Comparison of the Electron Spin Echo Envelope Modulation (ESEEM) for Human Lactoferrin and Transferrin Complexes of Copper(II) and Vanadyl Ion*

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Copper(II) and vanadyl ions were bound to human milk lactoferrin or serum transferrin with carbonate or oxalate as the synergistic anion. Electron spin echo envelope modulation (ESEEM) data were dependent on anion. When data in D2O/glycerol-D2 were compared with data in H2O/glycerol, the deep deuterium modulation indicated multiple exchangeable protons in the vicinity of the metals with at most one proton within about 2.9 Å of the metal. The distribution of exchangeable protons around the metals as probed by ESEEM was the same, within experimental uncertainty, for the copper or vanadyl complexes with either carbonate or oxalate as the anion. When 13C-labeled oxalate was used as the synergistic anion, 13C-ESEEM was observed for both the copper and vanadyl complexes of lactoferrin and transferrin. The deeper 13C modulation for copper and vanadyl transferrin 13Coxalate than for vanadyl transferrin 13Ccarbonate suggests that both ends of the oxalate are bound to the metal in the transferrin and lactoferrin complexes.

Transferrin, lactoferrin, and ovotransferrin are the major constituents of a group of iron binding proteins designated as siderophils. Although immunologically distinct, they share many physicochemical properties (2–4). The siderophils contain two spatially separated metal binding sites, and occupancy of these sites is dependent on interaction with an anion that is bound to a specific domain of the protein (2–4). Transferrin has been studied extensively because of its importance in iron transport (2–4). Recent studies have suggested a wide variety of biological roles for lactoferrin including regulation of myelopoiesis, immune response, microbical, and bacteriostatic activity, and the amelioration of inflammation (5–10). As with transferrin, functional activity of lactoferrin is related to its ability to bind iron (5, 10). Although in vivo function of these proteins is associated with iron, a wide range of metals has been shown to bind to the siderophils in vitro. Spectroscopic techniques that permit evaluation of the metal binding sites of lactoferrin and transferrin may help to elucidate the biological activity of these proteins.

The electron spin echo studies of lactoferrin and transferrin described in this paper were designed to compare three aspects of the metal binding sites in lactoferrin and transferrin: 1) coordination by nitrogen from a histidine imidazole, 2) interaction of the metal ion with exchangeable protons, and 3) coordination of oxalate as the synergistic anion. Previous ESEEM studies of copper transferrin had demonstrated coordination of a histidine imidazole and binding of the synergistic oxalate ion to the metal (11, 12). Similar studies had not been reported for lactoferrin. No ESEEM results had been reported for the vanadyl complexes of either transferrin or lactoferrin.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Nitrogen Modulation in Copper Complexes—The two-pulse ESEEM for CuLICO3 is shown in Fig. 1a. The data are similar to those reported for CuTfCO3 and CuOTfCO3 by Zweier et al. (11, 12). The Fourier transform of the data and a simulation are shown in Fig. 1, b and c, respectively. Corresponding three-pulse data are shown in Fig. 1, d–f. The modulation frequencies and values obtained from the simulations are summarized in Table 2. The nitrogen modulation frequencies for CuTfCO3 and CuOTfCO3 (0.8, 1.6, and 4.0 MHz) and for the analogous oxalate complexes (0.8, 1.6, and 4.1 MHz) are similar to the frequencies (0.7, 1.4, and 4.0 MHz) observed for the distant nitrogen of histidine imidazole coordinated to copper proteins including stellacyanin (39) and porcine kidney amine oxidase (35).

Nitrogen Modulation in Vanadyl Complexes—The two-pulse ESEEM data for VOlICO3 are shown in Fig. 2a. The nitrogen modulation is much less deep than the modulation in Fig. 1a. Similar ESEEM data were obtained for VOflox, VOICtCO3, and VOITfCO3. The Fourier transform and a simulation

1 The abbreviations used are: ESEEM, electron spin echo envelope modulation; Tf, lactoferrin; Tf, transferrin; ox, oxalate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CW, continuous wave; ENDOR, electron nuclear double resonance.

