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STRUCTURAL STATES OF THE BINUCLEAR COPPER CLUSTER OF CANCER MAGISTER METHEMOCYANIN

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SUMMARY

The binuclear cupric copper cluster of Cancer magister methemocyanin prepared from hemocyanin and hydrogen peroxide is diamagnetic (1). Upon treatment with azide, it is transformed into magnetic dipolar coupled (paramagnetic) Cu(II) pairs and then into magnetically isolated Cu(II) complexes. This progressive uncoupling of the binuclear cupric pairs in methemocyanin is interpreted in terms of a relaxation of superexchange through one or more bridging ligands.

INTRODUCTION

Three states of binuclear cupric methemocyanin are known: in diamagnetic oxyhemocyanin (2-4), in diamagnetic methemocyanin (1), and in paramagnetic methemocyanin (5-8). These states are formed by the action of O_2, H_2O_2, and NO plus O_2, respectively, on binuclear cuprous hemocyanin (2, 1, 8). The structural relationships among the three states are largely unknown because the native ligands, the stereochemical configurations, and the electronic structures are not understood except in part in the case of oxyhemocyanins which are bicupric μ-peroxide bridged complexes (9, 10) with two or more nitrogenous ligands per Cu (9, 14). The oxygen atoms of the peroxide dianion are spectroscopically equilvalent (10), but the Cu(II) ions are each in a non-equivalent coordination geometry of very low symmetry (27).

The reactions of azide with both molluscan and arthropodan methemocyanins have been studied with spectral, circular dichroic, and electron spin resonance criteria of mechanism, primarily by Lontie and his coworkers (11-13). However,

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because diamagnetic methemocyanin has only recently been described (1), their results have been difficult to interpret in terms of a unified theory (14). In this study, we have examined the anaerobic reaction of diamagnetic methemocyanin from Cancer magister (arthropodan) with N_3^-, using electron spin resonance spectrometry, to determine if it can be converted to paramagnetic forms. We observed a rapid conversion of diamagnetic methemocyanin to the magnetic dipolar coupled form, followed by slow conversion of the latter to mononuclear Cu(II).

MATERIALS AND METHODS

Hemocyanin was prepared from the hemolymph of Cancer magister by the method of Thomson et al. (15), followed by passage through a millipore filter into sterile serum bottles. The stock solutions were 3.510 mM in copper, 0.493 mM in calcium, and 14.2 mM in magnesium; they contained 137 mg of protein per ml. Methemocyanin was prepared by the method of Makino et al. (1) using 10 electron equivalents of H_2O_2 per gram atom of copper. The methemocyanin was dialyzed against 0.1 M Tris HCl buffer, pH 8.65, containing 1 mM EDTA to remove Ca^{2+}, Mg^{2+}, and residual H_2O_2. The protein was then dialyzed against deionized water to remove the salts, and sterilized by passage through an 0.47 micron Millipore filter. The methemocyanin so treated contained 2.54 mM copper, 2.76 mM Mg^{2+}, 0.248 mM Ca^{2+}, and 102 mg per ml of protein.

Diamagnetic methemocyanin, operationally defined as non-oxygenatable copper minus EPR-detectable copper, was determined by the method of Ke and Schubert (16). Copper, magnesium, and calcium contents of methemocyanin samples were determined by atomic absorption spectroscopy, using a Varian Techtron AA-5 atomic absorption spectrophotometer. Protein was measured from its absorbancy at 279 nm, $E_1^{1\%}$ cm = 14.7 (17), with a Zeiss PMQ-II spectrophotometer.

The reaction of azide with methemocyanin was followed by EPR spectroscopy. Dual side-arm EPR tubes containing methemocyanin and azide were made anaerobic by six cycles of vacuum and argon purging. Methemocyanin (0.2 ml in deionized H_2O) was mixed under these conditions with 0.2 ml 100 mM NaN_3 adjusted to pH 7.2 with 0.1 M phosphate buffer at 25°. The final concentrations of the reaction mixture were total Cu, 1.27 mM; protein, 51 mg/ml; azide, 50 mM; and phosphate, 50 mM. EPR spectra were recorded with a Varian V-4502 X-band spectrometer at -160° and 25 mW of microwave power.

The concentrations of mononuclear and binuclear Cu(II) were estimated by assuming that the EPR spectrum in Figure 1-B represented 100% binuclear Cu(II), and the EPR spectrum in Figure 1-E represented 100% of mononuclear Cu(II). These assumptions were based upon reported characteristics of the respective signals (6, 8). Computer simulated curves of intermediate mixtures of the two reference spectra were drawn up to approximate visually the experimental curves. The method is accurate to about ± 10%.

All reagents were of the best grade commercially available. Solutions were prepared with H_2O having a conductivity of 10^7 ohm-cm.

RESULTS

When methemocyanin reacted anaerobically with azide, its light green color turned brown within a few seconds. After one minute, the EPR spectrum was that of magnetic dipolar coupled Cu(II) pairs as already described (6) (Figure 1B). The EPR spectrum then changed slowly at 25°C to that of mononuclear Cu(II) (Figure 1E) with g-values similar to those reported for mononuclear Cu(II) from methemocyanin (6). No other major intermediate was detected. The maximum amount of EPR-detectable Cu(II) formed in the anaerobic azide reaction was about 30% of the total methemocyanin copper originally present, possibly because under our conditions of pH, temperature, and concentrations of Mg^{2+}, only 30% of the active sites of this giant molecule were accessible to reaction with azide, or were able to respond stereochemically.

