Binuclear Copper Electron Paramagnetic Resonance Signals of α-Methemocyanin of *Helix pomatia*

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The binuclear electron paramagnetic resonance signals of α-methemocyanin of *Helix pomatia* were investigated at liquid helium temperatures. These signals were completely eliminated by the addition of fluoride and enhanced in the presence of chloride, which did not alter the saturation behavior. The triplet nature of the signal at $g = 4.59$ was confirmed by the low power-saturation. The addition of nitrite at pH 5.0 and 0° C yielded slowly and reversibly a seven-line half-field signal. On lowering the temperature the intensity of the triplet signals increased to the lowest temperature reached 4.2 K, excluding a strong exchange coupling.

Introduction

Fresh deoxy- and oxyhemocyanins are diamagnetic [1]. On treatment with azide or fluoride at pH 5.0 the oxyhemocyanin of *Helix pomatia* yields methemocyanin [2], which shows binuclear EPR signals [3]. A dipolar coupling or exchange interaction in Cu(II) pairs would result in a triplet (S = 1) and a singlet (S = 0) state. For the triplet state EPR resonances are expected near $g = 2$ ($\Delta M_s = 1$) and near $g = 4$ ($\Delta M_s = 2$) [4]. The methemocyanin-inducing agents yield, moreover, partially mononuclear EPR signals due to broken copper pairs, *i. e.* magnetically isolated Cu(II) atoms.

The temperature dependence of the signal intensity of the $\Delta M_s = 2$ transition was measured in order to delineate the exchange coupling constant $2J$ for methemocyanin and for methemocyanin treated with chloride or nitrite. The probability of this transition was compared for the different samples by electron spin relaxation experiments.

Materials and Methods

Separation of α-hemocyanin

This component was separated as halves by preparative ultracentrifugation in 1.0 M NaCl at pH 5.7 according to Heirwegh *et al.* [5].

Preparation of methemocyanin

The samples were obtained by treatment of oxyhemocyanin with sodium azide 25 mM at pH 5.0 for 2 days at 37 °C [2]. The solutions were afterwards dialysed exhaustively against sodium acetate buffer, pH 5.0, I 0.1 M.

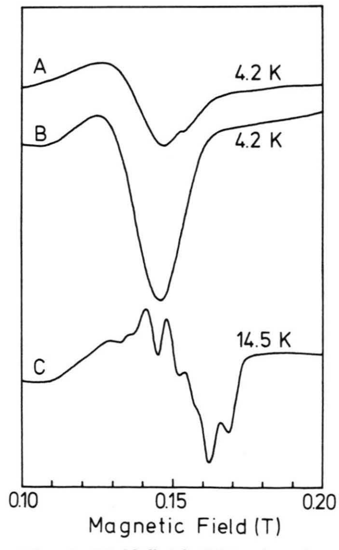

Fig. 1. Half-field EPR signals of α-methemocyanin of *H. pomatia* in 0.1 M sodium acetate buffer, pH 5.0. Microwave frequency 9.26 GHz, modulation amplitude 1 mT. A. Methemocyanin 132 mg/ml, gain 320, microwave power 20 mW, there is a very weak Fe signal at $g = 4.3$. B. Methemocyanin 136 mg/ml, 0.2 M NaCl ($Cl^-/Cu = 39$), gain 125, microwave power 20 mW. C. Methemocyanin 97 mg/ml, 0.36 M $NaNO_2$ ($NO_2^-/Cu = 100$), gain 400, microwave power 10 mW.

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Abbreviation: EPR, electron paramagnetic resonance.

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Samples used for Figs 1 – 3 were frozen in liquid nitrogen. An isopentane/methylcyclohexane (5/1, v/v) freezing mixture at 81 K was employed for freezing of the samples for Fig. 4.

EPR spectra

They were recorded on an E 4 spectrometer (Varian, Palo Alto, CA, USA) and stored on cassette tape using a HP 9830 calculator (Hewlett Packard, Palo Alto, CA, USA) interfaced to a digital oscilloscope 1020 A (Nicolet Instrument, Madison, WI, USA). For low-temperature work an ESR-9 continuous flow cryostat (Oxford Instruments, Oxford, UK) was used. The sample temperature was measured by a gold-iron/chromel thermocouple below the sample position. The thermocouple was calibrated against a carbon resistor thermometer. The carbon resistor was placed in a quartz EPR tube filled with silicone oil.

