Studies on the State of Copper in Native and Modified Human Ceruloplasmin*

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Several experiments reported in the preceding paper (1) have indicated that not all the copper atoms of human ceruloplasmin are present in the same state within this molecule. Furthermore, acid-base titrations of ceruloplasmin have shown distinct regions of instability. The absorbancy changes noted in the 610-μm range during these titrations indicated the involvement of the chromophoric copper atoms. It was of interest to correlate some of the results of physical-chemical studies with those obtained by methods more specifically designed to evaluate the copper constituent of ceruloplasmin. The studies utilized copper-chelating agents, methods for the analysis of total copper and of cuprous copper, and electron paramagnetic resonance spectroscopy. Measurements were made of the changes in optical absorption and in the well known oxidase activity of ceruloplasmin and were correlated with the results of the copper analyses on similarly treated protein.

EXPERIMENTAL PROCEDURE

Human ceruloplasmin was prepared from plasma or Cohn Fraction IV-1 (2) by methods previously described (3, 4). All procedures of fractionation and analyses utilized reagents made up in distilled water that had been passed through Amberlite MB-3 resin.

Protein concentrations were determined by a micro-Kjeldahl method and from absorbancy measurements. Carefully standardized solutions of ceruloplasmin have an E_mg absorbancy value of 14.0 (1).

A Radiometer model 22 pH meter with a scale expander was used for all pH determinations. A type B glass electrode was employed for pH readings in the alkaline range. The Cary model 14 and the Beckman model DU spectrophotometers were utilized.

Copper was measured by the CHD method of Peterson and Bollier (5) and by a modification of the 2,2'-bicoumarine method of Felsenfeld (6). The latter permits estimation of total and of cuprous copper. Direct reacting copper, defined as the copper that will react with CHD at neutral pH, was determined by the following method. One milliliter of a ceruloplasmin solution containing from 2 to 4 μg of copper was mixed with 0.20 ml of 25% (weight per volume) sodium acetate, and the absorbancy was read at 600 μm. To this solution was added, with mixing, 0.20 ml of a saturated solution of CHD in 50% (by volume) ethanol. The absorbancy was immediately determined against an appropriate blank and then measured every 5 minutes until a constant value was obtained. The increase in optical density was used to determine the amount of reactive copper. Residual ceruloplasmin was separated from the blue Cu-CHD complex by passage through a column of G-25 Sephadex (0.9 × 10 cm).

The oxidase activity of the various ceruloplasmin preparations was measured by the method of Scheinberg and Morell (7).

EPR spectroscopy was performed as described previously (8). The magnetic field increases to the right as shown by the arrow following the H. Measurement of g values was made with an accuracy of ±0.005 unit.

RESULTS

pH Titration of Ceruloplasmin—Few experiments in the acid instability region were carried out on ceruloplasmin because precipitates formed that obviated spectrophotometric measurements. Exposure of ceruloplasmin to pH 10.78 effects changes in its visible spectrum as shown in Fig. 1. Ultraviolet spectra in the 255- to 310-μm range were taken, but no time-dependent changes were observed. A decrease of approximately 50% in the absorption maximum at 610 μm was observed after 30 minutes of incubation. At 23 hours, the solution had lost all visible blue color and the absorbancy at 610 μm was 8% of the original. Adjustment of the pH to 7.81 at this time resulted in a return of blue color to approximately 42% of the original absorbancy at 610 μm (indicated by arrow in Fig. 1). Further neutralization to pH 7.18 did not result in any additional increase in absorption at this wavelength.

Fig. 2 shows the changes in EPR spectra as a function of time at pH 10.78. It was necessary to record the EPR spectra as rapidly as possible after freezing since, under the conditions employed, time-dependent spectral changes occurred. The samples were discarded after the EPR measurement.

It is apparent from Fig. 2 that at pH 10.78 there is the formation of a second peak in the first derivative curve at high field strength and a spreading out of the typical, narrowly spaced,
The latter color disappeared on adjustment of the pH to 7.75 and was this was the formation of a 530-μm absorbancy maximum. This sorption and complete loss of oxidase activity. Coincident with an immediate loss of blue color, a disappearance of 610-μm absorbancy but only 85% of its oxidase activity. A preparation incubated for 27 hours at pH 10.74 lost 96% of its 610-μm absorbancy but only 67% of its oxidase activity.

