M NaCl in D$_2$O at pH = 5.3 (where pD = meter reading + 0.4 units). Deuteration of the buffer removes the significant nuclear susceptibility signal of the slowing relaxing spin $I = \frac{1}{2}$ protons in water (14). Controls were drawn from the filtrate of the final concentration step during the buffer exchange. Samples and controls were degassed by repeated freeze-pump-thaw cycles to remove paramagnetic molecular oxygen. The reduced uteroferrin phosphate sample was prepared by addition of 100 mM phosphate at pH 4.9 to reduced uteroferrin which had previously been exchanged into deuterated buffer and degassed. Precise volumes (160 μl) of sample and control were loaded separately into matched Suprasil quartz buckets which had been thoroughly etched in hydrofluoric acid to remove ferromagnetic impurities (14).

Magnetization Data—Magnetization data were collected using a Quantum Design SQUID susceptometer over the temperature range from 1.9 to 200 K at fields of 0.31, 1.25, 2.5, and 5 tesla. Minor modifications of the instrument were required to obtain data of the quality reported here. The seal rings of the slide seal isolating the sample space from the atmosphere were changed often (every several days or every 500 data points) to prevent leakage of air. A small flow of clean helium gas was introduced at the top of the sample space, and a nitrogen-cooled trap was added to the pump line connecting the sample space to the fore pump to sweep potential contamination away from the sample region.

In Fig. 1A the sample magnetization difference data at 0.31, 2.5, and 5 tesla for the reduced uteroferrin sample minus its control are plotted as susceptibility (magnetization ($M$) divided by field ($H$)) against inverse temperature. A 1.8% spin $S = \frac{3}{2}$ impurity contribution has been subtracted from the data. The amount (1.6(2)% of this spin $S = \frac{3}{2}$ impurity was determined from the 5 tesla magnetization data (as in Refs. 16 and 17). The amount (3%) of spin $S = \frac{3}{2}$ impurity subtracted from the data for the phosphate complex shown in Fig. 3 was determined by comparing the integrals of the g = 4.3 signals of the EPR spectra of Fig. 2, A and B. The 1.25 tesla data sets have been omitted from Figs. 1 and 3 since they overlapped the 0.31 tesla data sets except at the very lowest temperatures where they differed only slightly.

The vertical location of this sample magnetization difference data set has been found through an iterative fitting process. The susceptibility data of Fig. 1A were first (arbitrarily) located to give zero intercept for the straight line low temperature data at 0.31 tesla. The knee region of the data (Fig. 1B) was fit to find a first estimate for $J$. The spin Hamiltonian parameter space was then searched with this value of $J$ to find the observed EPR g values. This set of spin Hamiltonian parameters (giving the correct g values) was used to calculate the susceptibility curves at the three observed fields. These theoretical curves lay above the data. The data set was translated vertically to align with the calculated saturation susceptibility curves at low temperatures. The newly located knee of the translated data was fit to find a second (improved) estimate for $J$ and the cycle repeated until the data did not have to be translated to align with the calculated susceptibility curves. Similarly the data set of Fig. 3 for the phosphate complex of reduced uteroferrin was located vertically by matching it to theoretical curves calculated from the spin Hamiltonian (1) using parameters which also gave the observed EPR g values.

The data in Figs. 1 and 3 are plotted per sample rather than per mol to separate uncertainties in the magnetization measurement (seen in the scatter of the data) from uncertainties in the measured spin concentrations. For clarity approximately one-third of the data points have been removed from the regions where the three fields overlap. This editing has been done without changing the apparent scatter in the data. The theoretical curves in these figures have been scaled using the spin concentration determined from fitting the susceptibility data.

RESULTS AND DISCUSSION

Reduced Uteroferrin—The antiferromagnetic coupling constant ($J_{\text{red}}$) for the binuclear iron center in the reduced purple acid phosphatases has been estimated to be small (10–20

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**Fig. 1.** A, the sample difference susceptibility (magnetization divided by field) in IU for reduced uteroferrin at three fixed fields (○, 5 tesla; Δ, 2.5 tesla; and □, 0.3125 tesla) plotted against inverse temperature. A 1.6(2)% spin $S = \frac{3}{2}$ impurity signal has been subtracted from the data. The theoretical curves were calculated from the spin Hamiltonian (1) using the parameters listed in Table I. These theoretical curves have been scaled to 152 nmol of spin corresponding to a protein concentration of 0.95 mM. B, the high temperature portion of the data and theory of A.
The stated uncertainty in \( J_{\text{red}} \) is based on the fitting process to a single data set. The other input parameters to the spin Hamiltonian (1) listed in this row are preliminary; no estimates of their uncertainties have been attempted.

