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Chicken Ceruloplasmin

EVIDENCE IN SUPPORT OF A TRINUCLEAR CLUSTER INVOLVING TYPE 2 AND 3 COPPER CENTERS*

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Ceruloplasmin was isolated to purity from chicken plasma by a single-step chromatography on aminoethyl-derivatized Sepharose. Molecular mass, as estimated by nonreducing sodium dodecyl sulfate-electrophoresis, was approximately 140 kDa, slightly higher than that found for ceruloplasmins from other sources. Specific activity as *p*-phenylenediamine oxidase was five times higher than that reported for mammalian ceruloplasmins. The copper content was estimated to be 5.01 ± 0.35 atoms per protein molecule, 50% of which was EPR-detectable. The EPR spectrum was completely devoid of any signal typical of the type 2 copper as seen in the other blue multicopper oxidases and in ceruloplasmin from mammalian species.

Anaerobic reduction of chicken ceruloplasmin resulted in the disappearance of the 330 nm optical band typical of type 3 copper, which was followed by the appearance of an EPR signal typical of type 2 copper. Subsequently, the type 1 copper and finally the newly formed type 2 copper were reduced. The original optical and EPR spectra were recovered within few minutes upon exposure of reduced ceruloplasmin to air. It is concluded that in oxidized chicken ceruloplasmin type 2 copper interacts with the diamagnetic pair responsible for the 330 nm absorption in such a way as to become EPR-undetectable and that the interaction is relieved by reduction of the pair. Whether this interaction is intrinsically weaker in other blue oxidases and ceruloplasmins studied or is lost with standard preparation procedures remains to be established.

Ceruloplasmin, the only blue copper oxidase of animal species, is present in the α_2-globulin fraction of vertebrate plasma. Over 95% of plasma copper is bound to it with different coordinations that characterize three classes of sites (Fee, 1975), as seen in plant copper-containing oxidases such as laccase and ascorbate oxidase. The type 1, or "blue" copper, is responsible for a very intense absorption band around 600 nm and is paramagnetic, with an unusually narrow parallel hyperfine coupling constant in the EPR[1] spectrum. The type 2 copper does not contribute appreciably to the visible spectrum, and its EPR spectrum displays values of the magnetic parameters typical of more regularly coordinated copper complexes. The type 3 copper is EPR-silent and is thought to consist of a pair of antiferromagnetically coupled Cu(II) ions. A number of unsolved problems, however, still exists for ceruloplasmin. (i) The stoichiometry of copper is apparently different with respect to other blue oxidases. The human system (Deinum and Vänngård, 1973; Ryden and Bjork, 1976) seems to contain 6–7 atoms of copper per protein molecule (a single chain of 132 kDa), approximately 40% of which are paramagnetic (two type 1 and one type 2 centers). (ii) Vänngård (1967) had proposed that the presence of type 2 copper might be an isolation artifact, but then he agreed that this class of copper centers is an essential constituent of ceruloplasmin, as well as of other multinuclear blue oxidases (Andreasson and Vänngård, 1970). However, at variance with laccase and ascorbate oxidase, selective removal of type 2 copper was never successful with ceruloplasmin (Aisen and Morell, 1965), thus making the analogy argument weaker. Syed *et al.* (1982) noticed the absence of the EPR features of type 2 copper in plaice ceruloplasmin. In a later report, Evans *et al.* (1985) claimed that type 2 copper might be absent even in mammalian ceruloplasmin. However, no indication of the total copper content nor of its distribution among the three different classes was reported for those ceruloplasmin preparations. (iii) Type 1 copper has always been found to consist of two distinct species in this protein, with different redox potentials (Deinum and Vänngård, 1973), EPR magnetic parameters (Gunnarsson *et al.*, 1973), and circular dichroism transitions (Hervé *et al.*, 1976). Furthermore, they are reduced by ascorbate at different rates, and only the one that is reduced more slowly can then be readily reoxidized by oxygen, while the other copper ion is reoxidized by H_2O_2 (Calabrese and Carbonaro, 1986). Therefore, the functional oxidase unit of ceruloplasmin might exclude one type 1 copper (Sakurai and Nakahara, 1986).