It should be pointed out that close examination of the spectrum of native ceruloplasmin (Fig. 2.1) shows a slight "shoulder" analogous to, but quantitatively much less than that of the reneutralized sample (Fig. 2H). This raises the question of whether the "shoulder" component of the EPR spectrum of native ceruloplasmin is representative of the native protein or whether it is a modification resulting from the handling or purification procedures.

The oxidase activity of ceruloplasmin is rapidly lost at the pH employed in the experiments recorded in Fig. 2. When ceruloplasmin is incubated at 25° for 11 hours at pH 10.74, it loses 93% of its 610-μm absorbancy but only 67% of its oxidase activity. A preparation incubated for 27 hours at pH 10.78 lost 96% of its 610-μm absorbancy but only 85% of its oxidase activity. A return of from 10% to 12% of the blue color on neutralization of these samples was not paralleled by a significant increase in oxidase activity. This disparity suggests that the oxidase activity of this protein is not under all circumstances dependent on the same structural features as 610-μm absorbancy. In this regard, it is important to consider the low turnover number of ceruloplasmin. It is conceivable that the copper of ceruloplasmin may be displaced from its normal environment to form non-specific cupric complexes that have lost the typical high absorp-

A solution of ceruloplasmin that was adjusted to pH 13 showed an immediate loss of blue color, a disappearance of 610-μm absorption and complete loss of oxidase activity. Coincident with this was the formation of a 530-μm absorbancy maximum. This latter color disappeared on adjustment of the pH to 7.75 and was probably due to the classical biuret reaction, the source of copper being ceruloplasmin. The EPR spectrum of ceruloplasmin at pH 13 shown in Fig. 3 resembles the spectrum of the sample aged at pH 10.78 (see Fig. 2G). Adjustment of the pH to 7.75 does not restore oxidase activity or the spectrum (Fig. 3B) to that of the native protein, although the same gm value is obtained. No evidence is found, however, that the narrow hyperfine structure of the native protein is reformed. On the contrary, a spread-out hyperfine structure typical of ordinary cupric complexes is clearly seen. Only two components of the nuclear hyperfine structure are shown in Fig. 3. However, additional hyperfine structure not seen in native ceruloplasmin appears on the main peak. This

![Fig. 1. Changes in the visible spectrum of ceruloplasmin with time at pH 10.78 at room temperature.](http://www.jbc.org/)

![Fig. 2. EPR spectra (first derivative) of ceruloplasmin (8.91 mg per ml) at pH 10.78, recorded at -170°. Modulation amplitude of 6 gauss, microwave energy of 25 milliwatts, and scanning rate of approximately 60 gauss per minute. Time of exposure (in minutes) for each spectrum was: A, 0; B, 4; C, 32; D, 77; E, 222; F, 382; G, 1372; and H, Sample G reneutralized to pH 7.8.](http://www.jbc.org/)
fore, that under the conditions of the described treatment, the copper complexes and with proteins (10, 11). It is likely, there-
spectrum expected from binding of a copper to 4 nitrogen atoms.

EDTA, recorded at 0.1 the amplification of A. EPR spectra were
Similar spectra have been also observed previously with simple
Copper-EDTA
Tris-treated ceruloplasmin
Ceruloplasmin denatured
Ascorbic acid-treated ce-
Native ceruloplasmin

FIG. 3. Effect of alkaline denaturation of the EPR spectrum of
ceruloplasmin. Spectra recorded as in Fig. 3. A, ceruloplasmin
(6.0 mg per ml) brought to pH 13.06; B, roneutralized to pH 7.76.

FIG. 4. EPR spectrums: A, the chromatographically isolated
blue component of ascorbate-treated ceruloplasmin (40.5 mg per
ml); B, the “colorless” ascorbate component (56.1 mg per ml)
recorded at 0.78 times the amplification of A; C, 2 mM copper-
EDTA, recorded at 0.1 the amplification of A. EPR spectra were
recorded as in Fig. 3.