Derived from a simulation of the EPR spectrum of reduced uteroferrin (not shown) collected at the University of Michigan for this study.

Ref. 20.

The principal values \((A_j)\) of the effective (spin \( S = \frac{1}{2} \) Hamiltonian) hyperfine tensor of the ferric site are strikingly anisotropic (20). These have been calculated from the isotropic intrinsic hyperfine tensor \((a_j)\) of the ferric site by equating the two expressions for the internal field to give

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\langle \frac{1}{2} A_j \rangle = \langle S_j a_j \rangle, \quad j = x, y, z
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where \((S_j a_j)\) are the spin expectation values of the ferric site derived from the Hamiltonian of Equation 1. The ferric site has been treated similarly.

The parameters used to calculate the susceptibility curves shown in Fig. 1 for reduced uteroferrin are listed in Table I. In addition, the calculated system \( g \) values and the components of the effective hyperfine tensor for each site are also compared with the values derived from EPR and Mössbauer data. This particular set of parameters gives very reasonable fits to the magnetization data (Fig. 1), the EPR \( g \) values (within 0.2\%) (Table I), and the effective hyperfine tensor of the ferric site (within 12\%) (Table I). The estimated uncertainty for \( J_{\text{red}} (19.8(5) \text{ cm}^{-1}) \) of ±2.5\% is based on fitting data for a single sample. The remaining spin Hamiltonian parameters in the top row of Table I may not be unique. Assumptions have been made and constraints applied (described in the preceding paragraphs) in order to achieve the exhibited fit. No estimates of the experimental uncertainties of these parameters have been attempted.

Improvements in these simulations may be achieved by freeing the Euler angles defining the orientation of the ferric site relative to the ferrous site from the present restriction to 90° rotations. This is required to improve the simulation of the effective \( A_j \) tensors of the ferric and ferrous sites while maintaining the existing match to the magnetization data and the observed system \( g \) tensor. Changes in the other parameters have been tried and will not accomplish this. The exhibited simulation whose parameters are given in Table I has an axial ferric site. With an axial ferric site there are three distinct

This was done to limit the number of free parameters after having made every effort to achieve a fit using \( g_1 = (2,2,2) \) for the ferric site. The fact that the fitting cannot be accomplished using \( g_1 = (2,2,2) \) for the ferric site suggests the unquenching of its orbital moment as is observed for rubredoxin (19). However, this is a tentative suggestion at this point for the ferric site of reduced uteroferrin. A firm conclusion on this matter must await further studies.

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phosphate complex of reduced uteroferrin in values for the components of the effective A tensor of the fields of spin corresponding to a protein concentration of has been subtracted from the data. The theoretical curves were against inverse temperature. Each yielded parameter sets which gave comparable quality samples as well as inorganic model complexes is in progress. The sample difference susceptibility of the protons in water is barely discernible in the vicinity of g = 2.3.

The similarity of these properties with those of reduced uteroferrin, and it is clear from a variety of spectroscopic and chemical results that the purple acid phosphatases are not iron-sulfur proteins (1-5, 8). Semimethemerythrin complexes, on the other hand, exhibit EPR signals with g, values of (1.06(15), 1.51(14), and 2.27(11)) as summarized in Table II. The broad, featureless signal of the phosphate complex of reduced uteroferrin so readily apparent under the conditions of C is barely discernible in B in the vicinity of g = 2.3.