In view of these problems, the object of the work reported here was the purification of ceruloplasmin from a new source, *i.e.* chicken plasma, with a very quick and mild procedure. The isolated protein displayed a number of novel properties. In particular, the EPR signal of type 2 copper became detectable only after reduction of the type 3 center, suggesting the presence of a type 2-type 3 cluster in the native protein.

EXPERIMENTAL PROCEDURES

All chemicals were reagent grade and were used without further purification. Sepharose 4B was purchased from Pharmacia LKB Biotechnology Inc., Uppsala. Purification of ceruloplasmin was performed in a single chromatographic step on a Sepharose derivative obtained by reaction of Sepharose with chloroethylamine. 300 ml of Sepharose 4B, previously treated at 70 °C for 2 h with 100 ml of 5 N NaOH and 25 ml of epichlorohydrine, and exhaustively washed, were suspended in 10 N NaOH and treated with 140 ml of 100% chloroethylamine for 2 h at 70 °C. The resulting derivatized Sepharose was equilibrated at pH 7.4 with 3 mM phosphate buffer. Freshly drawn chicken plasma was depleted of platelets and diluted with 20 mM 6-aminohexanoic acid to prevent proteolysis until the conducibility was as low as 7 ms at 18 °C. After adjusting to pH 7.4 by addition of small aliquots of H_3PO_4, the plasma was loaded on a column of the deriva-

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[1] The abbreviation used is: EPR, electron paramagnetic resonance.

tized Sepharose and washed with increasing concentrations of phosphate buffer at pH 7.4. Ceruloplasmin was eluted at 300 mM buffer and then concentrated by either Amicon concentrators or Centricon 30 microconcentrators. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969). Protein concentration was determined according to Lowry *et al.* (1951). Copper content was determined by atomic absorption with a Perkin Elmer 3030 spectrometer equipped with graphite furnace and chemically by the method of 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. Oxidase activity was measured spectrophotometrically as described by Osaki *et al.* (1966), in 0.1 M potassium phosphate buffer at pH 6.8, containing 50 μM EDTA, with *p*-phenylenediamine as the substrate. Optical spectra were recorded with a Perkin Elmer 330 spectrophotometer. X-band EPR spectra were recorded at 5 K on a Bruker ESP300 spectrometer and at 77 K on a Varian E9 spectrometer.

RESULTS

Molecular Properties of Chicken Ceruloplasmin—The nonreducing sodium dodecyl sulfate-electrophoresis pattern of the protein consisted of a single band which accounted for over 95% total protein content of the loaded mixture. The electrophoretic mobility was slightly lower than that of mammalian ceruloplasmins (Calabrese *et al.*, 1981; Calabrese *et al.*, 1983), corresponding to a molecular mass of ~140 kDa.

The copper stoichiometry was 5.01 ± 0.35 copper atoms/protein molecule as the average of 10 different preparations. Stoichiometry fluctuations did not reflect the degree of purity nor did they affect the spectroscopic and catalytic properties of the sample.

The oxidase activity of chicken ceruloplasmin, assayed between pH 6.0 and 8.0, was found to be five times higher than the value measured for mammalian ceruloplasmin under the same experimental conditions, with a maximum around pH 6.8.

Spectroscopic Properties of Chicken Ceruloplasmin—Fig. 1*A* (*curve a*) reports the absorption spectrum of chicken ceruloplasmin in the visible and near UV-visible region. For purpose of comparison, the spectrum of an equal concentration of the sheep protein, a good representative of mammalian ceruloplasmins (Calabrese *et al.*, 1983), is also reported (Fig. 1*A*, *curve b*). The spectroscopic parameters of the blue band differed slightly between the two species, the maximum absorption wavelength being appreciably blue-shifted and the extinction coefficient being higher in the chicken *versus* sheep ceruloplasmin (603 *versus* 610 nm and 9200 *versus* 8000 M^{-1} cm^{-1}, respectively). The higher intensity of the blue band is likely to be related to the absence of any reduced type 1 copper, as seen by the lack of reaction of chicken ceruloplasmin with H_2O_2 (Calabrese and Leuzzi, 1984). Furthermore, the intensity of the shoulder at 330 nm, which is associated with type 3 copper, is higher in the spectrum of chicken than in sheep ceruloplasmin.