2 Portions of this paper including "Experimental Procedures," Table 1, and Figs. 3–6 are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Fig. 1. ESEEM data for CuTfCO₃ obtained at approximately 5K with a microwave frequency of 9.19 GHz and a magnetic field of 3200 G which corresponds to a perpendicular line in the CW EPR spectrum. a, two-pulse ESEEM. b, cosine Fourier transform of the data in a, c, simulation of the two-pulse data obtained with $r = 4.2 \text{Å}$, $a = 1.7$ MHz, $eeqQ = 1.6$ MHz, and $\eta = 0.9$. d, three-pulse ESEEM. e, cosine Fourier transform of the data in d. f, simulation of the three-pulse data obtained with $r = 4.15 \text{Å}$, $a = 1.75$ MHz, $eeqQ = 1.55$ MHz, and $\eta = 0.90$.

**Table 2**

| Sample       | Observed frequencies | Calculated parameters |
|--------------|----------------------|-----------------------|
|              | $MHz$                | $MHz$ |
| CuTfCO₃     | 0.8, 1.6, 4.0        | 1.7, 1.6, 0.9         |
| CuLiCO₃     | 0.8, 1.6, 4.0        | 1.7, 1.6, 0.9         |
| CuTfox      | 0.8, 1.6, 4.1        | 1.8, 1.6, 0.9         |
| CuLiFox     | 0.8, 1.6, 4.1        | 1.8, 1.6, 0.9         |
| VOTfCO₃     | ~2, 4.6, 8.1         | 6.6                 |
| VOLTfCO₃    | ~2, 4.6, 8.1         | 6.6                 |
| VOLTfOX     | ~2, 4.8, 8.5         | 7.0                 |
| VOLLfOX     | ~2, 4.8, 8.5         | 7.0                 |
| VO(hfac)py  | ~2, 4.7, 8.4         | 7.1                 |
| VO(hfac)imid| ~2, 5.1, 9.3         | 7.6                 |

Uncertainties are ±0.1 MHz for the copper complexes and ±0.2 MHz for the vanadyl complexes.

Fig. 2. a, two-pulse ESEEM for VO(hfac)py obtained at 20K with a microwave frequency of 9.19 GHz and a magnetic field of 3250 G, which corresponds to a perpendicular line in the CW EPR spectrum. $b$, cosine Fourier transform of the data in a, c, simulated spectrum obtained with $r = 2.0 \text{Å}$, $a = 6.6$ MHz, $eeqQ = 1.5$ MHz, and $\eta = 0.5$. Proton modulation was not included in the simulation.

Since there are few data in the literature concerning ESEEM due to nitrogen bound to vanadyl ion, data were obtained for two nitrogenous-base complexes of vanadyl bis(hexafluorooctylacetonate), VO(hfac)₂. The two-pulse ESEEM for VO(hfac)₂L, L = pyridine, and imidazole, are shown in Fig. 3. The nitrogen modulation in these echoes is shallow, analogous to the modulation in Fig. 2a. Fourier transforms and simulations are shown in Figs. 4 and 5. The nitrogen modulation frequencies obtained for VO(hfac)py in the perpendicular region of the EPR spectrum (Table 2) are similar to those reported for vanadyl bis(acetylacetonate) pyridine, VO(acac)py, (4.45 and 8.1 MHz) in the perpendicular region of the EPR spectrum (40). The value of $a^N$ reported for VO(acac)py on the basis of an approximate analysis was 5.6 MHz (40).

The analysis of the ESEEM for the carbonate and oxalate complexes of vanadyl transferrin and lactoferrin indicated nitrogen hyperfine coupling constants of 6.6 and 7.0 MHz, respectively. These values are similar to the hyperfine coupling constants obtained by ESEEM in this study for VO(hfac)₂L, L = pyridine, and imidazole, and obtained by ENDOR for nitrogen bound equatorially to vanadyl ion (41–43). The nitrogen hyperfine coupling constants for the vanadyl transferrin and lactoferrin complexes are consistent with coordination of nitrogen, presumably a histidine imidazole, in the equatorial plane of the vanadyl ion.

