Electron Spin Echo Envelope Modulation Evidence for Carbonate Binding to Iron(III) and Copper(II) Transferrin and Lactoferrin

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Iron binding to transferrin and lactoferrin requires a synergistic anion, which is carbonate in vitro. The anion is thought to play a key role in iron binding and release. To understand better the iron-carbonate interaction, experiments were performed with iron(III) and copper(II) complexes of human milk lactoferrin and serum transferrin with carbon-13-labeled carbonate. Modulation frequencies were present in the Fourier transforms of two-pulse and three-pulse electron spin echo envelope modulation data for the Fe(III) and Cu(II) complexes, consistent with binding of carbonate to both metals. The metal-^13C interaction was similar for the lactoferrin and transferrin complexes. Spin coupling to the nitrogen of a coordinated histidine imidazole was observed for both metals. Both the metal-nitrogen and the metal-carbon spin coupling constants were about a factor of 5 smaller for the iron complexes than for the copper complexes, which indicated substantial similarity in the metal-carbonate and metal-imidazole binding for the two metals.

The transferrins are a family of iron-binding proteins that include transferrin (TF) and lactoferrin (Lf). These proteins are found in the physiological fluids of a wide variety of vertebrates (1, 2). The role of transferrin in iron transport has been studied extensively (1, 2). The roles of lactoferrin in regulation of myelopoiesis, immune response, microbicidal and bacteriostatic activity, and the anemia of inflammation have been reported recently (3-8). X-ray crystal structures have confirmed that both proteins have two metal-binding sites that are spatially separated (9, 10). Occupancy of these sites requires binding of an anion (1, 2). The proteins bind iron with extremely high equilibrium constants but readily release iron as needed by cells. The anion is thought to play a crucial role in iron binding and release, so it is important to elucidate the interaction with the iron.

The protein provides four ligands for the metal, leaving two open sites for coordination of the anion and/or water (9, 10). A variety of spectroscopic techniques has shown that the anion-binding site is in close proximity to the metal-binding site (1, 11). Some studies, especially those exploring steric effects, have suggested that the anion binds simultaneously to the metal and the protein. The resolution of the crystal structures is not sufficient to confirm this proposal. Most of the spectroscopic studies of anion binding have used metals other than iron. In a ^14C NMR study of Fe-Tf[^13C]CO₂, the absence of a peak that could be assigned to the carbonate carbon indicated that the iron-carbon distance was less than about 9 Å (12), which does not require that the carbonate be bound to the iron. It therefore would be desirable to obtain spectroscopic evidence to determine whether the carbonate is bound to iron in Fe-Tf-CO₂ and Fe-Lf-CO₂.

Electron spin echo envelope modulation (ESEEM) is a powerful tool to identify nuclear spins in the vicinity of a paramagnetic center (13). The metal-[^13C] coupling in Cu-Tf[^13C]oxalate (14, 15), Cu-Lf[^13C]oxalate (15), vanadyl-Tf[^13C]CO₂ (15), vanadyl-Lf[^13C]CO₂ (15), vanadyl-Tf[^13C]oxalate (15), and vanadyl-Lf[^13C]oxalate (15) confirmed the binding of the anion to the metal. In light of these reports it is surprising that metal[^13C] coupling was not observed in an early ESEEM study of Cu-Tf[^13C]CO₂ (14). In that study, data were presented in the time domain (14). More recently, Fourier transforms have been used to analyze ESEEM data, which facilitates detection of modulation frequencies that are difficult to observe in the time domain. We have therefore reexamined the ESEEM for Cu-Tf[^13C]CO₂ and have also obtained data for Cu-Lf[^13C]CO₂. To obtain information for the iron complexes directly we report ESEEM studies of Fe-Tf[^13C]CO₂ and Fe-Lf[^13C]CO₂.

EXPERIMENTAL PROCEDURES

Materials—Iron-free human transferrin and human milk lactoferrin were obtained from Sigma and used without further purification. The purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6%). For both proteins a single band was resolved. Heps buffer was obtained from Sigma. 99% enriched sodium[^13C] carbonate was purchased from Isotec Inc. Plastic disposable tubes and pipettes were used for all manipulations of the protein solutions except that EPR spectra were recorded in 4-mm (outer diameter) quartz tubes.

