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Presence of Coupled Trinuclear Copper Cluster in Mammalian Ceruloplasmin Is Essential for Efficient Electron Transfer to Oxygen*

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The reactivity with dioxygen of a mammalian (sheep) ceruloplasmin, anaerobically reduced with ascorbate, was found to depend on the state of the Type 2 and Type 3 copper centers, as monitored by EPR and optical spectroscopy.

A complete reoxidation by air after anaerobic reduction with ascorbate was observed with samples (A) purified by the single-step procedure described for chicken ceruloplasmin (Calabrese, L., Carbonaro, M., and Musci, G. (1988) *J. Biol. Chem.* 263, 6480–6483), while samples prepared by traditional multistep procedure (B) or subjected to freeze-thawing (C) displayed partial and very slow reoxidation, reflecting the functional nonequivalence of blue coppers which is considered a typical property of mammalian ceruloplasmin. The rate of reduction of the 330 nm chromophore was found to increase as a function of the extent and rate of reoxidation of different samples, while the 610 nm band displayed an opposite trend. Samples B and C showed a Type 2 copper signal in the EPR spectrum, while sample A showed practically no Type 2 copper in the oxidized protein, and a transient Type 2-like signal during reduction. The presence of a trinuclear Type 2-Type 3 cluster can therefore be proposed for all ceruloplasmins, and the integrity of the copper-copper coupling is essential for efficient oxidase behavior.

Multinuclear blue copper oxidases, ceruloplasmin, ascorbate oxidase, and laccase, are enzymes which mediate the four-electron reduction of oxygen by substrates without releasing O_2^- or H_2O_2 as intermediates. This property is related to the presence, in the same protein molecule, of at least four copper ions, divided into three spectroscopically distinguishable centers (Malmström *et al.*, 1975) which are referred to as the Type 1, or "blue," copper (with an intense optical absorption around 600 nm and a very small $A_{//}$ hyperfine splitting of the EPR spectrum), Type 2, or "non-blue," copper (with no apparent visible absorption and large $A_{//}$ values), and Type 3 copper (absorbing at 330 nm in the optical spectrum, and not detectable by EPR). The Type 3 copper is likely to be made of two antiferromagnetically coupled Cu(II) ions.

Ceruloplasmin, the blue copper oxidase of the vertebrate plasma, contains 5–7 copper atoms/molecule, 40–50% of which are visible by EPR (Ryden, 1984; Calabrese *et al.*, 1988a). Type 1 copper has repeatedly been shown to consist of two nonequivalent copper centers in mammalian ceruloplasmin: for the human species different redox potentials (Deinum and Vänngård, 1973) and EPR parameters (Gunnarsson *et al.*, 1973) have been measured. Moreover, in the course of redox titrations, it has always been observed that the two blue copper atoms of ceruloplasmin anaerobically reduced by ascorbate were reoxidized by oxygen at different rates, and one of them was practically nonreactive with oxygen (Carrico *et al.*, 1971; De Ley and Osaki, 1975). Subsequently, it was found that they were also reduced at different rates and that only the blue copper ion which was reduced more slowly could be reoxidized by oxygen, the other one being reoxidized by H_2O_2 (Calabrese and Carbonaro, 1986). These findings may support the suggestion that either one Type 1 copper is nonfunctional or that ceruloplasmin could be active in alternative functions involving H_2O_2.

Recently, we isolated a new ceruloplasmin from chicken plasma (Calabrese *et al.*, 1988a) and found it capable of complete and fast reoxidation by oxygen. The EPR spectrum of chicken ceruloplasmin was characterized by the absence of the signal typical of Type 2 copper. Since a Type 2-like copper signal reversibly appeared after reduction of the Type 3 center, the presence of a trinuclear cluster involving the three copper atoms was suggested. The aim of the present work was to reexamine the redox properties of sheep ceruloplasmin, as representative of mammalian forms, using protein samples with variable Type 2 copper content although with comparable enzyme activity. We found that samples with minimal Type 2 copper content displayed nearly complete and faster reoxidation of the reduced protein than samples with a higher amount of EPR-detectable Type 2 copper. The results obtained suggest that the redox properties of ceruloplasmin, irrespective of its source, are related to the presence of a trinuclear Type 2-Type 3 cluster, the integrity of which determines the behavior of the enzyme as a true oxidase and the functional effectiveness of both blue copper centers.