DISCUSSION

The bicupric forms of binuclear copper pairs in resting laccase, ceruloplasmin, tyrosinase, and methemocyanin (1, 19) are diamagnetic. We have suggested that diamagnetic Cu(II) of methemocyanin has a configuration related to the binuclear Cu(II) pair in oxyhemocyanin (1), which has a non-planar dioxygen bridged structure with a Cu ... Cu distance of 3.5-5.0 Å, depending upon the angle of rotation of the Cu ions from the plane of the peroxide anion (9). The azide-"sprung" forms of methemocyanin now observed are (1) a paramagnetic complex giving an EPR signal similar to that produced by NO + O_2 (5, 6, 8), ascribed to a magnetic dipolar coupled Cu(II) pair with Cu ... Cu distance of about 6.2 Å (5, 7); and (2) a mononuclear Cu(II) complex with an EPR spectrum similar to that previously observed as the result of anaerobic oxidation of hemocyanins with NO (5, 8, 20), and ascribed to Cu(II) sites with rhombic distortion, having two or more nitrogen ligands (18, 21, 23, 24, 25). This form of hemocyanin is called half-oxidized (8) or "half-met" (26). The mononuclear form observed in the present work with azide appears after the magnetic dipolar coupled binuclear form (Figure 1). This could in

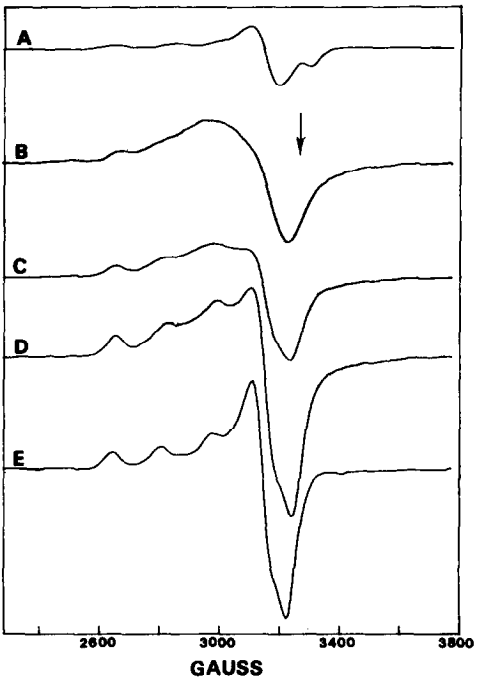

Figure 1. EPR spectra of successive stages in the anaerobic reaction of azide with diamagentic methemocyanin. Spectra were recorded at -160°C, 25 mW, and 8 gauss modulation amplitude. The arrow indicates the position of g = 2. Curve A is the spectrum of the methemocyanin itself. It shows 5.7% (of its total copper) as EPR-detectable Cu(II). This signal was subtracted from the subsequent reaction spectra. Curve A, methemocyanin, 1.09 mM, plus 0.11 mM oxygenatable copper. Curves B - E, plus 50 mM sodium azide and incubated for: B, 1 min at 25°; C, 30 min; D, 2 hrs; and E, 93 hrs. EPR detectable copper, corrected for the signal of untreated methemocyanin, was: B, 22% binuclear copper, 0% mononuclear; C, 15.6% binuclear copper, mononuclear, 10.4%; D, 11.8% binuclear copper, 17.6% mononuclear; and E, 0% binuclear copper, 19.9% mononuclear.

principle be due to an internal oxidation-reduction of the binuclear form, but because of the sequence and the completeness of conversion to the mononuclear form in the presence of azide, we consider it to be an end state of complete decoupling equivalent in magnetic character to the cupric copper of the half-oxidized state.

Metal ions in biological binuclear complexes share bridging ligands (e.g., hemerythrin, iron-sulfur proteins, superoxide dismutase); and it has been suggested that this is the case for the binuclear copper cluster in molluscan and arthropodan hemocyanins (2, 14, 22). In this case, superexchange

alone may give rise to very strong magnetic coupling of the cupric ions in the binuclear complex (2). We suppose this is what occurs in diamagnetic methemocyanin, through a bridging electron conductive ligand such as imidazole.

The uncoupling of the binuclear copper pair in methemocyanin in the presence of azide takes place in two discrete steps: (1) from strongly antiferromagnetic exchange coupling of the binuclear cupric pair to magnetic dipole-dipole coupling, accompanied by a separation of the cupric ions; and (2) from the latter to completely uncoupled cupric ions. No states intermediate to these three have been detected. Since superexchange is sensitive to the angle between the two cupric copper cations and the bridging ligand (28), effects of high azide concentrations upon the conformation of the hemocyanin molecules, or electrostatic effects of azide bound as a ligand to methemocyanin copper, could explain decoupling. Azide may also displace the bridging ligand(s) and bring about complete uncoupling by allowing the two cupric ions to separate to a distance of 8 Å or more.

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