Figs. 2. The EPR spectra (A) of reduced uteroferrin and (B) of its phosphate complex. These spectra were collected at the University of Minnesota on the same samples as used for the magnetization studies shown in Figs. 1 and 3. Both EPR spectra were recorded at X-band at 8 K (nominal), 10 G modulation amplitude, 0.2 milliwatts microwave power, at equivalent gain and sample concentration. C, the EPR spectrum (solid line) of the phosphate complex of reduced uteroferrin recorded at the University of Michigan at X-band at 8 K, 40 G modulation amplitude, and 200 milliwatts microwave power. The sample contained a small amount of uncomplexed reduced uteroferrin whose signal has been subtracted causing the wiggles in the region just below g = 2. The simulated spectrum (dashed line) has g values (linewidths) of (1.06(15), 1.51(14), and 2.27(11)) as summarized in Table II. The broad, featureless signal of the phosphate complex of reduced uteroferrin so readily apparent under the conditions of C is barely discernible in B in the vicinity of g = 2.3.

90° orientations of the ferric site relative to the ferrous site. Each yielded parameter sets which gave comparable quality fits to the magnetization data and the EPR g values. However, only one orientation (listed in Table I) also gave reasonable values for the components of the effective A tensor of the ferric site. Further work on site-specific 57Fe-enriched protein samples as well as inorganic model complexes is in progress.

The value obtained for J_red is significantly smaller than the lower limits previously determined from susceptibility measurements using either a Faraday balance (3) or (for the bovine enzyme) a SQUID susceptometer (5). It is consistent with values obtained from variable temperature NMR and EPR studies (2, 4, 9) but is more precise than these because a susceptibility measurement (of the appropriate precision) gives a more direct measure of J and extends over a wider temperature range. Previous susceptibility studies were limited by noise arising from the slow relaxation of the nuclear susceptibility of the protons in water (14). This noise source was eliminated in the present study by deuterating the buffer.

The value of J_red for reduced uteroferrin (19.8±5 cm⁻¹) can be compared with those of other mixed-valence binuclear iron-protein complexes. Reduced two-iron ferredoxins exhibit EPR spectra with g, values of 160 cm⁻¹ as a result of the bis(µ-sulfido)diiron core structure (21). These properties differ greatly from those of reduced uteroferrin, and it is clear from a variety of spectroscopic and chemical results that the purple acid phosphatases are not iron-sulfur proteins (1-5, 8).

Semimethemerythrin complexes, on the other hand, exhibit EPR signals with g, values of 1.8 and J values of 30-40 cm⁻¹ (22-24). The similarity of these properties with those of reduced uteroferrin suggests that these proteins share similar binuclear sites. EXAFS data on semimethemerythrin-N₃ indicate a (µ-hydroxy)bis(µ-carboxylato) diiron core (25), and EXAFS data of the reduced porcine⁴ and bovine (26) purple acid phosphatases are consistent with this structure.

Phosphate Complex of Reduced Uteroferrin—The addition of phosphate to reduced uteroferrin yields a complex that is purple in color, apparently EPR-silent (Fig. 2B), and catalytically active. The first two properties are usually associated with the oxidized form of the enzyme, while the third is characteristic of the reduced form. We have obtained magnetization data on this complex to gain further insight into its properties. From the process of simulating the magnetization data using the spin Hamiltonian (1) we learned that the EPR signal of the ground state was dramatically broad-

⁴ R. C. Scarrow and L. Que, Jr., unpublished observations.
phosphate complex of reduced uteroferrin under appropriate conditions of power, modulation, and temperature then yielded the signal \( g = (1.06, 1.51, 2.27) \) shown in Fig. 2C. Note that this signal spans a field range of more than 400 millitesla which is four times that of reduced uteroferrin (Fig. 2A). Simulations of the EPR signals of reduced uteroferrin and its phosphate complex (summarized in Tables I and II) indicate that the line widths have also increased by a factor of 4 upon complex formation. Thus it is not surprising that the signal had been missed previously. Double integration of the signal of the phosphate complex at 2.3 K yields 1.2(2) spins/mol of enzyme.

The sample difference susceptibilities of the phosphate complex of reduced uteroferrin measured at 0.31, 2.5, and 5 tesla are plotted against inverse temperature in Fig. 3. The spin Hamiltonian parameters used to calculate the theoretical curves shown in Fig. 3 are given in Table II. The exhibited simulation has an axial ferric site. Only one of the three distinct 90° orientations (that stated in Table II) of the axial ferric site relative to the ferrous site yielded a set of parameters which matched both the observed EPR \( g \) values and the form of the susceptibility data. This preliminary parameter set will be refined in conjunction with Mössbauer studies of \( ^{57}\text{Fe} \)-enriched samples of the phosphate complex of reduced uteroferrin. Based on simulating this one data set (Fig. 3) we conclude that the measured value of the exchange coupling \( \Delta = 6.0(5) \text{ cm}^{-1} \) has the stated uncertainty of ±5%.

