An e.p.r. study of the non-equivalence of the copper sites of caeruloplasmin

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The two Type 1 (blue) copper-binding sites of caeruloplasmin were spectroscopically differentiated by the kinetic analysis of the e.p.r. spectra during the redox cycle. One blue copper, with a hyperfine splitting constant ($A_{\parallel}$) of 6.8 mT, which was rapidly reduced, was not reoxidized by oxygen, whereas it was reoxidized by H_2O_2 . The other blue copper ($A_{\parallel} = 5.8$ mT), which was reduced slowly, was rapidly reoxidized by either oxygen or H_2O_2 . A conformational change of the Type 2 copper was concomitant with the fast reduction of Type 1 copper, whereas its reduction occurred during the slow phase. This sequence of events was reversed in the reoxidation step, that is, the Type 2 copper reappeared rapidly as the species with altered conformation and reverted to the symmetry typical of the native state in the slow phase. The specific reaction of a blue-copper site with the H_2O_2 can tentatively be related to the established ability of caeruloplasmin to prevent 'oxidative' attack of proteins and lipids.

INTRODUCTION

Caeruloplasmin, a blue copper oxidase, is the major copper-containing protein of the vertebrate plasma (Frieden & Hsieh, 1976). It is an α_1 -glycoprotein that binds 90–95% of blood plasma copper and has six or seven cupric ions per molecule (Fee, 1975). Various functions have been ascribed to this protein, including antioxidant defence (Al-Timini & Dormandy, 1977; Goldstein *et al.*, 1979; Gutteridge *et al.*, 1980; Gutteridge, 1983), copper transport and homeostasis and regulation of biogenic amines (Frieden & Hsieh, 1976). These functions may not be mutually exclusive and may be related to the heterogeneous nature of the copper atoms, which may make caeruloplasmin a multifunctional protein.

Blue copper oxidases (caeruloplasmin, ascorbate oxidase and laccase) contain at least four copper ions distributed in three types of spectroscopically distinguishable sites (Malmstrom *et al.*, 1975). The Type 1 (or blue) copper absorbs intensely at 610 nm and has an abnormally small hyperfine splitting constant ($A_{\parallel}$). The Type 2 copper has no optical absorption bands in the visible spectrum and has a higher hyperfine splitting value. The Type 3 copper is e.p.r.-non-detectable, absorbs at 330 nm and is likely to involve an antiferromagnetically coupled Cu(II) pair.

The stoichiometry of the three types of copper is not well established in caeruloplasmin. The protein contains six or seven copper atoms per molecule, and about 40% of the copper is e.p.r.-detectable and consists of one Type 2 and two Type 1 coppers (Fee, 1975). The role of the Type 2 copper is the most debated one. Its stoichiometry has been the object of conflicting reports (Deinum & Vanngard, 1973; Veldsema & Van Gelder, 1973; Calabrese *et al.*, 1981), and this has led to the conclusion that it may not be present *in vivo* (Syed *et al.*, 1982; Evans *et al.*, 1985). Spectroscopic and kinetic non-equivalence of the two blue copper atoms has been suggested on the basis of redox titrations (Deinum & Vanngard, 1973)

and analysis of the e.p.r. spectrum (Gunnarsson *et al.*, 1973). Recently it has been shown that part of the blue copper is in the reduced state in the native protein and is specifically reoxidized by H_2O_2 (Calabrese & Leuzzi, 1984).

Here we present a detailed analysis of the heterogeneous behaviour of caeruloplasmin copper, as detected by redox titration. It clearly appears that the two Type 1 copper atoms are reduced at different rates and that the Type 2 copper changes its symmetry when the first Type 1 copper is reduced. This behaviour is confirmed by reoxidation experiments, which also showed that the Type 1 copper, which is more rapidly reduced, is specifically reoxidized by H_2O_2 .