EXPERIMENTAL PROCEDURES

All reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany). Sepharose 4B was purchased from Pharmacia LKB Biotechnology Inc. (Uppsala) and DE22 from Whatman (Springfield Mill, Maidstone, Kent).

Sheep ceruloplasmin was isolated either according to the method of Calabrese *et al.* (1983), involving three steps, or by a single-step procedure (Calabrese *et al.*, 1988b), similar to that used for chicken ceruloplasmin (Calabrese *et al.*, 1988a). Ascorbate oxidase was a generous gift from Dr. L. Avigliano. Protein concentration was determined by the method of Lowry *et al.* (1951). Copper content was determined chemically according to Brumby and Massey (1967). The amount of paramagnetic copper was estimated by double integration of the EPR signal *versus* a standard solution of Cu-EDTA. Enzyme activity was assayed according to Osaki *et al.* (1966) with Cl^--free *p*-

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phenylenediamine as substrate. Linear reciprocal plots of $1/V$ *versus* $1/S$ to evaluate turnover kinetic parameters were obtained with the coupled assay of Løvstad and Frieden (1973), based on NADH consumption by the first oxidation product of *p*-phenylenediamine. The incubation mixture contained 0.25 mM diethylentriaminopentaacetic acid, 0.25 mM NADH, 1.3 μM ceruloplasmin, and enough NaCl to keep constant (9.6 mM) the Cl^- concentration, in 0.1 M phosphate buffer at pH 6.3. Anaerobic experiments were performed with Thunberg EPR tubes connected to a 2-mm pathlength optical cuvette. Anaerobic conditions were achieved by cycles of degassing and flushing with argon. Optical spectra were recorded with a Perkin-Elmer 330 spectrophotometer equipped with a Haake Mod. G temperature-control unit. Low temperature EPR spectra were recorded with a Varian E9 spectrometer operating at 9.15 GHz, interfaced to a Stelar Prometheus Data System for computer analysis and handling of the spectra. Simulations of the $M_I = -3/2$ hyperfine line of copper spectra were performed by summing different percentages of two spectra and visually evaluating the similarity of the obtained lineshape to that of the unknown species.

RESULTS

The EPR spectra of differently prepared samples of sheep ceruloplasmin are reported in Fig. 1 (*A–C*). Sample A was purified from fresh plasma by the single-step procedure (Calabrese *et al.*, 1988b). Sample B was isolated from fresh plasma by a method which proved to give the best preparations in shortest time by multistep procedures (Calabrese *et al.*, 1983). Sample C was isolated by the latter method from frozen plasma and also underwent several freezing-thawing cycles before use. The samples had comparable oxidase activity and turnover kinetic parameters (V_{max} and K_m), which were in line with the values previously reported for mammalian ceruloplasmin (Frieden and Hsieh, 1976). In particular, apparent K_m values for *p*-phenylenediamine of samples A–C were in the range 0.22–0.27 mM. The samples had the same copper contents, ~5.5 copper atoms/protein molecule. However, the EPR-detectable copper increased, from sample A to sample C (Fig. 1 and Table I). The Type 2 copper, as estimated by the height of the low field hyperfine line in the EPR spectra, was almost undetectable in sample A (Fig. 1*A*). In sample C, the Type 2 copper was more heterogeneous and its content higher than in sample B (Fig. 1, *B* and *C*).

