Pulsed Electron Paramagnetic Resonance Studies of the Copper Complexes of Transferrin*

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The nuclear modulation effect in pulsed EPR spectroscopy was used to probe the metal-protein bonds in Cu(II)-transferrin complexes as a function of pH. In preparations in equilibrium with air at pH 4.8 no modulation features other than those attributable to protons were observed, in keeping with conventional EPR studies indicating that at this pH only nonspecific binding takes place (Zweier, J. L., and Aisen, P. (1977) J. Biol. Chem. 252, 6090-6096). At pH 7.7, however, where each of the two specific sites of transferrin accommodates Cu(II) with carbonate as the associated anion, a modulation pattern is obtained which indicates that an imidazole ligand is coordinated to specifically bound Cu(II). This is consistent with the nitrogen superhyperfine structure in the EPR spectrum of Cu(II)-transferrin-carbonate, and corroborates chemical modification studies implicating histidine residues at the specific sites (Line, W. F., Grohlich, D., and Bezkorovainy, A. (1967) Biochemistry 6, 3393-3402). At pH 11.4, where the EPR spectrum shows three or four nitrogen ligands interacting with the first Cu(II) bound to transferrin in the presence or absence of carbonate or other binding anions, the modulation envelope continues to indicate that imidazole is a ligand to the Cu(II), suggesting that the metal is bound to a specific site and not to a bluetype complex.

A comparison of the envelopes of electron spin echoes of Cu(II)-transferrin-[13C]oxalate and Cu(II)-transferrin-[13C]oxalate reveals the presence of a Cu(II)-13C superhyperfine interaction in the latter complex. This exceeds the free precession frequency of 13C by a factor greater than 5 and is indicative of a contact interaction between Cu(II) and 13C. These results confirm that the anion is directly coordinated to the metal ion in the specific Cu(II)-transferrin-oxalate complex.

Because of its ability to bind to the two specific sites of transferrin, Cu(II) has been of great value as a spectroscopic probe of the metal-binding properties of the protein (1-3). In particular, the sensitivity to ligand structure of the hyperfine and superhyperfine splittings in the EPR spectra of Cu(II)-transferrin complexes has been exploited to demonstrate a spectroscopic difference between the two sites, the susceptibility of oxalate as the associated anion at only one site, and the probable involvement of only one specific site in Cu(II) binding at high pH in the absence of a bound anion (3). Recently, pulsed EPR methods have been applied to the study of metal-protein complexes to supplement information obtainable from conventional, continuous wave EPR spectroscopy (4-6). Pulsed EPR spectroscopy has been of particular value in identifying imidazole ligands of Cu(II) in copper proteins (4, 5, 7). We have, therefore, applied this technique to the study of several previously characterized Cu(II) complexes of transferrin. In addition to confirming the participation of histidine at the active sites, it has been used to demonstrate for the first time 13C superhyperfine structure (from [13C]oxalate) in the modulation pattern of the spin-echo envelope for the copper protein, thus establishing that a specifically bound metal ion is coordinated directly to its associated anion.

EXPERIMENTAL PROCEDURES

Materials—Chelate-free human apotransferrin was obtained by methods previously described (8). All complexes of transferrin with Cu(II) were prepared with 64Cu obtained as its oxide from Oak Ridge National Laboratory, and dissolved in 0.1 M HCl before use. Oxalic acid labeled with 13C (90%) was purchased from Stohler Isotopic Chemicals and used without further purification. Doubly distilled water was used for all experiments, and reagents were the highest quality commercially obtainable.

Methods—Procedures for the preparation of Cu(II) complexes of transferrin and its carbonate and oxalate derivatives followed those set forth in an earlier publication (3). Preparations were concentrated with a Schleicher and Schuell ultrafiltration apparatus to a final Cu(II) concentration of 1 to 2 x 10^-3 M. Oxalate complexes of Cu(II) were prepared by adding 0.2 ml of 0.1 M cupric acetate and 0.4 ml of 0.4 M oxalic acid, brought to pH 8.0 with sodium hydroxide, to 0.4 ml of water. After mixing, a further addition of 1.0 ml of glycine was made. The final solutions were 0.01 M in Cu(II) and had pH values in the range 7.2 to 7.4.