TABLE I
Parameters of EPR spectra of ceruloplasmin and derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>gXX</th>
<th>gYY</th>
<th>gZZ</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native ceruloplasmin</td>
<td>0 ± 0.005</td>
<td>0 ± 0.005</td>
<td>±0.001</td>
<td>2.056</td>
</tr>
</tbody>
</table>
| Ascorbic acid-treated ce-
uloplasmin                | 0.008 |
| Blue component            | 2.062 | 2.213 | 0.008 |
| Colorless component       | 2.060 | 2.204 | 0.019 |
| Ceruloplasmin denatured   | 2.060 | 2.204 | 0.019 |
| at pH 13 and then         |      |      |      |
| adjusted to pH 7          |      |      |      |
| Tris-treated ceruloplas-
min                        |      |      |      |
| Colorless component       | 2.054 | 2.260 | 0.018 |
| Copper-EDTA               | 2.090 | 2.237 | 0.015 |

a g value at maximal absorption, closely related to gXX.
b A1 is the hyperfine splitting constant related to gYY.

latter narrowly spaced structure is probably due to the nine-line
spectrum expected from binding of a copper to 4 nitrogen atoms.
Similar spectra have been also observed previously with simple
copper complexes and with proteins (10, 11). It is likely, there-
fore, that under the conditions of the described treatment, the
protein rearranges so that its cupric copper is now in a new en-
vironment and coordinated with nitrogen in a fashion analogous
to that observed in copper-amino acid complexes (11).

Treatment of Ceruloplasmin with Ascorbate or Tris—Two elec-
rophoretic components can be isolated from ceruloplasmin after
exposure to either ascorbate at pH 5 or to Tris buffer at pH 8 (1).
One of these components is colorless, whereas the other is disindis-
tinguishable from the native protein with respect to its visible
and ultraviolet spectrum, copper content, electrophoretic mo-
bility, sedimentation, and immunological properties (12). The
EPR spectrum of this material, shown in Fig. 4A is that of native
ceruloplasmin (see also Table I).
The colorless ascorbic acid reduction product of ceruloplasmin
contains approximately 4 g atoms of copper per mole (100,000)
of protein, as compared to 8 for the native protein. This product
has an increased electrophoretic mobility in alkaline buffers, and
its decreased rate of sedimentation indicates a molecule with an
increased asymmetry (1). The colorless component of ceruloplas-
min formed by the action of Tris buffer possesses similar
properties.

The EPR spectrum of the colorless ascorbic acid reduction
derivative, shown in Fig. 4B, is quite different from that of the
native protein. It is similar to the spectrum for ceruloplasmin
after exposure to pH 13 and neutralization. The previous
discussion of Fig. 3B is applicable to the spectrum of the ascorbic
acid reduction product of Fig. 4B. The hyperfine structure at
low field strength is well resolved and approaches the wide spac-
ing of ordinary cupric complexes such as the cupric-EDTA com-
plex (see Fig. 4C).

The value of g1 is an indication of the spread of the hyperfine
structure for complexes of similar gXX values. Considerable
spread is indicated by the data of Table I which gives a g1 for na-
tive ceruloplasmin of 2.209, and 2.269 for the colorless ascorbic
acid reduction product. The gXX values, however, change only slightly.
A similar change in the EPR spectrum is observed after treatment
with Tris (see Table I). EPR studies of ceruloplasmin in the
presence of ascorbate at neutral pH indicated that all of the
copper was in the cuprous form. Values for various parameters
obtained from the EPR measurements on native ceruloplasmin
and on various derivatives are given in Table I. Our values for
native ceruloplasmin agree very well with those previously pub-
lished by Malmström and Vangärd (11).

Even though the colorless ascorbic acid reduction product of
ceruloplasmin contains at least part of its copper in the cupric
state according to the EPR spectra, it no longer possesses oxidase
activity. The blue component isolated from the ascorbic acid
reduction mixture, however, has the same oxidase activity as
native ceruloplasmin.

Quantitative Determination of Copper in Ceruloplasmin—The
amount of cupric copper in ceruloplasmin was determined by
double integration of the EPR spectra. Total copper was meas-
ured by the CHD method. Table II summarizes the quantita-
tive data obtained on ceruloplasmin and some of its derivatives.
Repeated analyses of different samples of ceruloplasmin indicate
that approximately 40% of the copper in ceruloplasmin exists in
the cupric state. This is in close agreement with the values ob-
tained by other workers (13, 14).2

Measurements of cuprous and of total copper by the 2,2'-bi-
quinoline method (15) yielded an average value of 36% cuprous

2 J. Eisinger, Bell Telephone Laboratories, personal communi-
cation.
copper. The 2,2'-biquinoline reaction with ceruloplasmin in strong acetic acid solution proceeded only slowly. The color values after 15 minutes of reaction at room temperature were approximately 20% of the maximal values obtained after 24 hours. The data of Table II indicate that the fraction of cupric copper in the colorless components derived from ceruloplasmin does not vary appreciably from that found in the native protein.