Methods—The transferrin or lactoferrin metal-carbonate complexes were formed in 0.09 M Heps buffer at pH 3.5. The pH of the resulting solution was raised to ~7.5 with ammonia gas mixed with nitrogen. Two moles of metal ion were bound/mol of protein. Complexes with[^13C]carbonate were formed in a glove box with a nitrogen atmosphere to avoid contamination with[^13C]carbonate. The apoprotein buffer solution was purged with nitrogen gas to remove traces of[^13C]carbonate. Visible spectra and continuous wave (CW) EPR spectra were used to characterize the complexes (15). EPR Spectra—Pulsed EPR data were obtained with two-pulse and
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three-pulse sequences on a spectrometer that has been described previously (16). The time for a 90° pulse is 20 ns. ESEEM data were collected as follows: two-pulse data for Cu(II) complexes, 1500 points at 2-ns intervals; three-pulse data for Cu(II) complexes, 1024 points at 2-ns intervals; three-pulse data for Fe(III) complexes, 1700 points at 6 ns intervals. An Air Products cryostat or an Oxford ESR900 liquid helium cryostat was used to obtain sample temperatures of about 15 K for the copper complexes. An Oxford ESR910 pumped liquid helium cryostat was used to obtain sample temperatures of about 2-3 K for the iron complexes.

Analysis of ESEEM—The ESEEM data were cosine Fourier transformed to determine the characteristic modulation frequencies. The method of Mims (17) was used to extrapolate the data into the instrument “dead time.” The ESEEM data also were analyzed by omitting the instrument dead time and taking the power spectrum of the Fourier transform to ensure that signals were not due to artifacts caused by the estimates of the “missing” data.

Carbon-13 Modulation—The anisotropy of the carbon-13 modulation in the data for Cu-Tf-[13C]CO3 and Cu-Lf-[13C]CO3 was sufficiently small that the nuclear hyperfine coupling, \( a_\text{C} \), could be determined from Equation 1 where \( v_\text{m} \) is the observed modulation frequency, and \( \eta_\text{n} \) is the nuclear frequency at the observing magnetic field (18).

\[
v_\text{m} = |\eta_\text{n}| + 0.5a_\text{C}
\]

The higher frequency component was observed at all orientations examined, but the lower frequency component was obscured by the nitrogen modulation frequencies for some orientations of the molecule. The calculated values of \( a_\text{C} \) are summarized in Table I.

The equations reported in the literature for the analysis of ESEEM data are primarily for metal ions with a single unpaired electron \( (S = \frac{1}{2}) \) or metal ions with multiple unpaired electrons for which the zero-field splitting is sufficiently large that the ground state can be treated as a fictitious \( S' = \frac{1}{2} \) with anisotropic \( g \) values (13). For Fe(III) complexes, \( D = 0.23 \text{ cm}^{-1} \) and \( E/3 \) (19). Since the spectroscopic properties of Fe-Tf-CO3 and Fe-Lf-CO3 are similar, it is probable that the zero-field splittings are the same.

Determination of nuclear hyperfine splittings of the iron energy levels.

The Hamiltonian in Equation 2 was diagonalized at 1580 G (\( g = 4.2 \)) is due to transitions within the middle Kramer’s doublet (10, 20). The energy levels involved in this transition are combinations of wave functions with iron \( m_s = -\frac{1}{2} \) to \( +\frac{1}{2} \) (20). Thus, the equations in the literature are not appropriate to analyze the ESEEM modulation frequencies observed for Fe-Tf-CO3 and Fe-Lf-CO3. The Hamiltonian in Equation 2 was diagonalized at 1580 G to determine the nuclear hyperfine splitting of the iron energy levels.

\[
H = g_\text{e}B_\text{e}H \cdot S + g_\text{n}B_\text{n}H \cdot I + D[S^2 - \frac{3}{2}I(I + 1)]
\]

where \( g_\text{e} \), \( g_\text{n} \), and \( B_\text{e} \) are the electron and nuclear magnetons, respectively, \( D \) and \( E \) are the iron zero-field splitting parameters, and \( a \) is the electron-nuclear coupling constant.

Although the effective electron-nuclear coupling was orientation dependent, Equation 3 was found to be valid for a range of orientations of the molecule in the magnetic field. The variation in the coefficient of \( a_\text{C} \) was about 15% for the orientations examined.

\[
v_\text{m} = |\eta_\text{n}| + 0.5a_\text{C}
\]

The use of Equation 3 gave the 13C coupling constants for Fe-Tf-[13C]CO3 and Fe-Lf-[13C]CO3, which are listed in Table I.