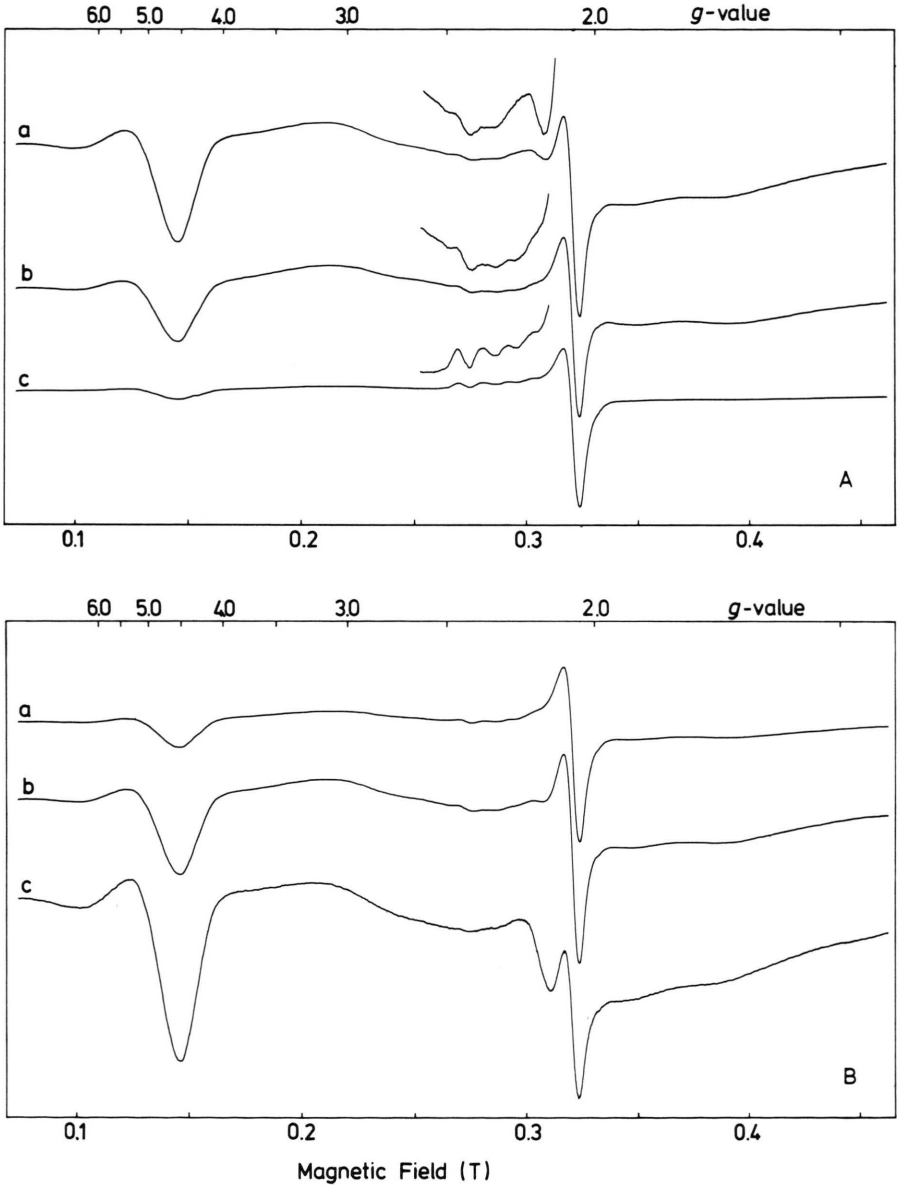

Fig. 2. EPR spectra of α-methemocyanin of *H. pomatia*, 112 mg/ml in 0.1 M sodium acetate buffer, pH 4.86, 0.2 M NaCl. Microwave frequency 9.26 GHz, modulation amplitude 1 mT. A. Influence of temperature, microwave power 10 mW: (a) 4.2 K, (b) 9.8 K, (c) 41.2 K. B. Influence of microwave power, 4.2 K: (a) 0.5 mW, (b) 5 mW, (c) 50 mW.

Microwave power saturation data

The experimental saturation data were fitted by a weighted least squares procedure to the equation given by Beinert and Orme-Johnson [6]. Calculations were carried out using a HP 9830 calculator, the data were plotted on a HP 7202 graphic plotter. For further details see microwave power saturation studies on iron-sulphur centers in ferredoxins and xanthine oxidase [7].

Results

Effect of fluoride

The EPR spectrum of α-methemocyanin of *H. pomatia*, prepared by azide treatment, is composed of mononuclear and binuclear signals. The binuclear signals vanish by the addition at pH 5.0 of 0.2 M fluoride to methemocyanin [3]. The remaining mononuclear signals amounted to 2.7% of the total copper. In these preparations no binuclear signals were detected by lowering the temperature from 77 to 4.2 K.