Vanadyl-nitrogen coupling constants are generally too small to resolve in CW EPR spectra. The absence of resolved nitrogen hyperfine coupling in the CW EPR spectra of VO–anion and VOLS–anion complexes (17, 26–28) could not be used to determine if there is a nitrogen bound to the vanadyl ion. Thus, the ESEEM studies provide information that is not accessible in the CW EPR spectra of these complexes.

Interaction with Exchangeable Protons—Several studies
have demonstrated the utility of comparing ESEEM data for proteins in D₂O and H₂O solutions (44–46). The ratio of two-pulse data in D₂O to data in H₂O separates the deuteron/proton modulation from other modulation frequencies. The deuteron modulation is deeper than the proton modulation, so the ratio is dominated by the deuteron modulation. The ESEEM for CuTfCO₃ in 1:1 D₂O:glycerol-d₃ divided by the ESEEM in 1:1 H₂O:glycerol is shown in Fig. 6a. The analogous ratio for CuLiFCO₃ is shown in Fig. 6b. Similar data were obtained for CuTfox and CuLiFoxy. The ratios of the ESEEM for VOTfCO₃ and VOLfCO₃ in 1:1 D₂O:glycerol-d₃ and 1:1 H₂O:glycerol are shown in Fig. 7, a and b. The deep deuteron modulation is similar for the four copper complexes and indicates similar interaction with exchangeable protons. Although there is not a unique set of distances that is consistent with the data, several conclusions can be reached. 1) There is at most one exchangeable proton within about 2.9 Å of the metal that contributes to the deuteron modulation. 2) There are multiple exchangeable protons within about 3.4 to 4.0 Å of the metal. 3) The exchangeable protons in the environment of the metal that are detected by ESEEM are indistinguishable for the copper and vanadyl complexes.

Nuclear magnetic relaxation studies of CuTfCO₃ and VOTfCO₃ found that the protons responsible for the relaxation effects were relatively distant from the metals (approximately 3.5 Å) and in rapid exchange with solvent (47). Nuclear magnetic relaxation studies of gadolinium(III) transferrin also indicated that second coordination sphere protons were responsible for the relaxation effect (48).

ENDOR studies of CuTfCO₃ indicated one exchangeable proton with an anisotropic interaction or two inequivalent exchangeable protons with couplings ranging from 9.4 to 17.6 MHz (49). Due to the difference in gyromagnetic ratios for protons and deuterons, the corresponding couplings for deuterons would be about 1.5 to 2.7 MHz. If the isotropic interaction is a substantial portion of the coupling observed in the ENDOR experiment, the coupling to these deuterons would be too large for detection by ESEEM. D₂O molecules coordinated to copper that have been detected by ESEEM have had isotropic coupling constants of 0.1 to 0.2 MHz (46). Thus, the ENDOR and ESEEM data for the copper transferrin complexes seem to provide complementary information: the ENDOR-detected protons are in the first coordination sphere of the copper and the ESEEM-detected protons are in the second coordination sphere. Since the unpaired electron for vanadyl iron is an orbital that does not participate in σ bonding to coordinated ligands, isotropic couplings are smaller for vanadyl than for copper(II) (50). An OD group, from water or a protein side chain, bound directly to vanadyl would be expected to have an impact on the ESEEM. The absence of such an interaction in the vanadyl ESEEM indicates that the OD group which the ENDOR experiment indicated was bound to copper(II) is not present in the vanadyl complex. This coordination site may already be occupied by the vanadyl oxygen.

**Coordination of Oxalate**—The observation of ¹³C modulation in the ESEEM data for ¹³C-labeled oxalate bound to CuTf and CuOTf (11, 12) indicated that the oxalate was bound to the copper. Since the ¹³C modulation is a small perturbation on the deep nitrogen modulation, the modulation was observed by taking the ratio of the ESEEM for the [¹³C]₁- and [¹³C]oxalate complexes (11, 12). Similar ¹³C modulation was observed in this study for the ¹³C-labeled oxalate complexes of copper lactoferrin and transferrin. The data for CuTf are in good agreement with the literature (11). The modulation frequency is shifted away from the free carbon frequency for the complexes of all three proteins due to the isotropic coupling, ₁₃ν = 1.0 MHz. Nonzero values of ₁₃ν arise when there is delocalization of the metal unpaired electron into orbitals of the atom that gives rise to the modulation, which occurs when there is a binding pathway between the metal and the nucleus.