MATERIALS AND METHODS

All commercial chemicals were supplied by Merck and were of the best grade available. All buffers and solutions used were treated with Chelex 100 (Bio-Rad) to remove metal impurities. Sheep caeruloplasmin was isolated as previously described (Calabrese *et al.*, 1983). Protein concentration was expressed on a molar basis and calculated by using an ϵ_{610} value of $8000 \text{ cm}^{-1} \cdot \text{M}^{-1}$ (Calabrese *et al.*, 1983). Copper content was determined by the method of Brumby & Massey (1967). Anaerobic experiments were performed with Thunberg cells connected to a 2 mm-pathlength optical cuvette and to a quartz e.p.r. tube. Anaerobic conditions were obtained by several cycles of degassing followed by flushing with argon.

Optical absorption spectra were recorded with a Perkin-Elmer spectrophotometer model 330. E.p.r. spectra were recorded with a Varian E9 spectrometer at 9 GHz and 110 K. The total signal intensities were measured by double integration of the spectra against a copper-EDTA standard. Double integrations and subtractions of e.p.r. spectra were performed with a Hewlett-Packard model 85 computer.

Fig. 1. Reduction of caeruleoplasmin: e.p.r. spectra

a, The native protein; b, 5 min after the anaerobic addition of 1.4 electron equivalents of NADH per copper atom plus $3\mu\text{M}$ -phenazine methosulphate; c, after 18 min; d, after 33 min. The low-field part of the spectra is also shown with $\times 4$ amplification (a' , b' , c' , d'). $a' - b'$ and $b' - c'$ are the corresponding difference spectra.

RESULTS

Reduction experiments

Reduction of caeruleoplasmin was performed by anaerobic addition of stoichiometric amounts of ascorbate or NADH. NADH was used in the presence of traces of phenazine methosulphate as mediator (Deinum & Vanngard, 1973). The reaction pattern was the same with both reductants, with some kinetic differences, the reaction with ascorbate being slightly slower.

Fig. 1 shows the time course of the disappearance of the e.p.r. spectrum of caeruleoplasmin (Fig. 1, spectrum a) after the addition of 1.4 electron equivalents of NADH per copper atom. The lineshape of the spectrum changed gradually during the reduction, thus allowing one to distinguish between the behaviours of the three paramagnetic copper atoms.

After 5 min incubation the e.p.r. signal intensity was 71%, the e.p.r. intensity of the native sample (Fig. 1, spectrum b). Analysis of the hyperfine pattern showed that the $-3/2$ hyperfine line of the Type 2 copper was not changed in intensity, although it moved to higher magnetic fields (Fig. 1, spectrum b'). The difference spectrum obtained by subtracting the spectrum b from the native spectrum contained a Type 1 hyperfine pattern with $A_{\parallel} = 6.8$ mT (Fig. 1, spectrum $a' - b'$).

After 18 min the signal intensity was approx. 30% that

of the original signal (Fig. 1, spectrum c), but Type 2 copper was still 70% oxidized, as judged by the intensity decrease of the first hyperfine line (Fig. 1, spectrum c'). The difference spectrum ($b' - c'$) displayed a Type 1 hyperfine pattern with $A_{\parallel} = 5.8$ mT (superimposed on the Type 2 component).

After 40 min the residual signal amount to $\approx 10\%$ (Fig. 1, spectra d and d') and was essentially due to the Type 2 copper atom.

Oxidation experiments

Exposure of the reduced caeruleoplasmin (Fig. 2, spectra a and a') to air caused an immediate 70% recovery of the total intensity of the e.p.r. spectrum (Fig. 2, spectra b and b'), but the lineshape of the signal was markedly different from that of the native enzyme. The difference spectrum ($b' - a'$) evidences, as the components of the recovered e.p.r.-detectable copper, the Type 2 copper with altered lineshape of Fig. 1, spectrum b' , and the Type 1 copper with $A_{\parallel} = 5.8$ mT. The spectrum did not change after 30 min incubation in air at room temperature. Addition of $2\text{ mM-H}_2\text{O}_2$ restored the original signal within 15 min (Fig. 2, spectra c and c'). The difference spectrum ($c' - b'$) showed that H_2O_2 caused the reoxidation of the other Type 1 copper ($A_{\parallel} = 6.8$ mT). Concidentally the original lineshape of the Type 2 copper was recovered. Parallel optical