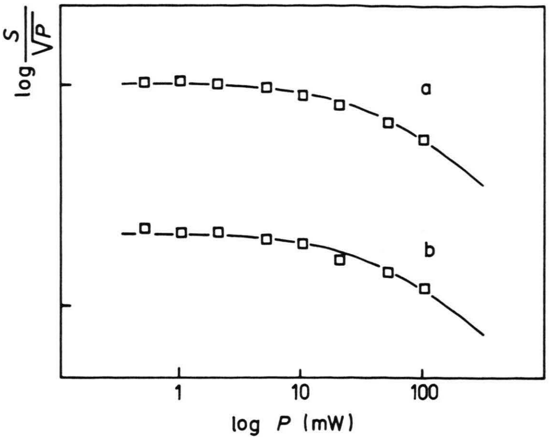

Fig. 3. Microwave power saturation data for α-methemocyanin of *H. pomatia:* (a) 115 mg/ml in 0.1 M sodium acetate buffer, pH 5.00, and (b) 112 mg/ml in 0.1 M sodium acetate buffer, pH 4.86, 0.2 M NaCl. The signal amplitude of the $g = 4$ feature was used, the data points were fitted by a weighted least squares procedure to a saturation function (7), the power for half-saturation $P_{1/2}$ was treated as a free parameter, the best fit was obtained using 1.0 as inhomogeneity parameter b. EPR conditions: microwave frequency 9.26 GHz, modulation amplitude 1 mT, 4.2 K. *S* is the derivative signal amplitude.

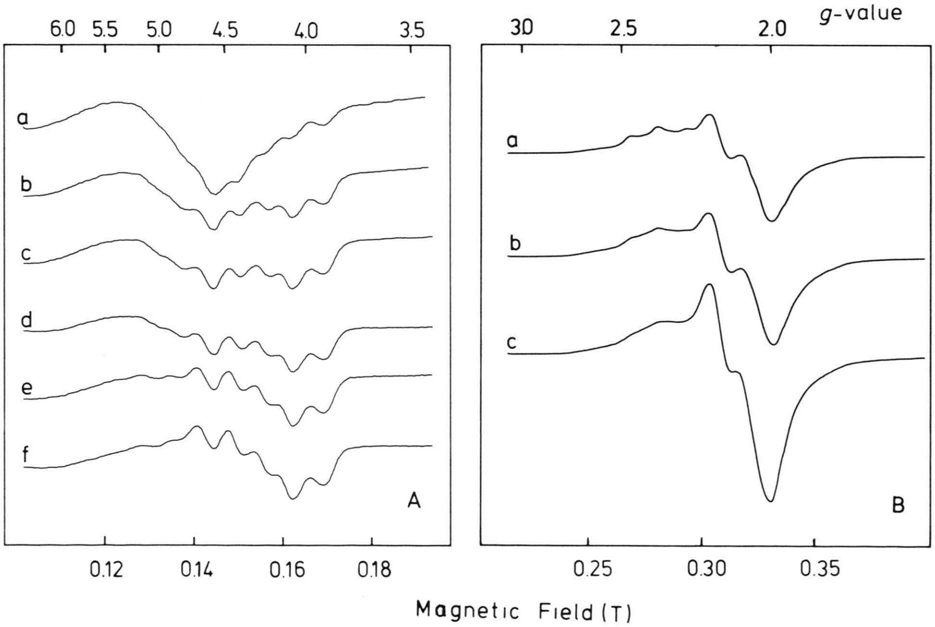

Fig. 4. EPR spectra of α-methemocyanin of *H. pomatia*, 97 mg/ml. A. Time-dependent spectral changes in the $g = 4$ region during the reaction with sodium nitrite 0.36 M at 0 °C (nitrite/Cu = 100). The reaction was carried out in quartz EPR tubes matched for internal diameter and was stopped by freezing in an isopentane/methylcyclohexane (5/1, v/v) freezing mixture at 81 K after (a) 15 s, (b) 1 min, (c) 2 min, (d) 5 min, (e) 30 min, and (f) 60 min after mixing. EPR conditions: microwave frequency 9.26 GHz, microwave power 10 mW, modulation amplitude 1 mT, 13.4 K. B. Influence of temperature for sample of Fig. 4A, f, which had reacted with sodium nitrite at 0 °C for 60 min: (a) 39.8 K, (b) 14.3 K, (c) 4.2 K. EPR conditions: microwave frequency 9.26 GHz, microwave power 10 mW, modulation amplitude 1 mT, the receiver gain was 25 compared to 320 for Fig. 4A, f.

Effect of chloride

The addition of chloride to methemocyanin increased the signal intensity by a factor of 5.6 at 4.2 K (Fig. 1 A and B). By lowering the temperature the mononuclear and the binuclear signals can also be readily discerned. Thus, at 41.2 K the mononuclear signal of isolated Cu(II) atoms predominates (Fig. 2 A, c) and at 4.2 K the spectrum of the Cu(II) pairs (Fig. 2 A, a). By increasing the microwave power at 4.2 K, a spectrum of the Cu(II) pair was obtained with only a small contribution from the broken Cu(II) pairs (Fig. 2 B, c).

