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EPR study of nitric oxide-treated reduced ceruloplasmin

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SUMMARY

EPR inactive reduced ceruloplasmin reacts reversibly with nitric oxide, to form paramagnetic Cu(II)–NO^- complexes which exhibit EPR absorptions centered around $g = 2$ and $g = 4$. The EPR absorption around $g = 4$ is caused by a 'forbidden' $\Delta m = 2$ transition within the triplet state of magnetic dipole–dipole coupled copper ion pairs.

Ceruloplasmin contains three types of copper; paramagnetic type-1 and type-2 copper and diamagnetic copper[1–3]. As has been described by us nitric oxide reacts reversibly with type-1 copper of oxidized ceruloplasmin, under formation of a diamagnetic charge–transfer complex[4]. In this communication we report on the effect of nitric oxide on reduced ceruloplasmin and will present evidence for dipole–dipole coupled copper atoms in the enzyme.

EPR signals of inorganic copper dimers and dimeric copper porphyrins have been studied extensively by Boas *et al.*[5] and Boyd *et al.*[6], respectively. As they have shown in these complexes dipole–dipole coupling or electron exchange between the two copper ions with spin $s = 1/2$ or a combination of both give rise to a singlet ($S = 0$) and a triplet ($S = 1$) state. Within the triplet state $\Delta m = 1$ transitions are allowed and originate an intense EPR signal around $g = 2$, but also 'forbidden' $\Delta m = 2$ transitions can occur, causing a weak EPR signal at half field ($g = 4$). Furthermore, when the value of the exchange constant (J) is small, $\Delta m = 1$ transitions between the singlet and the triplet state are also possible[7], giving rise to weak EPR absorptions around $g = 2$. In proteins these types of transitions, ascribed to copper–copper dimers have also been observed by Schoot Uiterkamp[7,8] and Schoot Uiterkamp and Mason[9] in NO-treated tyrosinase and hemocyanin.

Abbreviation: PMS, phenazine methosulphate.

Ceruloplasmin (EC 1.12.3.1) was isolated from human Cohn fraction IV by a procedure essentially according to Veldsema and Van Gelder[10]. Reduction of ceruloplasmin, which could maximally accept 4 reducing equivalents, was carried out with an excess of NADH in the presence of a catalytic amount of phenazine methosulphate (PMS)[11,12]. Chemicals were analar grade, obtained from British Drug Houses, except for NADH and PMS which were purchased from Sigma Chemical Co.

Reduced ceruloplasmin showing no EPR absorption was incubated anaerobically with approximately 0.5 atm NO (Matheson) for 5 min at 20 °C. The sample was frozen in liquid nitrogen and the excess of NO was removed by repeated evacuation. The EPR spectrum arising after this treatment is shown in Fig. 1.

A broad signal of about 1100 gauss wide, centered at 3050 gauss ($g=2$), and a smaller signal at half field ($g=4$) are observed. The appearance of this EPR spectrum indicates an oxidation of diamagnetic Cu(I) by NO to a paramagnetic Cu(II)–NO^- complex. It is interesting to note that the proposed electron transfer between nitric oxide and the copper ions in the reduced enzyme is opposite to that in the oxidized enzyme. In the latter case an electron transfer takes place from NO to the metal ion, resulting in a positively charged ligand. Recently it has also been reported by Yonetani *et al.*[13] and Enemark and Feltman[14] that NO can act either as an oxidant or as a reductant.

Like in oxidized ceruloplasmin the reaction of NO with reduced ceruloplasmin is reversible. This is demonstrated by the disappearance of the EPR signal after removal of NO by repeated evacuation of the cuvette and flushing with oxygen-free nitrogen gas. Reoxidation of this sample with oxygen restores the original EPR spectrum of oxidized ceruloplasmin.

The $g=2$ signal (Fig. 1) shows a complicated hyperfine splitting of approximately 70 gauss superimposed on a broad signal with a width of 1100 gauss. In the $g_\perp$ region a not yet identified signal at $g=2.021$ is observed which is saturated at the conditions used in Fig. 1. The broad signal is a $\Delta m=1$ absorption originating from magnetically coupled copper pairs and the narrow signal with the hyperfine splitting is ascribed to single copper sites reacting with NO. A power dependence study between 0.5 and 80 mW at 12 °K of these signals show that the narrow signal is saturated at a lower power than the broad one. It is interesting to note that these types of signals are also found by Schoot Uiterkamp[7,8] and Mason and Schoot Uiterkamp[9] in NO-treated hemocyanins from *Cancer magister* and *Helix pomatia*.

The temperature dependence of the $g=2$ signal of NO-treated reduced ceruloplasmin, measured at 230, 180, 123 and 83 °K at a power of 20 mW, and at 10 °K at a power of 0.5 mW exhibits normal Curie behaviour of the signal intensities (not shown). The fact that there is no deviation from normal Curie behaviour between 230 and 10 °K limits the exchange constant (J) between the singlet and triplet state of the two Cu(II) ions to values lower than 7 cm^{-1}. Therefore, the exchange contribution is neglected in computer simulations of the $\Delta m=2$ signal, now in progress. A power-dependence study of the signal at half field at 12 °K shows in contrast to the signal around $g=2$, no saturation between 0.5 and 160 mW. This is consistent with the small transition probability of the $\Delta m=2$ signal.