Nitrogen Modulation—In the data for Cu-Tf-CO3 and Cu-Lf-CO3, the copper-nitrogen coupling constant was estimated from the frequency of the peak at 3.5 to 4.1 MHz using the relationship in Equation 4 (13).

\[
v_\text{m} = 2(|\eta_\text{n}| + 0.5a_\text{C}) + \frac{1}{2}(3 + 3 + \eta_\text{n})
\]

where \( v_\text{m} \) is the observed modulation frequency, \( \eta_\text{n} \) is the nuclear Zeeman frequency at the observing magnetic field, and \( \alpha_\text{N} \) and \( \eta_\text{n} \) are the quadrupolar parameters for the nitrogen.

The quadrupolar parameters for the imidazole nitrogens are similar to the iron in Fe-Tf-CO3 and Fe-Lf-CO3, the analog of Equation 4 for nitrogen coupling to iron in Fe-Tf-CO3 and Fe-Lf-CO3 requires replacement of the term \( (\eta_\text{n} \pm 0.5a) \) in Equation 4 with \( (\eta_\text{n} \pm 1.0a) \) to give Equation 5.

\[
v_\text{m} = 2(|\eta_\text{n}| \pm 1.0a) + \frac{1}{2}(3 + 3 + \eta_\text{n})
\]

where variables are defined as in Equation 4.

RESULTS AND DISCUSSION

13C Modulation—The cosine Fourier transform of the two-pulse ESEEM for Cu-Tf-CO3 (Fig. 1a) at a magnetic field of 3180 G is in good agreement with that obtained previously (15). This magnetic field corresponds to a peak in the perpendicular region of the CW EPR spectrum. The observed frequencies are due to coupling to the distant nitrogen of a coordinated imidazole as discussed below. When [13C]CO3 was used to prepare the complex, an additional peak at 6.6 MHz was observed (Fig. 1b). Similarly, a peak at 6.7 MHz was observed for Cu-Lf-[13C]CO3. This frequency is due to interaction of the copper electron spin with the 13C nuclear spin. A corresponding peak was not observed in three-pulse ESEEM for either Cu-Tf-[13C]CO3 or Cu-Lf-[13C]CO3 at this magnetic field. The peak may be sufficiently broad that the modulation decays within the minimum dead time (\( \tau + T \)) of the three-pulse experiment, which is about 500 ns on our instrument (16).

2615 G is near the low field extreme of the CW EPR spectrum. The observed frequencies are primarily for metal ions with a single unpaired electron \( (S = \frac{1}{2}) \) or metal ions with multiple unpaired electrons for which the zero-field splitting is sufficiently large that the ground state can be treated as a fictitious \( S' = \frac{1}{2} \) with anisotropic \( g \) values (13). For Fe(III) complexes, \( D = 0.23 \text{ cm}^{-1} \) and \( E/3 \) (19). Since the spectroscopic properties of Fe-Tf-CO3 and Fe-Lf-CO3 are similar, it is probable that the zero-field splitting parameters are the same. The use of Equation 3 gave the 13C coupling constants for Fe-Tf-CO3 and Fe-Lf-CO3, which are listed in Table I.

**Results**

**Table 1**

| Sample         | Magnetic field | Observed frequenciesa | Calculated coupling constant |
|----------------|----------------|------------------------|-----------------------------|
|                | G MHz          | Nitrogen MHz           | Carbon MHz                  |
| Cu-Tf-CO3      | 3185 0.74 0.86 | 1.60 4.05 6.6           | 1.5 6.4                     |
| Cu-Lf-CO3      | 2910 0.74 0.86 | 1.56 3.7 3.9 1.3 6.8    | 1.7 7.6                     |
|                | 2615 0.74 0.86 | 1.53 3.5 2.0 7.0        | 1.6 0.4                     |
|                | 3170 0.65 0.81 | 1.60 4.1 6.7            | 1.8 6.5                     |
|                | 2800 0.64 0.84 | 1.56 3.7 3.9 3.5 7.0    | 1.7 6.2                     |
| Fe-Tf-CO3      | 1580 0.70 0.85 | 1.50 2.3 3.2 3.2        | -0.4 1.5                    |
| Fe-Lf-CO3      | 1580 0.75 0.85 | 1.50 2.3 3.2 3.2        | -0.4 1.5                    |

a Uncertainties: ±0.05 MHz for sharp peaks, ±0.1 MHz for broad peaks.

b Observed for [13C]carbonate. These frequencies are absent in samples prepared with [13C]carbonate.
The cosine Fourier transforms of data for a, Cu-Tf-CO$_3$ and b, Cu-Tf-$^{13}$C|CO$_3$.

