EPR Study of Cu(II) in *N*-Terminal Lobe Fragment of Hen Ovotransferrin

Teruyuki Kawabata,* Masayoshi Fujisawa, Akira I. Hida, and Shigeru Okada
Department of Pathological Research on Diseases, Medical and Dental Graduate School, Okayama University, 2-5-1 Shikata-cho, Okayama 700-8558

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The *N*-terminal lobe fragment of hen ovotransferrin was prepared by trypsin digestion and the following gel filtration and ion-exchange chromatography. EPR spectra of Cu(II) complex of the *N*-terminal fragment were recorded independently from the *C*-terminal lobe and a major spectrum of the copper(II) complex showed $g_{//} = 2.300$ and $g_{\perp} = 2.061$ with $A^{Cu}_{//} = 14.0$ mT, $A^{Cu}_{\perp} = 2.20$ mT, which had a superhyperfine splitting ($A^{N}_{\perp} = 1.44$ mT) of a nitrogen ligand, which may correspond to type A-1 site of copper binding position in the *N*-terminal lobe of hen ovotransferrin.

Transferrin family proteins, such as serum transferrin, lactoferrin and ovotransferrin, play important roles in iron metabolism and host defense mechanism in our body.[1–3] The proteins have two metal-binding sites and chelate several kinds of metals, such as iron, copper, aluminum, zinc and nickel.[4–7] The iron-binding structure of the transferrins has been most intensively studied with physicochemical and biological techniques and the crystallized structures of *N*-lobes of recombinant and mutant human serum proteins are available.[8,9] The proteins are composed of *N*-terminal and *C*-terminal lobes, which are joined by a hinge region and each lobe has a very similar, but a little different iron-binding environment. EPR offered a significant insight to the iron-binding structures and we reported an EPR study of Fe(III) in the *N*-terminal lobe of ovotransferrin.[10] In addition to iron, the copper-binding structure was also well documented.[11–13] The human lactoferrin was crystallized as copper-binding form[14] which has square pyramidal geometry in *N*-terminal lobe and distorted octahedral one in *C*-terminal. On EPR study of the copper of ovotransferrin, the two binding sites showed different spectra as type A-1 and type B-1 at physiological pH, and an additional type B-2 at alkaline solution.[15,16] In these studies, copper complexes in the *N*-terminal and *C*-terminal lobes were separately prepared by using chemical properties different from each copper-binding site and the EPR spectra were recorded as a whole protein. In this study we prepared *N*-terminal lobe fragment of hen ovotransferrin with proteolytic digestion and measured EPR spectra of the copper complex independently from the *C*-terminal lobe.

The *N*-terminal fragment was prepared by the previously described method.[17,18] Hen ovotransferrin (Wako Pure Chemical Industries, Osaka, Japan) was digested with trypsin in 0.1 mol dm^{-3} Tris-HCl buffer (pH 8.0) containing 10 mmol dm^{-3} $CaCl_2$ after the iron saturation of both iron-binding sites. After the digestion *N*-terminal fragment was separated by gel chromatography with Sephacryl S200 and the following ion-exchange chromatography (CM-Sepharose CL6B). The purified *N*-terminal fragment was analyzed with a protein sequencer (Applied Biosystems Model 477/120A) according to the manufacturer's protocol. The fragment of ovotransferrin had an amino acid sequence of APPKSVI-, which was the same sequence of *N*-terminal of ovotransferrin. EPR study was done after copper saturation of the *N*-terminal fragment with $CuSO_4$ in HEPES buffer (pH 7.4). The iron-binding structure in the *N*-terminal lobe was kept intact by the digestion and showed a specific UV–vis absorption that is ascribed to LMCT of tyrosine ligand and a characteristic EPR spectrum.[10]

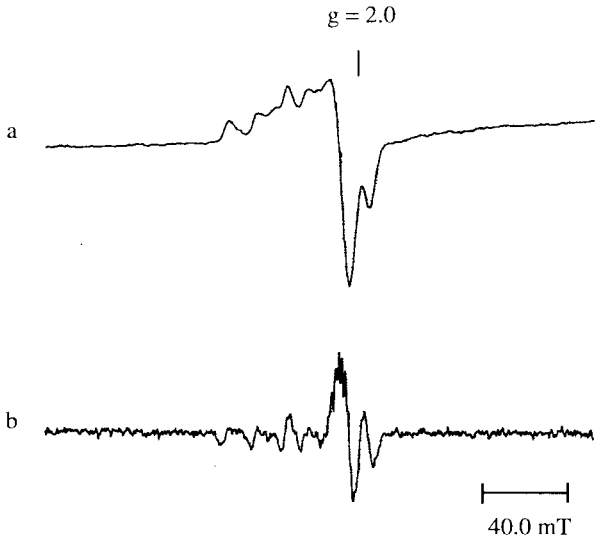

Figure 1. EPR spectra of Cu(II) complex of *N*-terminal fragment of hen ovotransferrin. EPR spectra were recorded at 120 K equipped with 100 kHz magnetic field modulation and Mn^{2+} in MgO was used as a standard g marker. a: the first-derivative and b: the second-derivative EPR spectra.