Sample A was anaerobically reduced by addition of 2-electron equivalents of ascorbate/copper atom, and the reduction course was followed by EPR. A Type 2-like copper signal ($A_{//}$ ~ 130 G) grew up concomitantly to the disappearance of the Type 1 copper (Fig. 2, *curves a–c*), as previously described for chicken ceruloplasmin (Calabrese *et al.*, 1988a). This type of copper was distinguishable from that already present in unreacted enzyme, samples B and C, according to the different position and linewidth of the low field hyperfine line. The new signal reached an intensity amounting to ~1.0 Cu/molecule, as estimated by double integration of the EPR signal (Fig. 2, *curve c*) and was reduced more slowly than the other copper centers by ascorbate (Fig. 2, *curves c* and *d*). Upon reoxidation, an immediate 75% recovery of the total intensity was observed (Fig. 2, *curve e*), followed by a slower increase of the spectral intensity that reached the original one after 1 h of incubation to air (Fig. 2, *curve f*). At the end of the reoxidation, the lineshape of the EPR signal was different from the native one, as monitored by the appearance of a broad resonance around 0.26 tesla (Fig. 2, *curve f'*), typical of a Type 2 copper species. The asymmetric lineshape of this low field feature was diagnostic of its heterogeneous nature. Computer simulation indicated that it could be a mixture of a "normal" Type 2 copper (*e.g.* the Type 2 copper of unreacted sample B, Fig. 1*B*) and of the transient Type 2-like copper (Fig. 2, *curve c'*), with contributions to the total area amounting to 80 and 20%, respectively. The anaerobic reduction of sample B has been reported elsewhere (Calabrese and Carbonaro, 1986) and can be summarized as follows: concomitantly with the fast reduction of the first blue copper atom the Type 2 copper changed its lineshape to that of the transient species described above and was reduced only concomitantly with the second blue copper ion. This sequence of events was reversed in the reoxidation step. The Type 2 copper recovered its intensity with altered lineshape together with the reoxidation of the "fast" blue copper atom and

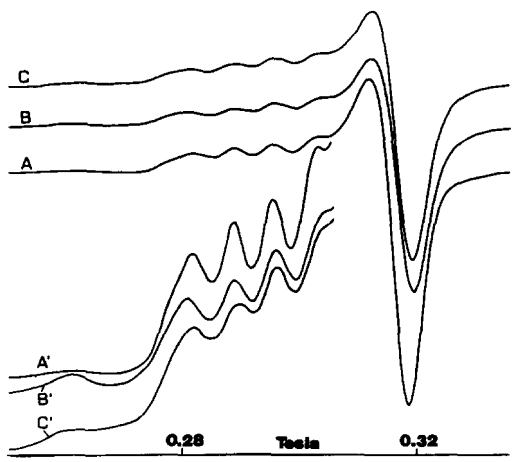

FIG. 1. **EPR spectra of sheep ceruloplasmin.** *A*, 2.8×10^{-4} M sample A; *B*, 1.4×10^{-4} M sample B; *C*, 8×10^{-5} M sample C. Nomenclature of samples is given in the text. All samples were in 50 mM phosphate buffer, pH 7.0. EPR conditions: field set, 3000 G; scan range, 1000 G; microwave power, 20 mW; modulation amplitude, 10 G; microwave frequency, 9.151 GHz; temperature, 130 K; amplifier gain, 50 (*A*), 80 (*B*), and 125 (*C*). Primed spectra were recorded at 6.3 times higher gain.

TABLE I

Copper stoichiometry of the sheep ceruloplasmin samples used in this work

The figures are approximate values based on an experimental error amounting to ~5%.

	Atoms of copper (130 kDa)	EPR detectable copper	EPR nondetectable copper
Sample A	5.5	2.1	3.4
Sample B	5.5	2.5	3.0
Sample C	5.5	3.0	2.5

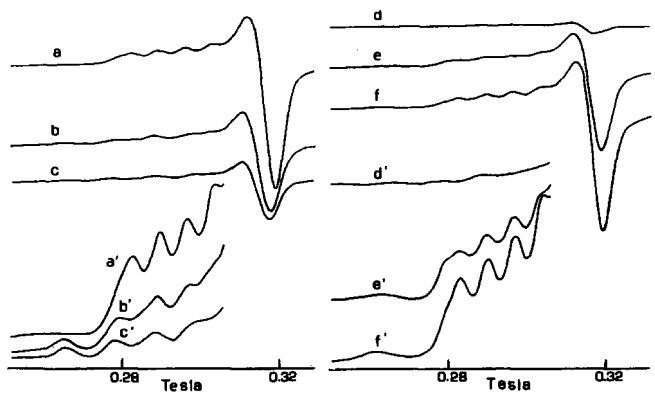

FIG. 2. **EPR spectra of the ceruloplasmin sample A of Fig. 1 during a redox cycle.** The spectra were recorded after anaerobic addition of 2-electron equivalents ascorbate/copper at 0 (*a*), 30 (*b*), 90 (*c*), and 120 (*d*) min, and after exposure to air of the reduced protein for 1 (*e*) and 70 (*f*) min. Primed spectra were recorded at 8 times higher gain. Experimental conditions as in Fig. 1.

returned to its original lineshape during the "slow" reoxidation phase. In sample C only a fraction of Type 2 copper was active in the redox cycle.

