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THE REACTION OF NITRIC OXIDE WITH CERULOPLASMIN

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SUMMARY

The reaction of nitric oxide with oxidized ceruloplasmin has been studied. It is shown that nitric oxide is capable of forming a diamagnetic charge-transfer complex with type-1 copper whereas type-2 copper is unaffected. From a comparison of the intensities of the EPR signals before and after treatment with NO it is concluded that, depending on the number of copper atoms per molecule, ceruloplasmin contains one type-2 and two or three type-1 copper atoms per molecule.

INTRODUCTION

In ceruloplasmin, a blue copper-containing protein, three different types of copper can be distinguished: type 1 with a typical absorption band at 610 nm and an EPR signal with a small hyperfine splitting, and type 2 with a larger hyperfine splitting and probably anti-ferromagnetic spin-paired copper(II) couples[1].

Although the reaction of NO with hemoproteins[2–5] has been studied extensively, little is known about its effect on copper-containing proteins, except for hemocyanin[6], which shows an EPR signal when the ligand is added to the protein[7]. In this paper we report on the effects of NO on the EPR and optical spectra of oxidized ceruloplasmin. It will be shown that NO is capable of forming a reversible diamagnetic charge-transfer complex with type-1 copper.

MATERIALS AND METHODS

Ceruloplasmin was isolated from an enriched Cohn fraction, a gift from the Netherlands Red Cross, essentially according to Deutsch *et al.*[8]. The final preparation had an absorption ratio at 610/280 nm of 0.043. All experiments were carried out anaerobically in Thunberg cuvettes or modified EPR tubes equipped with a special gas holder for anaerobic addition of NO (Matheson gas products). Anaerobiosis was achieved by repeated evacuation and flushing with nitrogen gas washed through an

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alkaline pyrogallol column, containing catalytic amounts of copper and iron ions. The ceruloplasmin concentration was calculated from $A_{610\,nm}$ using an absorbance coefficient of 10.9 $mM^{-1} \cdot cm^{-1}$ (ref. 8).

Absorption spectra were measured with a Cary-17 spectrophotometer at 20 °C and EPR spectra with a Varian E-3 spectrometer at −180 °C. The magnetic field was measured with an AEG EPR field meter (GA 11-21). Frequency was determined with the aid of a Hewlett Packard frequency counter (524 C) and frequency converter unit (525 B) in combination with a transfer oscillator (540 B).

RESULTS AND DISCUSSION

The reaction of NO with oxidized ceruloplasmin under anaerobic conditions was complete within the mixing time of the gas and liquid phases. Fig. 1 shows that it causes a decrease of the 610-nm maximum and the appearance of a broad band

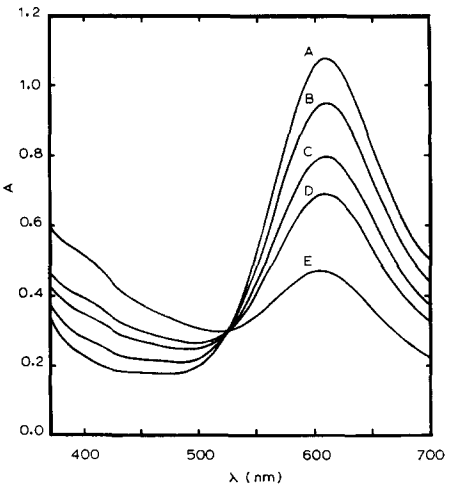

Fig. 1. The effect of NO on the optical absorption spectrum of oxidized ceruloplasmin. A, no nitric oxide, B–E equilibrated under approximately 0.1, 0.3, 0.5 and 1.0 atm NO, respectively, at 20 °C. The reaction medium (pH 7.0) contained 99 μM ceruloplasmin, 100 mM sodium acetate and 250 mM NaCl.

at 400 nm. An isosbestic point is found at 525 nm. As can also be seen in Fig. 1 the absorbance decrease at 610 nm is dependent on the NO tension, saturation being nearly reached at 1 atm. The binding is reversible, the 610-nm band being nearly completely restored after repeated flushing with N_2 and evacuation. Thus, the decrease in the absorbance at 610 nm cannot be explained by a reduction of the enzyme. Since the 610-nm band is characteristic of type-1 copper[1], it is concluded that NO reacts with this type.