Fig. 2. Oxidation of caeruloplasmin by oxygen: e.p.r. spectra

a, Protein anaerobically reduced with 1.4 electron equivalents of NADH per copper atom; b, after 2 min incubation in air (oxygen); c, 16 min after the addition of four electron equivalents of H_2O_2 . The low-field part of the spectra is also shown with $\times 4$ amplification (a' , b' , c'). $b' - a'$ and $c' - b'$ are the corresponding difference spectra.

experiments indicated that 70% of the 610 nm band and 90% of the 330 nm band were recovered in the presence of air. Subsequent addition of H_2O_2 resulted in further increase of the 610 nm absorption up to a value 10% higher with respect to the original spectrum, as previously reported (Calabrese & Leuzzi, 1984).

Fig. 3 shows the results obtained in experiments where reduced caeruloplasmin was reoxidized anaerobically by stoichiometric amounts of H_2O_2 (1 electron equivalent per copper atom). Samples were incubated at room temperature before freezing for spectra recording. The time course of the reaction was very similar to that observed in the experiments shown in Fig. 2. An immediate 60% increase of the e.p.r. signal was obtained (Fig. 3, spectrum b). The hyperfine pattern (Fig. 3, spectrum b') consisted of the Type 2 copper with altered lineshape and of the Type 1 copper component with $A_{\parallel} = 5.8$ mT (Fig. 3, spectrum $b' - a'$). Then the intensity of the signal increased (Fig. 3, spectra c, d and e) to match the original spectrum in 60 min (Fig. 3, spectrum e) with a gradual change of the lineshape (Fig. 3, spectra c' , d' and e'). The difference spectrum ($e' - b'$) showed that in this slow phase there was the appearance of the Type 1 copper with $A_{\parallel} = 6.8$ mT concomitant with the recovery of the remaining 50% Type 2 copper which appeared with the original spectral parameters.

The incubation temperature of samples of reduced caeruloplasmin subjected to anaerobic by H_2O_2 were maintained at 0 °C before freezing for spectra recording, to slow down (5 min) the first rapid step. Even under these conditions the signal was seen to increase gradually with the same lineshape until it regained 60% of the intensity. After that there was a change in the lineshape to the original one.

DISCUSSION

It has been repeatedly suggested that the two Type 1 copper atoms of caeruloplasmin are non-equivalent, on the basis of kinetic and equilibrium redox titrations (Carrico *et al.*, 1971; Deinum & Vanngard, 1973; Manabe *et al.*, 1973) of the optical visible band of the protein. Carrico *et al.* (1971) reported that the blue band was recovered in two phases on exposure of the completely reduced protein to oxygen. The first phase, accounting for about 50% of the band, had a rate constant of $3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. The second phase was much slower and independent of oxygen concentration. Deinum & Vanngard (1973) interpreted the redox titration of the 610 nm band in terms of two non-identical Type 1 Cu(II) atoms, having E_0 values of 490 and 580 mV respectively. The only e.p.r. study in line with

Fig. 3. Oxidation of caeruloplasmin by H_2O_2 : e.p.r. spectra

a, The protein anaerobically reduced with 1.3 electron equivalents of ascorbate per copper atom; b, 1 min after the anaerobic addition of 1.1 electron equivalents of H_2O_2 ; c, after 5 min; d, after 30 min; e, after 60 min. The low-field part of the spectra is also shown with $\times 6$ amplification (a' , b' , c' , d' , e'). $b' - a'$ and $e' - b'$ are the corresponding difference spectra.

these results is the computer simulation of the spectrum of the resting enzyme, which was accounted for by one Type 2 and two Type 1 copper sites (Gunnarsson *et al.*, 1973).