The ¹³C/¹²C ratio data for VOTfOx and VOLfox are shown in Fig. 8, a, c and b, d, respectively. In the vanadyl complexes, the ¹²C modulation is at the free carbon frequency which indicates that ₁₃ν = 0.0 MHz. Simulated spectra were obtained for one ¹³C at 2.5 to 2.7 Å from the vanadyl (Fig. 8a and b) and for two ¹³C at 2.8 to 2.9 Å from the vanadyl (Fig. 8c and d). The simulations with one ¹³C at a shorter distance agreed slightly better with the data at short values of τ than the simulations with two ¹³C at a longer distance. Due to uncertainties concerning the assumptions inherent in the simulations, these small differences in the agreement between the calculated and observed data may not permit a distinction between one and two ¹³C nuclei interacting with the vanadyl. However, much weaker ¹³C modulation was observed for VOTf(¹³C)oxalate (Fig. 8e) than for VOTf(¹²C)oxalate (Fig. 8a). Since there is only one labeled carbon in the carbonate and the metal to ¹³C distances are similar for coordinated oxalate and carbonate, the deeper modulation for the oxalate complex argues strongly for two interacting carbons. The interaction with two equivalent, or nearly equivalent, carbon nuclei indicates that the two ends of the oxalate are bound to the metal.

¹³C NMR spectra for GaTf(¹³C)oxalate and AlTf(¹³C)oxalate showed peaks at 166 and 169 ppm for coordinated oxalate (51). The nonequivalence of the two carbons of the oxalate was attributed to coordination of one end of the oxalate to the trivalent metal and interaction of the other end with positively charged protein residue(s) (51). A single broad

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**Fig. 7.** Ratio of the two-pulse ESEEM in 1:1 D₂O:glycerol-d₃ to that in 1:1 H₂O:glycerol at 20K for VOTfCO₃ (a) and VOLfCO₃ (b). The data were obtained at a magnetic field of 3250 G which corresponds to a perpendicular line in the CW EPR spectrum. The simulated spectra were obtained with one deuteron at 3.0 Å and eight deuterons at 3.4 Å. Other parameters used in the simulations were γ = 0.85741, α = 0.0, eeQ = 0.22 MHz, and τ = 0.10.
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peak at 170 ppm was obtained in the $^{13}$C NMR spectrum of ZnTf($[^{13}$C]oxalate) (51). It was proposed that the oxalate was again bound to the divalent metal through one end, but that the interaction of the other end with the protein resulted in a chemical shift that was similar to that for the end that was coordinated to the metal (51). Our results suggest another interpretation for these results. Both ends of the oxalate could be coordinated to the metal, but one end of the oxalate also interacts with the positively charged protein residue. The additional interaction with the protein could make the two carbons nonequivalent. A chemical shift difference between two coordinated ends of the oxalate could also arise from the different trans ligands. These effects may be different for the trivalent and divalent metals, resulting in a larger shift difference for the Ga(III) and Al(III) complexes than for the Zn(II) complex.

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Supplementary Material to
Comparison of the Electron Spin Echo Envelope Modulation (ESEEM) for Human Lactoferrin and Transferrin Complexes of Copper(II) and Vanadyl Lactoferrin

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EXPERIMENTAL PROCEDURES

Materials. Iron(III) lactoferrin (IF) and human milk lactoferrin (IF) were obtained from Sigma Chemical Co. and used without further purification. The purity of the transferrin and lactoferrin was checked by SDS-PAGE (44). For both proteins a single band was resolved. Hemagglutinating (Hg(Hg-Hg)2) complex was obtained from Sigma Chemical Co. 99%-enriched 125I-labeled and 99%-enriched and iodine-labeled (64) human transferrin (HGM Biomedicals Inc. and Biosearch, respectively) 99%-deuterated glycine-3 was obtained from Cambridge Isotope Laboratories. Vanadyl (vanadium(IV))-conjugated 1H NMR spectra were reported in the characteristic (32). All radioactive disposables and pipetters were used for all manipulations of the protein solutions. The protein solutions were obtained in 4 petals to tubes.