The effect of NO at a pressure of about 0.5 atm on the EPR spectrum of oxidized ceruloplasmin is shown in Fig. 2. The signal of type-1 copper with the narrow hyperfine splitting vanishes, leaving a characteristic type-2 signal with $g_{\parallel} = 2.28, g_{\perp} = 2.06$ and hyperfine splitting of 166 G. The narrow hyperfine structure is restored after removal of the NO by evacuation and flushing with N_2. The inset of Fig. 2 shows a

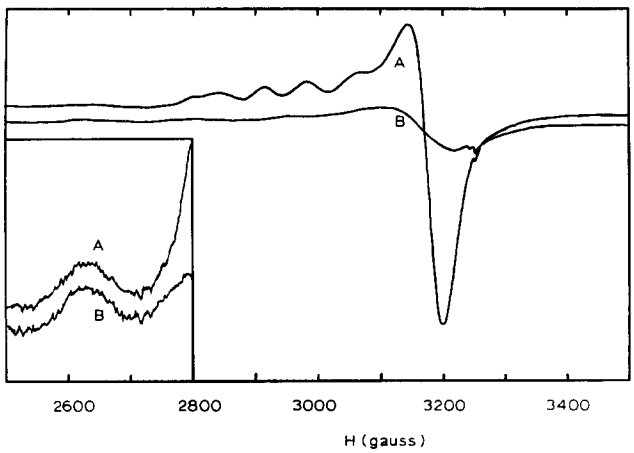

Fig. 2. EPR spectra in the absence (A) and in the presence (B) of approx. 0.5 atm NO. Microwavepower, 20 mW; modulation amplitude, 10 G; scanning rate, 125 G/min; time constant, 1.0 s; temperature, 93 °K; frequency, 9.14 GHz. Ceruloplasmin (99 μM) was dissolved in same reaction medium as in Fig. 1. The inset shows a magnification (15.5 ×) of the broad low-field hyperfine line.

magnification of the broad low-field line before and after treatment with NO. At this or at higher pressure (1.0 atm) of NO, no effect is found either on the position or the intensity of this hyperfine line. This indicates that type-2 copper is unaffected and that NO reacts only with type 1. In this context, it is noteworthy that NO also fails to produce any change in the EPR spectrum of superoxide dismutase, an enzyme containing only type-2 coppers (Wever, R., unpublished).

In contrast to the observation shown in Fig. 1, that the maximal effect on the absorption spectrum is found with a little more than 1 atm NO, the maximal decrease of the EPR signal occurs at a pressure of approximately 0.5 atm. Since on cooling the sample from room temperature to −180 °C a colour change takes place from greenish-blue to yellowish-brown the difference can be explained by a higher affinity of NO for the protein at lower temperatures. Another possibility is that the oxidized unliganded type-1 copper is reduced by a temperature-dependent intramolecular electron shift. It is interesting to note that bleaching of the blue colour has also been reported by Carrico *et al.*[9] for partially reduced ceruloplasmin.

The disappearance of the type-1 copper signal after NO addition can be explained either by spin pairing, magnetic dipolar interaction or a charge transfer between NO and the copper. Evidence for charge transfer is the formation of the broad band at 400 nm and the decrease of the 610-nm band. A comparable transfer of electrons from NO to a metal atom giving rise to diamagnetic compounds is also found for the NO derivatives of ferricytochrome *c* (ref. 10) and iron(III), manganese(II), cobalt(II) protoporphyrin–apohemoprotein complexes[5].

The type-2 EPR signal present after NO treatment (Fig. 2) has an intensity of 25–26% relative to the original EPR signal, as determined by integration of the signals. Depending on the number of copper atoms per molecule of ceruloplasmin, 4 out of 8 (ref. 11) or 3 out of 7 (ref. 12) copper atoms are paramagnetic. From this it is concluded that ceruloplasmin in the former case contains three type-1 and one

type-2 and in the latter two type-1 and one type-2 copper atoms, in agreement with G. Bemski as cited in ref. 12. The stoichiometry of type 1 and type 2, found in the case of 4 paramagnetic copper atoms per molecule is inconsistent with that reported by Andréasson and Vänngård[11]. They obtained from computer simulations of their EPR spectrum of ceruloplasmin two type-1 coppers and two different type-2 coppers with an $A_{\parallel}$ of 145 and 180 G, respectively.

From optical reductive titration data Veldsema and Van Gelder[13] suggested that the ratio of type-1 to type-2 copper is 1:3. The suggestion was based on the finding that only this ratio gives a straight line in a Nernst plot of type-1 against type-2 copper. However, as they mentioned, a higher ratio could not be excluded from these experiments and indeed it is to be expected if the type-1 copper atoms differ in redox potential. Since the results in this paper indicate that ceruloplasmin contains more type-1 than type-2 copper atoms it must be concluded that the type 1 can be subdivided at least with respect to their redox potential.

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