To prove that the observed spectroscopic features of ceruloplasmin after redox cycling were not due to or affected by residual unreacted ascorbate, ascorbate oxidase at catalytic concentrations (~10^{-8} M) was added to reduced ceruloplasmin just before readmitting air into the sample. No difference could be detected with either sample A, B, or C with respect to samples reoxidation in the absence of ascorbate oxidase.

Fig. 3, *A–C* reports the optical changes of the protein chromophores (at 610 and at 330 nm) during the anaerobic reduction course of the three ceruloplasmin samples of Fig. 1. In sample A (Fig. 3*A*) the 610 and 330 nm bands decreased in a parallel fashion at earlier times, while later on the decrease of the 330-nm band became faster than that of the other chromophore. The changes of the chicken ceruloplasmin chromophores were comparable in trend to those of sample A and are reported in the same figure as *broken lines*. Readmission of air to the cell caused the recovery of ~70% of the 610 nm optical density, within the shortest time of manual mixing (~1 min), concomitantly with full recovery of the 330 nm absorption. Full reoxidation of the optical density at 610 nm took place in approximately 1 h. In sample B, (Fig. 3*B*), the intensity at 330 nm decreased at a rate much slower than that of the 610 nm band. After exposure of the reduced protein to air, the 610 nm band regained only 60% of the original intensity and remained essentially stable even after prolonged incubation to air. The recovery of the 330 nm band was, as observed for sample A, completed within 1 min, thus not allowing to evaluate, under these experimental conditions, eventual differences between samples A and B, with respect to the rate of reoxidation of the 330 nm chromophore. In sample C (Fig. 3*C*), the 330 nm optical density did not change during the whole reduction process, while the 610 nm band decreased almost immediately to 60% of the original value and reached a plateau at 40% after 40 min. Full reduction of the 610 nm band could be achieved in this case with excess reductant, *i.e.* ~12 electron equivalents/copper atom. In both cases reexposure to oxygen, in the presence or absence of ~10^{-8} M ascorbate oxidase, did not lead to any recovery of the 610 nm optical density. At variance with sample B, where the complete reoxidation of Type 1 copper could be achieved by adding 4-electron equivalents of H_2O_2 (Calabrese and Carbonaro, 1986), in this case a slow and incomplete recovery of the 610 nm absorbance could be observed only when large excess of either H_2O_2 or ferricyanide (20- and 200-electron equivalents, respectively) were added to the samples, irrespective of the presence of ascorbate oxidase.