Electron spin-echo measurements were performed at X-band (~9 GHz) and a temperature of 4.2 K. The spectrometer, and the system used for tracing the envelope of the spin echo, have been described by Mims and Peisach (4).

RESULTS AND DISCUSSION

The envelope of electron spin echoes was obtained by plotting the amplitude of the spin echoes as a function of the time interval between echo-generating microwave pulses. This envelope is commonly observed to be modulated by frequencies characteristic of the nuclei coupled to the electron spin, i.e. by the ENDOR frequencies of the system (9). It can, therefore, under favorable circumstances, provide insights into the ligand structure of a paramagnetic metal complex which is not obtainable from conventional EPR spectroscopy.

Fig. 1 displays the electron spin-echo envelopes of complexes of transferrin and Cu(II) at three different values of pH. At pH 4.8, in preparations in equilibrium with air, the
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Fig. 1. Electron spin-echo envelopes for Cu(II)-transferrin obtained at pH 4.8 (A), pH 7.7 (B), and pH 11.3 (C). In A, the major features consist of two periods, of 36 ns and 72 ns which arise from the interaction of Cu(II) with protein-bound and solvent protons (11). In B and C the low frequency periods arise from the interaction of Cu(II) with the remote nitrogen of an imidazole ligand. In A, the envelope was obtained at a Zeeman field, $H_0$, of 3230 G and a frequency $\nu$ of 9.341 MHz. In B, $H_0 = 3720$ G and $\nu = 9.460$ MHz; while in C, $H_0 = 3271$ G and $\nu = 9.385$ MHz.

most prominent features in the electron spin-echo envelope are those due to protons (10, 11) (Fig. 1A). The corresponding EPR spectrum in the presence or absence of added carbonate is inhomogeneously broadened and lacks indication of nitrogen superhyperfine structure (2, 3). By pH 7.7, however, each specific site of transferrin can accommodate a cupric ion with carbonate as its associated anion (3). The EPR spectrum of monocupric transferrin at pH 9.6 or higher shows a complex superhyperfine structure which must arise from three, or even four, nitrogen ligands (2, 3), and which is anion-independent (3). At pH 11.3, the presence of a low frequency modulation pattern in the spin-echo envelope (Fig. 1C), which is essentially the same as seen at pH 7.7 (Fig. 1B), unequivocally confirms that the Cu(II) binding site in monocupric transferrin is not structurally analogous to biuret. (Ni-

residues at the active sites of transferrin (12). The present spectroscopic studies corroborate this assignment, and also suggest that the nitrogen ligand contributing to the superhyperfine pattern in the EPR spectra of Cu(II)-transferrin-carbonate is derived from the imidazole group of a histidine residue. It should be noted, however, that the superhyperfine splitting in the EPR spectrum, and the low frequency modulation in the electron spin-echo decay envelope, do not originate from the same hyperfine interaction. The splitting in the EPR spectrum is 9 G (2), or about 25 MHz, and arises from interaction of the Cu(II) electron spin with the nuclear spin of a nitrogen ligand directly coordinated to the copper. Nitrogen directly coordinated to Cu(II) does not, however, contribute to the modulation pattern (4, 5). Rather, modulation of the electron spin-echo envelope can be observed when the interaction energy is comparable to the nuclear Zeeman and nuclear quadrupole terms, a condition fulfilled by the coupling between the Cu(II) spin and the remote nitrogen of a coordinated imidazole group. This has recently been shown to arise from a contact interaction of the type $A \cdot I \cdot S$, where $A = 1.75$ MHz (0.6 G) (13). At present, it cannot be determined with confidence whether only a single imidazole group contributes to the modulation pattern, corresponding to the superhyperfine splitting which arises from a single nitrogen ligand in the EPR spectrum of Cu(II)-transferrin-carbonate.