Methods. The transferrin or lactoferrin metal-amine complexes were formed in 0.05 M Hepes buffer, CuCl2 and CuCl2 were used for the Hepes buffer at pH 7.4. For CuCl2 and CuCl2 were used for the Hepes buffer at pH 7.4. In an nitrogen atmosphere to avoid oxidation of vanadyl ions to vanadium(V) (16, 17). The solution was then placed in a glove box with a nitrogen atmosphere to avoid contamination with carbonate as the pyrogenic anion (18, 19). The appropriate buffer solution was purged with nitrogen gas to remove traces of CO2 or O2. In the solution of nitrogen gas was added to the apropiate solution. The pH of the resulting solution was adjusted to 7.4–7.5 with sodium gas mixed with nitrogen. The anion/nitrogen gas stream was passed through a 2.5 cm 0.10 mm. Samples for the FFR studies were prepared by mixing the aqueous solutions with an equal volume of glycine. The protein concentration in the aqueous solutions was 0.1 to 0.10 mg/ml. The mixtures were frozen immediately and stored in liquid nitrogen until the spectra were obtained.

Visible Spectra. A Cary-14 with an On-Line System 3920c modulacions was used. The visible spectra were obtained at 0.1 cm light path and an air gap was used.

EPR Spectra. The EPR spectra were obtained at 0.1 cm on an IBM ER2000 with 100 MHz modulation at modulation amplitudes and microwave power of 0.1 to 1.0 mW. The temperature was 77.0 K. All spectra were obtained with 0.1 cm light path and an air gap. The samples were obtained with two-pulse or three-pulse sequences on a home-built spectrometer that has been described previously (21). The time for a pulse was 20.0 ns. A 200 MHz liquid-helium cryostat was used to obtain sample temperatures of 5–50 K. The data shown in the figure were obtained at 4.0 K. All EPR data were obtained for all of the samples and 3-pulse data were obtained for selected samples.

Characterization of Complexes. Formation of the copper(II) lactoferrin and transferrin complexes was monitored by visible spectra and continuous wave (CW) EPR spectra. The visible spectral data are summarized in Table 1. The band in the visible spectrum at 430–440 nm is characteristic of complex formation and has been assigned to charge-transfer interaction with coordinated tyrosine (22). The EPR spectra were monitored in six different samples with the EPR spectra, CuCl2 and CuCl2 were identically in experimental uncertainty and are in agreement with those reported in the literature for CuCl2 (23, 24). The EPR spectra are shown in Fig. 1A, 1B, and 1C. The EPR spectra for CuCl2 and CuCl2 are essentially identical to each other and exhibit slight differences in the relative line intensities, which are in agreement with published spectra (23). Consistent with published results for CuCl2 and CuCl2, CuCl2 was found to be specifically bound to either transferrin or lactoferrin (23).

Table 1

| EPR spectroscopic data for CuCl2 transferrin and lactoferrin complexes and proteins | Reference | Protein | Electronic excitation | One electron transfer | Complex excitation | Complex stability |
| --- | --- | --- | --- | --- | --- | --- |
| EPR spectroscopic data for CuCl2 transferrin and lactoferrin complexes and proteins | Reference | Protein | Electronic excitation | One electron transfer | Complex excitation | Complex stability |
| EPR spectroscopic data for CuCl2 transferrin and lactoferrin complexes and proteins | Reference | Protein | Electronic excitation | One electron transfer | Complex excitation | Complex stability |

Six different proteins were monitored by EPR spectroscopic data for CuCl2 transferrin and lactoferrin complexes and proteins. The position of the bands and maxima are given in Table 1 and the nitrogentation in parentheses are per mol of metal.