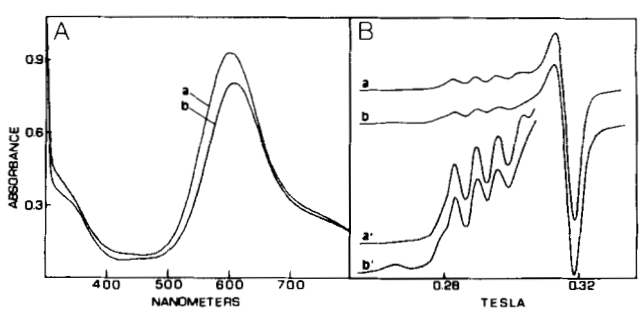

FIG. 1. **Spectroscopic properties of chicken ceruloplasmin.** *A*, optical spectra of 10^{-4} M chicken (*a*) and sheep (*b*) ceruloplasmin. *B*, low temperature X-band EPR spectra of chicken ceruloplasmin. *a*, 1.4 × 10^{-4} M protein sample examined at the end of the isolation procedure. *b*, 1.7 × 10^{-4} M protein, stored at −20 °C and examined 40 h after the isolation. The latter sample had suffered two cycles of freezing-thawing. Experimental conditions: modulation amplitude, 10 G; microwave power, 20 milliwatts; microwave frequency, 9.13 GHz; temperature 77 K. Spectra *a'* and *b'* were recorded at 6.25 and 8 times higher gain, respectively.

Fig. 1*B* (*curves a* and *a'*) shows the X-band EPR spectrum of chicken ceruloplasmin at 77 K. Half the total copper was EPR-detectable, regardless of the copper content of different samples.

The most important feature to be noted in the EPR spectrum is the absence at lower magnetic field of the −3/2 hyperfine line typical of type 2 copper, which is always present in previously studied ceruloplasmins and in other blue oxidases. The absence of this line was observed in all preparations. To test whether the type 2 copper EPR signal could not be detected because of relaxation time broadening effects, the EPR spectrum was measured also at 5 K, but it was still devoid of the −3/2 hyperfine of type 2 copper. On the other hand, "aging" of the protein (freeze-thawing, standing in solution over long time) irreversibly led to the appearance of type 2-like copper, as revealed by the clear appearance of the −3/2 hyperfine line at low field (Fig. 1*B*, *curves b* and *b'*).

Another important feature of the EPR spectrum is the linewidth of the type 1 copper hyperfine lines, which is sharper than in spectrum of ceruloplasmin from other sources. This suggests a higher degree of homogeneity of the blue copper centers in the chicken protein.

Reduction and Reoxidation of Chicken Ceruloplasmin—The EPR spectra of chicken ceruloplasmin anaerobically reacted with 10 protein equivalents of ascorbate at 25 °C are reported in Fig. 2. The type 1 copper was slowly and steadily reduced. A line at field values typical of type 2 copper developed after 20–30 min (*curve b*) and then slowly disappeared. When, at the end of the reduction process (*curve c*), oxygen was readmitted in the cell, a fast and substantially complete recovery of the native EPR signal was observed (*curve d*), in contrast with that observed with mammalian ceruloplasmins (Calabrese and Carbonaro, 1986). The same full reoxidation was obtained when oxygen was readmitted at the stage of curve b. The amount of the transient type 2 copper that appears during the reduction process was estimated to be ~0.5 Cu ions per ceruloplasmin molecule by the method of Vänngård (1967). Fig. 3 correlates the time courses of appearance of the type 2 copper EPR signal, the optical absorbances at 330 and 603 nm, and the amount of EPR-detectable copper during the redox process. The appearance of this type 2 signal in the