EPR spectra were recorded at 120 K by a JEOL EPR spectrometer JES-1XG (Tokyo, Japan), equipped with 100 kHz magnetic field modulation and Mn^{2+} in MgO was used as a standard g marker. The simulation of EPR spectra was done with Bruker's WIN-EPR software packages (Rheinstetten, Germany). Figure 1 shows the first- (Figure 1a) and second-derivative (Figure 1b) EPR spectra of Cu(II) in *N*-terminal fragment, which had a well-documented spectrum of $g_{//} > g_{\perp}$ with $A^{Cu}_{//} = 14.0$ mT and the superhyperfine splitting of a nitrogen ligand on $g_{\perp}$ was clearly observed on the second derivative spectrum (Figure 1b). In addition to the major complex, we observed smaller another spectrum in Figure 1, which may be non-specific or contaminated one. Figure 2 shows a magnified second derivative EPR spectrum around $g_{\perp}$ of Figure 1b and a simulated splitting with hyperfine and superhyperfine structures. Table 1 summarizes our EPR parameters and other

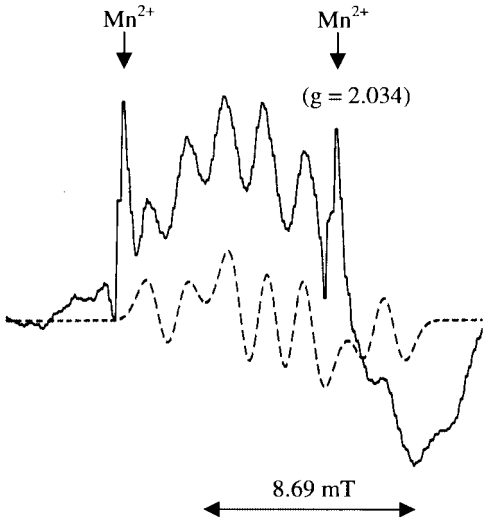

Figure 2. EPR spectrum of Cu(II) complex of *N*-terminal fragment around $g_{\perp}$ and the simulated line. ——: the second-derivative EPR spectrum around $g_{\perp}$; -----: the simulated line with hyperfine and superhyperfine structures.

Table 1. EPR parametcrs of Cu(II) complexes of transferrins

Cu(II)-complex[a,b]	$g_{//}$	$g_{\perp}$		$A^{Cu}_{//}$ (mT)	$A^{Cu}_{\perp}$ (mT)	$A^{N}_{\perp}$ (mT)
		g_x	g_y			
N-terminal ovoTf (this study)	2.300	2.061		14.0	2.20	1.44
ovoTf(A-1)[16]	2.313	2.066		14.6	n.d.	n.d.
serum Tf[19]	2.313	2.062		16.7	2.04	1.26
serum Tf[20]	2.312	2.043	2.060	15.6	< 3.0	< 3.0
lactoTf[13]	2.315	2.057		15.1	n.d.[c]	n.d.

[a]ovoTf: ovotransferrin; Tf: transferrin; lactoTf: lactoferrin. [b]The superscript numbers indicate the referenees where the parameters cited from. [c]n.d.: not determined.

reported ones.[13,16,19,20] The major EPR spectrum of Cu(II) complex of *N*-terminal lobe fragment had similar EPR parameters to ones of Cu(II) in transferrins with a nitrogen superhyperfine splitting, which may correspond to type A-1 site of copper-binding positions of ovotransferrin.

EPR spectrum of Cu(II) in the *N*-terminal lobe fragment of hen ovotransferrin was first reported independently from *C*-terminal lobe, and showed similar EPR spectra to ones of Cu(II) in *N*-terminal lobe of whole transferrin proteins. However we did not study the pH dependence and the effect of synergistic anions. Further detailed studies should be done to elucidate the coordination environment around copper in the *N*-terminal lobe fragment.

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