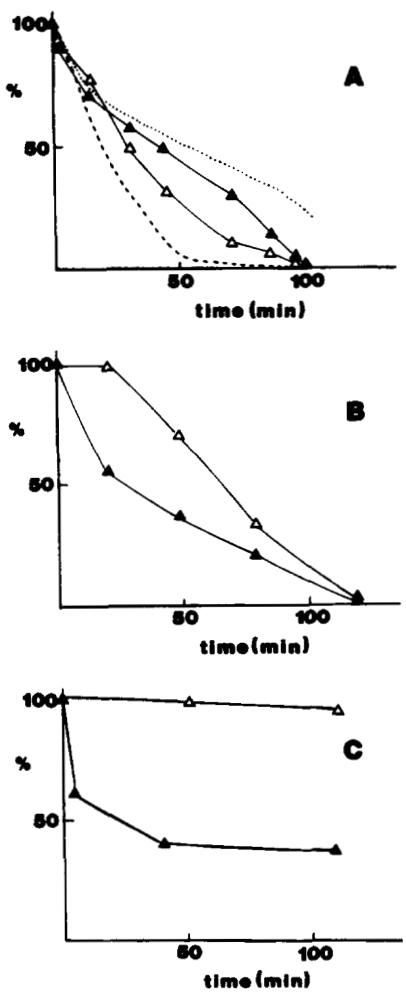

FIG. 3. **Time courses of the optical absorbances at 330 nm (△) and at 610 nm (▲) during anaerobic reduction of sheep ceruloplasmin with ascorbate.** *Panels A–C* refer to samples A–C of Fig. 1, respectively. The corresponding changes of chicken ceruloplasmin are reported in *panel A* as *dashed* (– – –) and *dotted* (· · · ·) *lines* for the 330 and 610 nm chromophores, respectively. Changes are expressed as percent of the difference between the optical densities of the fully oxidized and the fully reduced protein. The samples, ~1 × 10^{-4} M in 50 mM phosphate buffer, pH 7.0, were reduced by 2-electron equivalents of ascorbate/copper atom.

TABLE II

Ratio of initial rates of reduction of the 610 nm and 330 nm cromophores and percent of reoxidation after 1 and 70 min of ceruloplasmin samples

Sample	$\frac{rate_{610\ nm}}{rate_{330\ nm}}$	% reoxidation	
		1 min	70 min
Chicken	0.83	97	100
Sheep A	1.07	70	100
Sheep B	1.71[a]	60	65
Sheep C	>100	0	~5

[a] This rate was calculated after an initial lag phase of ~15 min.

The ratio between the relative rates of reduction of the two chromophores (A_{610nm} on A_{330nm}) in the various samples is reported in Table II.

DISCUSSION

The results presented here show that sheep ceruloplasmin obtained with the same quick preparation used for chicken plasma had practically no Type 2 copper EPR signal, although the absence of this feature was not as total as in the chicken protein, and underwent complete reoxidation after reaction with ascorbate (sample A). In fact, a small fraction of blue copper centers (~30%, see Table II) reoxidized more slowly in the sheep protein as compared with chicken ceruloplasmin, thus indicating that the proteins from the two sources still maintain some subtle structural differences even when prepared with the same mild and quick procedure. Anyway, these samples did fully recover the blue absorption band within 1 h (Table II), contrary to the general finding of past work on mammalian ceruloplasmin showing that one of the blue copper ions is reoxidized by oxygen, the reoxidation of the other one being not complete after several hours (Carrico *et al.*, 1971; De Ley and Osaki, 1975; Sakurai and Nakahara, 1986). Actually, sheep ceruloplasmin, when prepared (sample B) by the shortest multistep method in use so far for mammalian ceruloplasmins (Calabrese *et al.*, 1983) also displayed a Type 2 copper signal and a very long reoxidation time for half the