A point of some controversy concerns the ability of transferrin to bind Cu(II) at a specific site at high pH (3, 14). The EPR spectrum of monocupric transferrin at pH 9.6 or higher shows a complex superhyperfine structure which must arise from three, or even four, nitrogen ligands (2, 3), and which is anion-independent (3). At pH 11.3, the presence of a low frequency modulation pattern in the spin-echo envelope (Fig. 1C), which is essentially the same as seen at pH 7.7 (Fig. 1B), unequivocally confirms that the Cu(II) binding site in monocupric transferrin is not structurally analogous to biuret. (Ni-

Fig. 2. Electron spin-echo envelope for Cu(II)-transferrin-oxalate prepared with $^{13}$C-oxalate (A) and $^{15}$C-oxalate (B) at pH 8.9. In both samples the low frequency period arises from a histidine imidazole nitrogen interacting with Cu(II). The new features in the modulation envelope obtained with $^{13}$C-oxalate, which, for example, can easily be seen near $\tau = 1.18$ and 1.35 ns arises from $^{13}$C interaction with Cu(II). For the $^{15}$C-oxalate sample, $H_0 = 2830$ G and $\nu = 8.793$ MHz; while for the $^{15}$C-oxalate sample, $H_0 = 2830$ G and $\nu = 8.728$ MHz.
When \(^{14}C\)oxalate serves as the binding anion a new low frequency modulation, in addition to that associated with imidazole nitrogen, is detected (compare Fig. 2, A and B). It has been shown that when several nuclei are coupled to an electron spin the resulting modulation pattern is given by the product of the modulation patterns due to each nucleus considered separately (9, 15). It should therefore be possible to isolate the contribution due to \(^{13}C\) by plotting the ratio of the electron spin-echo envelope of Cu(II)-transferrin-[\(^{13}C\)]oxalate to that of Cu(II)-transferrin-[\(^{14}C\)]oxalate. A ratio plot of this kind is shown in Fig. 3 To establish that the period in the resulting modulation pattern is due to the \(^{13}C\)-nucleus, a model complex of Cu(II) and [\(^{13}C\)]oxalate was prepared and its spectrum recorded. A period of 149 ns is seen (Fig. 4B), which is not present in the [\(^{13}C\)]oxalate complex (Fig. 4A), and which, therefore, must be attributed to a (Cu(II)-\(^{13}C\)) superhyperfine interaction. This period exceeds that due to the free precession frequency of \(^{13}C\), 3.0 MHz at 2830 G, and probably arises from a contact interaction of the same order of magnitude as the Zeeman interaction. For the ratio plot (Fig. 3), a period of 158 ns was observed. This is within experimental error of the period observed in Cu(II)-[\(^{13}C\)]oxalate. For reasons which are as yet not completely understood a comparable interaction is not observable when \(^{12}C\)O functions as the associated anion; it may be that the Cu(II)-\(^{12}C\) coupling is smaller in this case so that modulation is too weak to be detected in the presence of the modulation patterns due to \(^{14}N\) and \(^{1}H\). It is also conceivable that both carboxyl groups of the oxalate are metal-bound, thereby producing an enhanced effect in the modulation envelope not seen with carbonate. Another possibility is that carbonate is not directly coordinated to the specifically bound metal ion as is oxalate, but this seems unlikely since both anions function similarly in stabilizing the metal-protein bond. There seems little doubt, however, that the oxalate is coordinated directly to the Cu(II), an inference previously reached with studies of transferrin based on \(^{13}C\) NMR spectroscopy (16) and on EPR spectroscopy with a spin-labeled derivative of oxalate as the obligated anion for Fe(III)-binding (17).

**FIG. 4.** Electron spin-echo envelopes for Cu(II) formed in a complex with \([^{13}C\)]oxalate (A) and \([^{14}C\)]oxalate (B). The pattern shown in the upper curve arises from the interaction of Cu(II) with protons from bound and solvent water, while superimposed on the lower curve is an additional modulation pattern, with a period of 149 ns or 6.7 MHz which arises from the Cu(II)-\(^{13}C\) interaction. For the \(^{13}C\) complex, \(H_{0} = 2830\) G and \(\nu = 8.946\) MHz; while for the \(^{12}C\) complex, \(H_{0} = 2830\) G and \(\nu = 8785\) MHz.

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