The reduction experiments reported here permitted one to demonstrate, by differential e.p.r. spectroscopy, that the two distinct e.p.r. spectra of Type 1 copper, with $A_{\parallel} \approx 7$ mT and $A_{\parallel} \approx 6$ mT, correspond to species that are reduced quickly and slowly respectively. The two $A_{\parallel}$ values are slightly lower than those reported by Gunnarsson *et al.* (1973) for human and porcine caeruloplasmin (7 and 9 mT respectively), but are consistent with the different lineshape of sheep caeruloplasmin in the $g_{\parallel}$ region. The sequence of events during the reduction of the e.p.r.-detectable copper also showed that the fast reduction of the Type 1 copper atom characterized by $A_{\parallel} \approx 7$ mT is accompanied by a symmetry change of the Type 2 copper, reflected in the shift of the first ($-3/2$) hyperfine line to higher magnetic fields. Then the second Type 1 copper, with $A_{\parallel} \approx 6$ mT, and finally the modified Type 2 copper atom, are reduced. These results are in line with the concept, developed on the basis of evidence obtained with laccase, that reduction of Type 1 copper might cause a conformational change of the Type 2 copper site that would in turn affect

the rate of reduction of this metal (Reinhammar, 1983). The novelty of the present data is that it may suggest that both Type 1 copper atoms of caeruloplasmin are involved in this effect.

The fact that oxygen is unable to reoxidize all the caeruloplasmin copper (Carrico *et al.*, 1971; De Ley & Osaki, 1975) is a very surprising situation for an oxidase. However, the recent finding that purified caeruloplasmin from ox or sheep has part of the blue copper in the reduced state (Calabrese & Leuzzi, 1984) supports the idea that caeruloplasmin has very peculiar properties with respect to oxygen. In fact the fraction of the blue copper in the reduced state was specifically reoxidized by H_2O_2 (Calabrese & Leuzzi, 1984). In other oxidases, H_2O_2 preferentially reacts with the Type 3 copper (Strothkamp & Dawson, 1978; Penner-Hahn *et al.*, 1984). The case of caeruloplasmin seems to be a more complex process. In a very rapid initial phase the modified Type 2 copper and the Type 1 copper with $A_{\parallel} = 5.8$ mT appeared at the same time. Also 90% of the 330 nm absorption was recovered. The second phase was not observable in air, since it took several hours and was obscured by some protein denaturation. With H_2O_2 it was completed in 60 min and was characterized by reoxidation of the blue copper with $A_{\parallel} = 6.8$ mT and reappearance

of the original spectroscopic feature of the Type 2 copper.

In conclusion, the results of the present work can be summarized as follows.

(1) The two Type 1 copper sites are clearly differentiated by their kinetic behaviour in both reduction and oxidation experiments; oxygen is an efficient oxidant for the 'slowly reducible species', whereas H_2O_2 was efficient at both sites.

(2) The reoxidation by H_2O_2 is not associated with inactivation of the protein and appears to be kinetically selective for the 'fast reducible' Type 1 copper. Whether it is functionally important it is impossible to state from the present data, but it is tempting to relate this reaction to the reported 'antioxidant' properties of caeruloplasmin (Taylor & Oey, 1982).

(3) A conformational change of the Type 2 copper occurs at the same time as the Type 1 copper is reduced or reoxidized, such that it regulates the electron flow between the two Type 1 copper atoms. This casts some doubts on the idea that only one of the Type 1 copper ions forms an active unit together with one Type 2 and two Type 3 copper ions (Reinhammar, 1983). On the basis of these results, Type 2 copper seems to be a good probe for functional events occurring in caeruloplasmin, irrespective of its real stoichiometry and nature in the native protein. This property may represent a further approach to the problem of its role in caeruloplasmin.

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