It is noteworthy that chloride affected only the size of the signal of the Cu(II) pairs and not significantly its shape. Furthermore, the relaxation properties of the triplet spin system were unchanged as demonstrated by the microwave power saturation of the $g = 4.59$ signal (Fig. 3). The power for half-saturation $P_{1/2}$ amounted to 43.8 and 42.9 mW for methemocyanin in 0.1 M acetate buffer, pH 5.0, respectively in the absence and in the presence of 0.2 M NaCl. The transition probability did not change and the lines were in both cases inhomogeneously broadened (Gaussian line shapes) with an inhomogeneity parameter $b = 1.0$ [7, 8].

Effect of nitrite

The addition of 0.36 M sodium nitrite to methemocyanin yielded reversibly a seven-line signal [9], which was also enhanced at liquid-helium temperatures and showed a hyperfine splitting of 6.25 mT (Fig. 1 c). This signal, reported for the action of NO on deoxyhemocyanin [10], was shown to be due to the presence of traces of dioxygen during the NO treatment [11]. The $\Delta M_s = 2$ signal shifted from $g = 4.59$ to $g = 4.44$. The reaction, followed at 0 °C, was complete after about 1 h (Fig. 4 A). The broad binuclear $\Delta M_s = 1$ signals became relatively narrower after the addition of nitrite. By lowering the temperature, the mononuclear signal near $g = 2$ became strongly saturated. The signal attributable to $\Delta M_s = 1$ binuclear transitions (Fig. 4 B) agrees well with the spectrum obtained by manual subtraction of the mononuclear signal [9, 12].

Influence of temperature on the intensity of the half-field binuclear signal

The signal intensities times the absolute temperature normalized at 40 K are presented in Fig. 5 as a function of the absolute temperature. In accordance with the saturation experiments (Fig. 3) no significant differences were observed between methemocyanin in the absence and in the presence of chloride. There is apparently no antiferromagnetic exchange coupling. The temperature dependency of the half-field signal of the nitrite derivative of methemocyanin allows at the utmost only a very weak antiferromagnetic coupling.

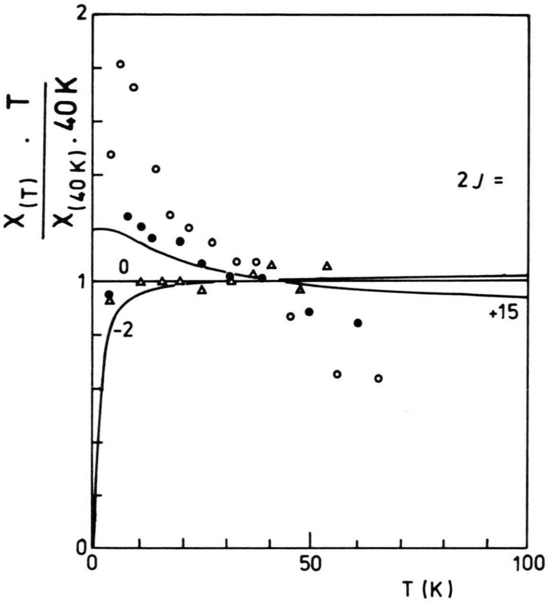

Fig. 5. The EPR signal intensity times the absolute temperature, normalized at 40 K, plotted against the absolute temperature for the methemocyanin preparations of Fig. 1: ●, in 0.1 M sodium acetate buffer, pH 5.0; ○, id., 0.2 M NaCl; △, id., 0.36 M sodium nitrite. The full lines represent the susceptibilities times the absolute temperature, normalized at 40 K, plotted against the absolute temperature. The susceptibilities were calculated according to the equation of Bleaney and Bowers [13].

Discussion

Methemocyanin can be defined as a form of hemocyanin, containing a pair of Cu(II) atoms, which does not bind oxygen and can, for molluscan methemocyanins, be regenerated with traces of hydrogen peroxide [14].

The α-methemocyanin of *H. pomatia*, prepared by the action of azide, which was removed by dialysis, showed binuclear EPR signals. These signals disappeared by the addition of fluoride, even at liquid helium temperatures, which indicates a strong antiferromagnetic coupling, analogous with the binding of peroxide in oxyhemocyanin. The enhancement of the triplet signals by chloride leveled off at a chloride concentration of 5.2 mM for a methemocyanin concentration of 57.5 mg/ml ($Cl^-/Cu = 2.5$) [14]. This points to a direct binding of chloride. Neither the line shape nor the electron spin relaxation were changed significantly.

The addition of nitrite shifted the $\Delta M_S = 2$ transition at $g = 4.59$ to the seven-line signal at $g = 4.44$, characteristic for a hyperfine splitting due to the nuclear spins of a copper pair. This might indicate a nitro bridge as demonstrated for μ-nitro-bis-cobalt complexes [15].

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