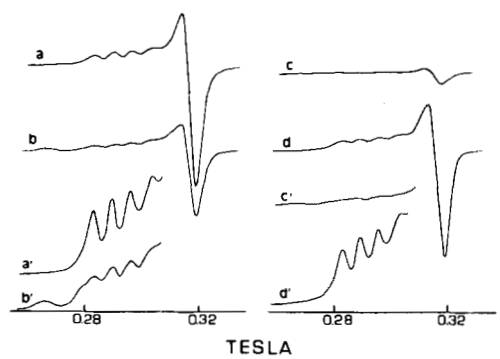

FIG. 2. **Low temperature X-band EPR spectra of 9 × 10^{-5} M chicken ceruloplasmin anaerobically reduced with 4.5 × 10^{-4} M sodium ascorbate at 25 °C.** *a*, native protein; *b*, after 50 min; *c*, after 130 min; *d*, 7 min after re-exposure to the air. Primed spectra were recorded at 6.25 higher gain. Experimental conditions were as in Fig. 1.

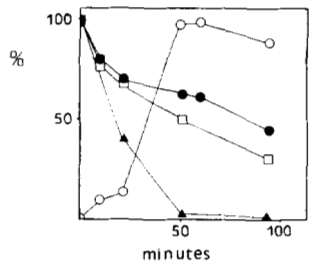

FIG. 3. **Time courses of the type 2 copper EPR signal (○), the optical absorbances at 330 nm (▲), and at 603 nm (□), and the amount of EPR-detectable copper (●) during the anaerobic reduction of chicken ceruloplasmin by ascorbate.** Both optical absorbances are expressed as percent of the difference between the optical density at the specified wavelength of the fully oxidized and the fully reduced protein. The percent of type 2 copper EPR signal is expressed as fraction of the height of the −3/2 hyperfine line at time t over the maximum intensity attained by the same band in the reduction course.

EPR spectrum occurs after the 330 nm band has almost completely disappeared. The absorption at 603 and 330 nm were completely recovered after reoxidation in air.

DISCUSSION

Ceruloplasmin purified from chicken plasma with the quick and mild single-step procedure adopted in this work, although resembling ceruloplasmin isolated from other sources, as far as molecular mass and optical spectrum are concerned, displays some points of difference that are of special relevance to major open questions in the field. (i) The EPR spectrum of chicken ceruloplasmin is marked by the absence of the type 2 copper features even at liquid helium temperature. However, the absence of the type 2 copper is in contrast with the good oxidase activity of chicken ceruloplasmin (5-fold that of sheep ceruloplasmin). In fact, laccase (Morpurgo *et al.*, 1980) and ascorbate oxidase (Sakurai *et al.*, 1987) lose their oxidase activity when their type 2 copper is selectively removed. Furthermore, the spectral homogeneity of type 1 copper and the capability of oxygen to restore all the reduced type 1 copper of chicken ceruloplasmin make this protein more similar to true oxidases with respect to human, ovine, and bovine ceruloplasmin, where only half of the blue sites were reoxidized by oxygen (De Ley and Osaki, 1975; Calabrese and Carbonaro, 1986; Sakurai and Nakahara, 1986). Finally, from the copper content, the number of EPR-silent copper centers in chicken ceruloplasmin exceeds a single antiferromagnetic pair but is less than two pairs, suggesting that extra copper might be present, although spectroscopically undetectable in the isolated protein. (ii) Anaerobic reduction of the protein by ascorbate leads to the appearance of a type 2-like copper EPR signal after the 330 nm band in the optical spectrum has disappeared. This EPR signal accounts for about half copper ion per ceruloplasmin molecule and disappears again upon exposure to air, regardless of the redox state of the other chromophores and concomitantly with the recovery of the 330 nm absorption (Figs. 2 and 3). (iii) Freezing-thawing and/or standing irreversibly transform chicken ceruloplasmin into a protein displaying an EPR spectrum (Fig. 1*B*) with the heterogeneous hyperfine pattern that is typical of mammalian ceruloplasmin and other blue oxidases.