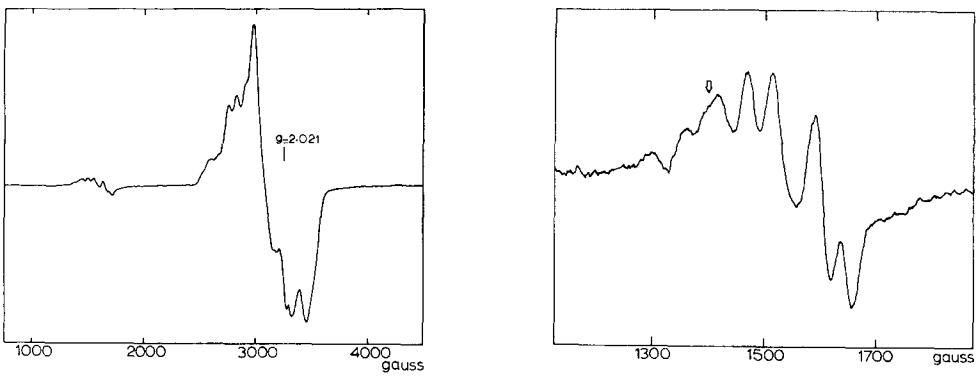

Fig. 1. EPR spectrum of reduced ceruloplasmin in the presence of NO. Ceruloplasmin (500 μM; $A_{610\ nm}/A_{280\ nm}$ = 0.046) was dissolved in 100 mM sodium acetate and 250 mM NaCl (pH 7.0). The protein was reduced with NADH (3 mM) and PMS (20 μM) and incubated anaerobically with NO at 20 °C for 5 min, after which the sample was frozen. Spectrum was recorded on a Varian E-3 EPR spectrometer equipped with a helium-flow system (Air Products Inc.). The temperature was measured with a carbon resistor, placed just below the sample and previously calibrated against a calibrated germanium resistor situated at the sample position. Magnetic field was measured with an AEG EPR-field meter (GA 11-22.2). Frequency was determined with a Hewlett–Packard frequency counter (HG 5246 L) with a frequency convertor (5255 A). Microwave power, 20 mW; modulation amplitude, 10 gauss; frequency, 9.105 GHz; temp., 12 °K.

Fig. 2. Magnification (12.4 ×) of $\Delta m=2$ absorption shown in Fig. 1 of NO-treated reduced ceruloplasmin EPR conditions as described in Fig. 1.

The seven hyperfine lines, which are expected for a $\Delta m=2$ transition in the case of a coupling between two copper ions with a nucleus spin L=3/2 are well resolved. As shown in Fig. 2, which is a magnification of the signal at half field of Fig. 1, the hyperfine splitting of this signal is irregular and besides in one of the lines a further splitting can be clearly observed as indicated by the arrow in the figure. This suggests that the $\Delta m=2$ signal is composed of different types of EPR absorptions.

With oxidized ceruloplasmin we have demonstrated that NO reacts specifically with type-1 copper. At this moment, however, it is not yet possible to decide which of the three types of copper in reduced ceruloplasmin reacts with NO, and is responsible for the observed $\Delta m=1$ and $\Delta m=2$ signals. Computer simulations of the shown EPR spectra are in progress in order to obtain more information about the distance between and the geometry of the copper atoms.

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REFERENCES

1 Vänngård, T. (1967) in *Magnetic Resonance in Biological Systems* (Ehrenberg, A., Malmström, B.G. and Vänngård, T., eds), pp. 213–219, Pergamon Press, Oxford
2 Andréasson, L.-E. and Vänngård, T. (1970) *Biochim. Biophys. Acta* 200, 247–257
3 Malkin, R. and Malmström, B.G. (1970) *Adv. Enzymol.* 33, 177–244
4 Wever, R., Van Leeuwen, F.X.R. and Van Gelder, B.F. (1973) *Biochim. Biophys. Acta* 302, 236–239
5 Boas, J.F., Dunhill, R.H., Pilbrow, J.R., Srivastava, R.C. and Smith, T.D. (1969) *J. Chem. Soc. A*, 94–108
6 Boyd, P.D.W., Smith, T.D., Price, J.H. and Pilbrow, J.R. (1972) *J. Chem. Phys.* 56, 1253–1263
7 Schoot Uiterkamp, A.J.M. (1973) Ph.D. thesis, University of Groningen
8 Schoot Uiterkamp, A.J.M. (1972) *FEBS Lett.* 20, 93–96
9 Schoot Uiterkamp, A.J.M. and Mason, H.S. (1973) *Proc. Natl. Acad. Sci. U.S.* 70, 993–996
10 Veldsema, A. and Van Gelder, B.F. (1973) *Biochim. Biophys. Acta* 293, 322–333
11 Van Gelder, B.F. and Slater, E.C. (1963) *Biochim. Biophys. Acta* 73, 663–665
12 Van Gelder, B.F. and Veldsema, A. (1966) *Biochim. Biophys. Acta* 130, 267–269
13 Yonetani, T., Yamamoto, H., Erman, J.E., Leigh, Jr, J.S. and Reed, G.H. (1972) *J. Biol. Chem.* 247, 2447–2455
14 Enemark, J.H. and Feltham, R.D. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 3534–3536