blue copper, and even no reoxidation at all if repeatedly freeze-thawed (sample C). No gross alteration of the protein structure apparently occurred as a function of the preparation and/or storage conditions used, as evident from the comparable values of the blue band and turnover kinetic parameters in all samples. Fig. 3 and Table II show that the lower the ratio between the rates of bleaching at 610 and 330 nm in various samples the higher was the extent of protein eventually reoxidized by oxygen. The EPR spectrum of sample A was similar to that of chicken ceruloplasmin also after reaction with ascorbate (Fig. 2). In both cases a transient copper species with Type 2-like EPR features appeared during the reduction process, although in the case of the sheep protein it partially remained spectroscopically evident after the reoxidation step. This indicates that the suggestion of a Type 2-Type 3 trinuclear cluster accounting for the lack of Type 2 EPR spectrum in the oxidized protein also holds for mammalian ceruloplasmin, in spite of the minor differences observed. We actually took advantage of the apparently weaker coupling of the cluster in the sheep protein, in order to correlate the redox behavior with the extent of decoupling observed. A minimal scheme of interpretation can be constructed on the basis of the data reported here and in the previous work on chicken ceruloplasmin. It involves two Type 1 copper ions as initial acceptors of reducing equivalents (as proposed for this copper Type in other blue oxidases (Reinhammar, 1984)). In the optimally coupled protein, they are able to immediately redistribute their electrons to the other redox sites (Figs. 2 and 3*A*) until all the copper centers are reduced by slight excess of ascorbate. In the protein with uncoupled cluster, the two Type 1 copper ions become not equivalent in both reduction and reoxidation (Fig. 3, *B* and *C*, and Calabrese and Carbonaro, 1986). One of them, however, which might be referred to as Type 1A, is still able to communicate with a copper-copper pair in the cluster, identical, under these conditions, to the conventional Type 3 copper. The loosened interaction within the cluster of the third Cu(II)-Type 2 ion would be associated with loss of communication between Type 1B and the oxygen-binding site. In these circumstances faster reduction of Type 1A is also observed (Fig. 3*B*) as would be expected for a redox center accessible to substrate electrons but kinetically constrained by the availability of Type 1B to concerted electron transfer to oxygen via the trinuclear cluster. From the point of view of protein assembly and conformation, this means that two structurally equivalent (or very similar) Type 1 sites are located on different domains (A and B, respectively), as sketched in Fig. 4, with domain A shared by Type 1A and two ions of the cluster (to be the Type 3 in decoupled samples). The third copper ion of the cluster becomes the conventional Type 2 copper in uncoupled samples of ceruloplasmin, as it is in the other blue oxidases. In these latter cases the intrinsically weaker coupling of the cluster is evidenced by the availability of well-characterized preparations of the so called "Type 2-depleted oxidases," while ceruloplasmin has repeatedly been shown to lose its copper in all-or-none fashion. Many observations on laccase and ascorbate oxidase are in line with the results presented here, although it should be kept in mind that laccase possesses just one Type 1 copper. An inversion of the bleaching rates of the 610 and 330 nm chromophores has been observed in laccase as consequent to impaired electron transfer from Type 1 to Type 3 due to an OH^- ion bound to the Type 2 copper, above pH 7.0 (Reinhammar, 1984). Type 2 copper has also been suggested as being close to the diamagnetic Type 3 pair to explain results on the binding of exogenous ligands like azide to Type 2-depleted (Morpurgo *et al.*, 1982) or native (Solomon *et al.*, 1987) laccase. A catalytic role for a mixed Type 2 Cu(I)-Type 3 Cu(II) complex in stabilizing an oxygen radical intermediate has been recently proposed (Koudelka and Ettinger, 1988) on the basis of the effects of F^- on the activity of laccase.

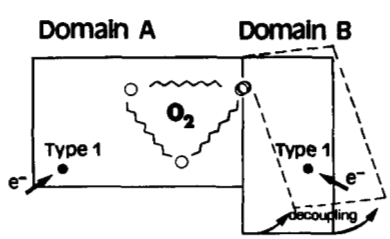

FIG. 4. **Schematic drawing of possible spatial relationship of copper atoms in ceruloplasmin.** *Lines* joining *circles* indicating copper atoms do not refer to real distances.

The picture outlined above for ceruloplasmin is in agreement with available information from amino acid sequence of both human ceruloplasmin (Takahashi *et al.*, 1984) and plastocyanin (Ambler, 1980), which represents the best up-to-date model for a Type 1-binding domain (Ryden, 1982, 1984). It appears that a plastocyanin-like Type 1 and three non-sulfur coordinated copper ions (reasonably the conventional Type 2 and Type 3 copper centers) reside on a COOH-terminal domain of 109 amino acids (out of 1046 of the full chain length) which is quite homologous to plastocyanin. Another plastocyanin-like Type 1-binding site is found in the middle region of the protein sequence and might be the location of the Type 1 copper here referred to as Type 1B.

A further point deserves a final comment. In a previous report (Calabrese and Carbonaro, 1986) a substantial shift in the spectral position of Type 2 copper was interpreted as a rearrangement of the ligands of Type 2 copper during the reduction of Type 1 copper. We here show that this modified form of Type 2 copper appears during the reduction process irrespective of the presence of EPR-detectable Type 2 copper in the oxidized protein. This transient Type 2-like species may well not originate from the Type 2 copper seen in decoupled samples of oxidized ceruloplasmin, but may be related to some half-oxidized species of Type 3 copper, often referred to as the "half-met" form in other binuclear metal centers of proteins (Spira *et al.*, 1982). Further work is in progress to characterize this additional "uncoupled" state of ceruloplasmin.

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