A general and striking behavior observed throughout our simulations of the magnetization data of Fig. 3 was the increased sensitivity of the calculated system \( g \) values to the zero field splitting parameters when the exchange coupling is comparable to the zero field splittings. This result is summarized in Fig. 4. In this figure the range of each of the three calculated \( g \) values \( (g_1, g_2, g_3) \) for reduced uteroferrin \( (\text{thin bars}) \) and its phosphate complex \( (\text{broad bars}) \) are plotted for defined changes (stated along the abscissa) in each of five spin Hamiltonian parameters.

When the exchange coupling is large relative to the zero field splittings of the iron sites (as it is for reduced uteroferrin, \( \text{thin bars} \) in Fig. 4) the relative change in the system \( g \) values is small for defined changes in the spin Hamiltonian parameters. This stability of the predicted \( g \) values to changes in the spin Hamiltonian parameters is required in the face of protein heterogeneity (19) if an EPR signal is to be readily observed. However, when the exchange coupling is comparable to the zero field splittings (as it is for the phosphate complex of reduced uteroferrin, \( \text{broad bars} \) in Fig. 4) the relative change in the system \( g \) values is large for defined

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**TABLE II**

| Phosphate complex of reduced uteroferrin | Ferrous | Euler angles \( \alpha, \beta, \gamma \) | Ferric |
|----------------------------------------|---------|--------------------------------------|--------|
| \( J_{\text{red}, \rho} \) cm\(^{-1} \) | \( D_1 \) | \( E_1/D_1 \) | \( a_1 \) | \( g_1 \) | \( D_2 \) | \( E_2/D_2 \) | \( a_2 \) | \( g_2 \) |
| Observed \( g \) (± 0.01)\(^a\) | \( 6.0(5) \) | 0.08 | -15 | 2.0 | (90,90,90) | \( 0.0 \) | -20 | (2.0,2.07,2.04) |
| Calculated \( g \) | \( (1.06, 1.51, 2.27) \) | (0.15, 0.14, 0.11)\(^b\) | (1.17, 1.51, 2.30) | None available | None available | None available |

\(^a\) The stated uncertainty in \( J_{\text{red}, \rho} \) is based on the fitting process to a single data set. The other input parameters to the spin Hamiltonian (1) listed in this row are preliminary; no estimates of their uncertainties have been attempted.

\(^b\) Derived from the simulation exhibited in Fig. 2C.

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**FIG. 4.** The ranges in the individual calculated \( g \) values \( (g_1, g_2, g_3) \) for reduced uteroferrin \( (\text{thin bars}) \) and its phosphate complex \( (\text{broad bars}) \) are plotted for defined changes (stated on the abscissa) of each of five parameters of the spin Hamiltonian of Equation 1. Superscript \( a \) indicates the ratio \( \Delta g_1/g_1 \) which expresses the increased sensitivity of the calculated \( g \) value of the phosphate complex relative to that of reduced uteroferrin to a defined change in a spin Hamiltonian parameter. The logarithmic average of these three ratios for \( g_1, g_2, g_3 \) the increased "effective \( g \) strain" of the phosphate complex over reduced uteroferrin, is stated at the top of the figure for each of the spin Hamiltonian parameters. These ratios (the numbers at the top of the figure) do not depend on the magnitude of the change in the spin parameters (the percentage indicated at the bottom of the figure).
changes in the spin Hamiltonian parameters. It is this increased sensitivity of the system $g$ values (increased "effective $g$-strain") to the same protein heterogeneities combined with the increased anisotropy of its $g$ tensor which results in an EPR signal for the phosphate complex (shown in Fig. 2, B and C) that is extremely broad and difficult to detect.