Unlike the copper complexes, the VO(V)-enol and VO(V)-enol complexes are more complex. The VO(V)-enol complexes consist of a single band with CW EPR spectra (17, 28). Two bands of vanadyl bond to each mole of copper and vanadyl complex (23) with either carbonate or carbonate as the pyrogenic anion.

Analysis. The EPR experiments were pursued in order to obtain the characteristic modulation frequencies for the spectra of EPR spectra (23, 24). The position of the bands and maxima are given in Table 1.

"dead-time." Simulations were performed on a MicroVAX II and typically used 500 orientations of the molecule in the magnetic field at intervals of 8 ps along the time axis. Parameters for individual simulations are given in the figure captions: $r$ is the distance between the metal and the nitrogen, $s$ is the isoelectronic hyperfine constant, $e$ is the quadrupole coupling constant, and $o$ is the quadrupole symmetry parameters. The spectra were analyzed by first-order perturbation theory (30). Simulated spectra including quadrupole interactions in the transfromion of signals at the about 0.8 MHz in the two-pulse data split into two peaks in the three-pulse data. As noted in the analysis of metal-ligand complexes of histidines imidazoles, the lower frequencies are almost purely quadrupolar in origin. This occurs when the nuclear Zeeman frequency is about one-half the isotropic coupling (31, 32). The calculations presented in the analysis indicate that the quadrupolar coupling constant of 1.7 to 1.8 MHz for the copper-lactoferrin complex, which is consistent with the assignment of the low frequencies as nitrogen quadrupole frequencies. First-order analysis of the nitrogen frequencies (Table 2) with the assumption that the frequencies were approximately superimposed at 0.9 MHz gave values of the quadrupole parameters that were in good agreement with the results obtained from the simulations.

Combination frequencies were not observed in the Fourier transform of the three-pulse EPR spectra shown in Fig. 1A, 1B, and 1C. The absence of combination frequencies indicates that there were no deuteron atoms less than 2.5 Å from the metal. The deuteron frequencies in the VO(V)-enol complexes and the nitrogen frequency were far from the center of the quadrupolar hyperfine interaction. The frequency of the nitrogen frequency and the quadrupolar hyperfine interaction is about 0.8 MHz in the two-pulse data split into two peaks in the three-pulse data. As noted in the analysis of metal-ligand complexes of histidines imidazoles, the lower frequencies are almost purely quadrupolar in origin. This occurs when the nuclear Zeeman frequency is about one-half the isotropic coupling (31, 32).
in the perpendicular region of the EPR spectrum. Thus some caution must be exercised due to the failure of the simulations to account for "angular selection".

**Figure 3.** Two-pulse ESEEM obtained at 20 K for A) VO(hfac)$_2$pyridine in toluene solution at 3200 G and B) VO(hfac)$_2$imidazole in 1:1 toluene:chloroform solution at 3235 G. The magnetic fields correspond to perpendicular lines in the CW EPR spectra.

**Figure 4.** A) Cosine Fourier transform of the ESEEM data for VO(hfac)$_2$pyridine. B) Simulated spectrum obtained with $r = 2.0$ Å, $a = 9.3$ MHz, $\varepsilon = 0.1$ MHz and $\eta = 0.2$. Proton modulation was not included in the simulation.

**Figure 5.** A) Cosine Fourier transform of the ESEEM data for VO(hfac)$_2$imidazole. B) Simulated spectrum obtained with $r = 2.0$ Å, $a = 7.6$ MHz, $\varepsilon = 1.3$ MHz, and $\eta = 0.3$. Proton modulation was not included in the simulation.

**Figure 6.** Ratio of the two-pulse ESEEM in 1:1 D$_2$O:glycerol-d$_3$ to that in 1:1 H$_2$O:glycerol for A) CuTFCO$_3$ and B) CuO$_3$CO$_3$ at 5 K. The data were obtained at a magnetic field of 3200 G which corresponds to a perpendicular line in the CW EPR spectrum. The simulated spectra were obtained with one deuteron at 3.0 Å and eight deuterons at 3.4 Å. Other parameters used in the simulations were $\hbar \omega = 0.3974$ T, $a = 0.0$, $\varepsilon = 0.22$ MHz, and $\eta = 0.3$. Other