**FIG. 2.** Three-pulse ESEEM for Cu-Tf-CO$_3$ obtained at approximately 15 K with a microwave frequency of 9.202 GHz and a magnetic field of 2810 G, which corresponds to an intermediate orientation for molecules with copper $m_l = 3/2$ and is near the parallel orientation for molecules with copper $m_l = 1/2$. The spectra are cosine Fourier transforms of data for a, Cu-Tf-CO$_3$ and b, Cu-Tf-$^{13}$C|CO$_3$.

The cosine Fourier transform of the three-pulse ESEEM for Cu-Tf-$^{13}$C|CO$_3$ at this magnetic field is assigned to the parallel orientation. The peak at 1.9 MHz which was resolved for Cu-Lf-$^{13}$C|CO$_3$ is assigned as the difference frequency in Equation 1.

In the CW EPR spectrum of Cu-Tf-CO$_3$ and Cu-Lf-CO$_3$ and corresponds to the parallel orientation for molecules with copper $m_l = 1/2$. Thus, the $^{13}$C modulation frequency of 7.0 MHz observed in the Fourier transforms of two-pulse ESEEM for Cu-Tf-$^{13}$C|CO$_3$ and Cu-Lf-$^{13}$C|CO$_3$ at this magnetic field is assigned to the parallel orientation. The peak at 1.9 MHz which was resolved for Cu-Lf-$^{13}$C|CO$_3$ is assigned as the difference frequency in Equation 1.

The frequency of the peak at 3.5-4.1 MHz depends on the number of unpaired electrons on the metal (21, 22). Since Cu(II) has $n = 1$ and Fe(III) has $n = 5$, the observed decrease in the coupling constant by a factor of 5 indicates that the bonding remains approximately constant, the coupling constant is expected to vary as $1/n$ where $n$ is the number of unpaired electrons on the metal (21, 22). Since Cu(II) has $n = 1$ and Fe(III) has $n = 5$, the observed decrease in the coupling constant by a factor of 5 indicates that the bonding between the metal and the carbonate is similar for the two metals.

**Nitrogen Modulation**—The nitrogen modulation frequencies for Cu-Tf-CO$_3$ and Cu-Lf-CO$_3$ at a magnetic field of 3020 G have been reported previously (15). For the three magnetic fields examined in this study, the nitrogen frequencies in the range of 0.7-1.6 MHz were approximately constant (Table I). As noted in the analysis of ESEEM of other copper complexes of histidine imidazoles, these frequencies are almost purely quadrupolar, which occurs at “near cancellation,” i.e. when the nuclear Zeeman frequency is about half the isotropic contribution consistent with these values is 0.7 MHz, which corresponds to a copper-$^{13}$C dipolar interaction at 3.1 Å. The ESEEM data were obtained for the magnetic field along the copper z axis, including the copper yz plane, and at an intermediate orientation with respect to the copper axis system. Since the orientation of the copper-$^{13}$C vector within the copper axis system is not known, the data may not have sampled the maximum dipolar interaction. Thus, 3.1 Å is an upper limit on the copper-$^{13}$C distance. The magnitude of the isotropic interaction requires through-bond interaction between the copper and the carbonate carbon. The copper-$^{13}$C distance is consistent with direct coordination of the carbonate to the copper.

The cosine Fourier transform of the three-pulse ESEEM for Fe-Tf-CO$_3$ at a magnetic field of 1580 G is shown in Fig. 3a. When $^{13}$C|carbonate was used to prepare the complex, an additional peak at 3.2 MHz (Fig. 3b) was observed. Similarly, a peak at 3.2 MHz was observed for Fe-Lf-$^{13}$C|CO$_3$, (Fig. 3c) but not for Fe-Lf-CO$_3$. The frequencies of these peaks indicate (Equation 3) that the iron-$^{13}$C coupling was about 1.5 MHz. This value is approximately one-fifth of the value observed for the analogous copper complex. If a single unpaired electron dominates a metal-nuclear spin-spin interaction and the bonding remains approximately constant, the coupling constant is expected to vary as $1/n$ where $n$ is the number of unpaired electrons on the metal (21, 22). Since Cu(II) has $n = 1$ and Fe(III) has $n = 5$, the observed decrease in the coupling constant by a factor of 5 indicates that the bonding between the metal and the carbonate is similar for the two metals.

**Nitrogen Modulation**—The nitrogen modulation frequencies for Cu-Tf-CO$_3$ and Cu-Lf-CO$_3$ at a magnetic field of 2810 G have resonances for a nearly parallel orientation of molecules with copper $m_l = 1/2$ and $m_l = 3/2$. The two frequencies for the parallel orientation are due to the observation of both the sum and difference frequencies predicted by Equation 1.