This evidence strongly suggests that, in native oxidized chicken ceruloplasmin, type 2 copper is magnetically interacting with the type 3 copper pair. Trinuclear antiferromagnetic clusters may involve strong non-Heisenberg exchange interactions, which can give rise to anomalous linewidth behavior (Benelli *et al.*, 1984). This may explain, on one hand, the presence of 3 EPR-undetectable copper atoms in chicken ceruloplasmin and, on the other hand, the non-integer stoichiometry (~2.6) of this trinuclear cluster, with a fraction of copper still EPR detectable, yet lacking a resolved hyperfine structure. Uncoupling is observed during aging processes, which are very likely to disrupt the cluster by conformational changes. The cluster is magnetically broken by reduction of type 3 (Fig. 3), as monitored by the disappearance of the 330 nm band. Since a half-reduced type 3 center, *i.e.* Cu(II)–Cu(I), does not necessarily have a 330 nm absorption, the kinetics of the appearance of a type 2 copper EPR signal during the reduction process (Fig. 3) does not rule out the possibility that the signal may correspond to a mixed valence type 3 site of the kind produced by reduction of type 2-depleted laccase (Spira *et al.*, 1982; Reinhammar, 1983). However, since the amount of the type 2 copper transient EPR signal in chicken ceruloplasmin never exceeds unity, this competing hypothesis requires that the third EPR-undetectable copper of the native enzyme either be always in the reduced state or be reduced concomitantly with one copper of the type 3 pair. The former possibility seems unlikely, in view of the stoichiometry of the EPR-detectable copper in this protein, while the latter one implies that a formal distinction between type 2 and 3 does not apply to this kind of trinuclear cluster. Other considerations disfavor the possibility of a mixed valence species. Half-reduced type 3 pair in laccase is obtained by reducing samples depleted of type 2 (Spira *et al.*, 1982; Reinhammar, 1983). Furthermore, while in chicken ceruloplasmin the transient type 2 species is formed by reduction in the absence of oxygen and disappears upon exposure to air with recovery of the 330 nm absorption band, half-met, type 2-depleted laccases are stable derivatives and can regenerate the oxidized protein only after treatment with H_2O_2 (Spira *et al.*, 1982). Previous studies have proposed that type 2 and 3 sites may be located close together in laccase (Spira-Solomon *et al.*, 1986) and ascorbate oxidase (Sakurai *et al.*, 1987). However, the evidence in these reports was obtained essentially by the use of type 2-depleted preparation and/or the reaction with an exogenous binding ligand such as azide. In the case described in the present work the more direct approach of coupling-uncoupling in the course of a reduction-reoxidation process was apparently successful for the first time in a native multicopper oxidase. Moreover, it is clear that chicken ceruloplasmin would represent a case of much stronger interaction in the trinuclear cluster, as to lead to disappearance of the type 2 copper EPR signal. This kind of interaction may be physiologically important, as coupled redox activity in the three partners in the cluster is essential to the multielectron reduction of oxygen to water typical of multicopper oxidases (Spira-Solomon *et al.*, 1986). As a matter of fact, reduced type 3 copper centers alone, as in hemocyanin, are able to bind oxygen, but unable to reduce it to water.

As a matter of fact, chicken ceruloplasmin is, to date, the only ceruloplasmin capable of complete reoxidation by oxygen. It is tempting to ascribe this property to the intact trinuclear cluster and the "anomalous" behavior of the mammalian proteins to a looser state of this cluster leading to uncoupling of the type 2 copper in the oxidized protein.

The question now arises why chicken ceruloplasmin displays a much stronger interaction of its non-blue centers, compared with mammalian ceruloplasmins and other blue oxidases. It may represent a new class of ceruloplasmin with a different molecular architecture around the non-blue copper sites. Alternatively, the chicken protein may be more apt to keep its native properties after the quick method of isolation adopted here. A fascinating question is whether magnetically

coupled trinuclear clusters are present also in laccase and ascorbate oxidase, but have never been identified because those proteins cannot be purified in such a quick and nonperturbative way as described here for ceruloplasmin.

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