The increased sensitivity of the calculated $g$ values to changes in the spin Hamiltonian parameters for the phosphate complex relative to that of reduced uteroferrin can be expressed as the ratio of the fractional changes in $g((\Delta g)/g_{red})/(\Delta g/g_{red})$ for each of the three $g$ values. These can be averaged for each spin Hamiltonian parameter using logarithms. The resulting average factor of increased sensitivity of the phosphate complex over reduced uteroferrin is stated at the top of Fig. 4 for each spin Hamiltonian parameter. The predicted overall average increase in "effective $g$ strain" of the phosphate complex over reduced uteroferrin is factor of 9, nearly an order of magnitude. This leads to the prediction that the EPR signal of the phosphate complex will be an order of magnitude broader than that of the reduced protein if each has the same microheterogeneities in the spin Hamiltonian parameters. Comparison of the measured line widths of the observed EPR spectra (Tables I and II) indicate an increase by a factor of 4 in going from reduced uteroferrin to its phosphate complex.

In hindsight, there are a number of reasons the EPR signal of the spin $S = \frac{1}{2}$ ground state of the phosphate complex of reduced uteroferrin was difficult to detect. The smaller value of the exchange coupling ($J_{red,P} = 6.0(5) \text{ cm}^{-1}$) resulted in 1) an unexpected increase in EPR line width (see Fig. 4 and text), 2) a dramatic increase in the anisotropy of the $g$ tensor (the lower $g_s$ and $g_i$ components reduce the signal amplitude associated with $g_s$), and 3) depopulation of the ground state through thermal excitation of the spin ladder at the temperatures ($T > 88 \text{ K}$) typical of EPR studies. In addition, 4) samples of the phosphate complex routinely contain small amounts of uncomplexed protein whose intense EPR signals mask that of the complex and 5) common cavity and preparative contaminants such as copper and/or oxygen can give comparable small signals over the same field span.

The change by a factor of 3 in the exchange coupling in going from reduced uteroferrin ($J_{red} = 19.8(5) \text{ cm}^{-1}$) to its phosphate complex ($J_{red,P} = 6.0(5) \text{ cm}^{-1}$) may indicate a significant change in the nature of the bridging group(s) between the iron atoms. A likely consequence of the phosphate binding is the protonation of the hydroxo bridge in reduced uteroferrin. It has been demonstrated that protonation of the oxo bridge in $(\mu$-oxo)bis$(\mu$-carboxylato)diiron(III) complexes results in the $J$ value decreasing from 242 cm$^{-1}$ (27) to 34 cm$^{-1}$ (28). Thus a decrease in exchange coupling could also be expected for the conversion of a hydroxo bridge to an aquo bridge.

CONCLUSIONS

The exchange coupling of reduced uteroferrin has now been measured directly using techniques recently developed for metalloprotein magnetization measurements (14). The result ($J_{red} = 19.8(5) \text{ cm}^{-1}$) is substantially less than the lower limit set by previous susceptibility measurements employing either a Faraday balance or a SQUID susceptometer. Also, the result is more precise than but consistent with the values derived either from room temperature NMR measurements or from the temperature dependence of the EPR signal at low temperatures.

The susceptibility of the phosphate complex of reduced uteroferrin (Fig. 3) verifies the paramagnetism of the complex first established by Mössbauer spectroscopy (12). The exchange coupling of the phosphate complex of reduced uteroferrin ($J_{red,P} = 6.0(5) \text{ cm}^{-1}$) is substantially smaller than that of reduced uteroferrin. This reduction in the exchange coupling by a factor of 3 may indicate a significant structural change in the binuclear iron center, possibly the protonation of the hydroxo bridge.

A set of spin Hamiltonian parameters has been presented for reduced uteroferrin (Table I) which yields a satisfactory fit to the low and high field magnetization data, to the EPR $g$ values, and to the highly anisotropic effective hyperfine tensor of the ferric site. Likewise, a set of spin Hamiltonian parameters has been presented for the phosphate complex of reduced uteroferrin (Table II) which yields a fit to its magnetization data and to the $g$ values of its newly identified EPR spectrum.

A major puzzle concerning the phosphate complex of reduced uteroferrin was its apparent EPR silence (Fig. 2B). This apparent EPR silence arises from the increase in the "effective $g$ strain" caused by the decrease in the exchange coupling (to a value comparable to the zero field splittings) which gives rise to increased sensitivity of the system $g$ values to existing heterogeneities in the protein's zero field splittings. This and the increased $g$ anisotropy ($g = (1.06, 1.51, 2.27)$) gives rise to an EPR signal which is extremely broad and difficult to detect (Fig. 2B) but which we have now identified (Fig. 2C).

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