Analysis of the observed $^{13}$C modulation frequencies with Equation 1 gave the copper-$^{13}$C coupling constants shown in Table I. The observed values of 6.4-8.4 MHz imply an isotropic contribution of 7.4 ± 0.5 MHz. The smallest anisotropic contribution consistent with these values is 0.7 MHz, which corresponds to a copper-$^{13}$C dipolar interaction at 3.1 Å. The ESEEM data were obtained for the magnetic field along the copper z axis, in the copper yz plane, and at an intermediate orientation with respect to the copper axis system. Since the orientation of the copper-$^{13}$C vector within the copper axis system is not known, the data may not have sampled the maximum dipolar interaction. Thus, 3.1 Å is an upper limit on the copper-$^{13}$C distance. The magnitude of the isotropic interaction requires through-bond interaction between the copper and the carbonate carbon. The copper-$^{13}$C distance is consistent with direct coordination of the carbonate to the copper.

The cosine Fourier transform of the three-pulse ESEEM of Iron(III) and Copper(II) Transferrin and Lactoferrin...
frequency and the quadrupole parameters (Equation 4). The peak at 4.05–4.10 MHz at 3185 G is from molecules with the magnetic field in the perpendicular plane of the copper. At 2615 G the 3.5-MHz peak is due to the parallel orientation of molecules with copper \( m_1 = \frac{1}{2} \). At 2800–2810 G the nitrogen frequency of 3.5 MHz is assigned to a nearly parallel orientation of molecules with copper \( m_1 = \frac{1}{2} \) on the basis of the similarity with the frequency observed at 2615 G, and the 3.7- and 3.9-MHz frequencies are assigned to intermediate orientations of molecules with copper \( m_1 = \frac{1}{2} \). The orientation dependence of the copper-nitrogen coupling is relatively small (Table I). This is consistent with assignment of the coupling to interaction with the distant nitrogen of the coordinated histidine imidazole since the long distance would result in a small dipolar contribution to the interaction.

The three nitrogen frequencies at 0.70–0.75, 0.89, and 1.57 in the ESEEM data for Fe-Tf CO\(_3^--\) and Fe-Lf CO\(_3^--\) are within experimental uncertainty of the three frequencies observed for the analogous copper complexes. By analogy, these frequencies are assigned as predominately quadrupolar, arising from interaction with the distant nitrogen of the coordinated histidine imidazole. Apparently the conditions for near cancellation are also satisfied for the iron complex. As discussed under “Methods,” near cancellation for the middle Kramer’s doublet of Fe-Tf CO\(_3^--\) requires that the nuclear Zeeman frequency be approximately equal to the nuclear hyperfine coupling. At 1580 G, the nitrogen nuclear Zeeman frequency is 0.49 MHz so \( \alpha_N \) is -0.5 MHz. Alternatively, the nitrogen hyperfine coupling can be estimated from the observed frequency of 2.3 MHz and Equation 5, which gave \( \alpha_N \sim 0.35 \) MHz. Together, the two approaches suggest \( \alpha_N \sim 0.4 \) MHz. This value also is about one-fifth of the value observed for the copper complex, which indicates that the metal-imidazole binding is similar for the two metals.

**CONCLUSION**

\(^{13}\)C modulation was observed in the ESEEM for Fe-Tf [\(^{13}\)C]CO\(_3\), Fe-Lf [\(^{13}\)C]CO\(_3\), Cu-Tf [\(^{13}\)C]CO\(_3\), and Cu-Lf [\(^{13}\)C]CO\(_3\). The large isotropic contribution and the magnitude of the anisotropic contribution to the interaction for Cu-Tf [\(^{13}\)C]CO\(_3\) and Cu-Lf [\(^{13}\)C]CO\(_3\) are consistent with coordinated carbonate anion. The similarity in the quadrupolar nitrogen modulation frequencies for the iron and copper complexes is consistent with interaction with the distant nitrogen of a coordinated histidine imidazole for both metals. The iron-nitrogen and iron-carbon coupling constants were about a factor of 5 smaller than the analogous copper-nuclear coupling constants, which implies substantial similarity in the metal-ligand bonding for the two metals. This spectroscopic study provides strong evidence that the carbonate anion binds directly to the metal in iron and copper complexes of transferrin and lactoferrin.

The spectroscopic data indicate substantial similarity between the metal-anion-binding sites for transferrin and lactoferrin. X-ray crystal structures have shown that the overall topologies also are very similar for the two proteins and that the metal-anion-binding sites occur at the interface between two dissimilar domains (9, 10). Iron release is known to occur with a large change in protein conformation. Whether this change is triggered by events at the metal-anion-binding site or at the protein surface remains to be determined.

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