The possibility exists that some of the copper of ceruloplasmin is not directly associated with the spectrophotometric or biological properties of the molecule and could possibly be removed without altering them. Pertinent data are shown in Table III.

The passage of ceruloplasmin in 0.05 M sodium acetate, pH 7, through Dowex A-1, a chelating resin with a high affinity for copper, resulted in only a small loss of copper and had essentially no effect on the absorbancy ratio and oxidase activity. However, on treatment with CHD over a period of 1 hour, ceruloplasmin was found to lose 1 to 1.5 atoms of copper. The major losses took place within a few minutes after the addition of the chelating agent. This result suggests that approximately 15% of the copper in ceruloplasmin is not tightly bound. The ceruloplasmin was separated from the blue CHD copper chelate complex by passage through G-25 Sephadex. No appreciable change in absorbancy ratio or oxidase activity was found to have occurred as the result of the chelation experiment. The ceruloplasmin recovered from the CHD chelation experiment was again passed over a column of the Dowex A-1 resin. An additional atom of copper per molecule of ceruloplasmin was removed without significantly affecting the absorbancy ratio or the oxidase activity. Similar results were obtained with three different crystalline preparations having an absorbancy ratio at 280: 260 nm of 22, which is characteristic of "pure" ceruloplasmin. The ceruloplasmin used in the experiments reported in Table III was crystalline material in which the absorbancy ratio had increased on standing at 2°C. The similarity of the chelation results for the aged and the fresh preparations show that the protein-bound copper is not converted to a form capable of reacting with CHD on loss of blue color. However, all of the copper of the colorless ascorbic acid reduction product is rapidly chelated by CHD. These preliminary data indicate that the spectral and oxidase properties of ceruloplasmin are not seriously modified by the removal of 2 to 2.5 of the 8 copper atoms in ceruloplasmin.

**DISCUSSION**

Comparisons of the EPR spectrum of ceruloplasmin with those of cupric copper complexes have been made previously by Malmström and Vännigård (11). Since our observations agree with theirs, we shall only briefly state the essential features. Ceruloplasmin and also laccase, as these authors found, stand out among copper proteins because of the unusually narrow spacing of their hyperfine structure. This is expressed in a low value of $g_1$ (≃2.20) and a low hyperfine splitting constant $A$ of 0.008 cm$^{-1}$, a value approximately half that observed with other copper complexes. Malmström and Vännigård (11) raise the point as to whether this unusual feature may not be related to the oxidase function of these enzymes. Too few proteins of this type are known at present to decide this question.

In an any more detailed account of the properties of copper in ceruloplasmin, these main features of the EPR spectrum will have to be explained and correlated with the results of optical measurements, which show an unusually high absorption at 610 mp ($\lambda_{max}$). Based on the level of cupric copper in ceruloplasmin, it is approximately 300 times higher than the comparable absorption at 800 mp ($\lambda_{max}$) of ordinary cupric complexes. A unique or obvious explanation of all of these phenomena does not exist at present.3

3 An earlier suggestion made by one of us (8) that the observations may be explained on the basis of electron exchange between closely adjacent cupric-cuprous ion pairs appears untenable, since a search for three additional components in the hyperfine structure, which are required by such a model, has been unsuccessful. In view of these negative results, and the actually resolved spectral details, exchange interaction could only be thought to exist in the case of extreme inequivalence of the copper atoms involved. A more satisfactory postulate may then be that of a strong covalent component in the bonding of the copper of its ligands. Since the experiments reported in this paper were not designed to answer these questions and since considerable work more closely related to these problems has been done in other laboratories (13, 14), this will not be discussed further.

**Table II**

<table>
<thead>
<tr>
<th>Copper concentration</th>
<th>Cu$^{2+}$</th>
<th>Cu$^{2+}$</th>
</tr>
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</table>
| Native ceruloplasmin | 1.00 x 10$^{-3}$ | 2.50 x 10$^{-2}$ | 40
| Blue component | 0.78 x 10$^{-4}$ | 1.08 x 10$^{-4}$ | 40
| Colorless ascorbate reduction product | 1.20 x 10$^{-4}$ | 2.98 x 10$^{-4}$ | 44
| Colorless Tris | 3.51 x 10$^{-4}$ | 0.37 x 10$^{-4}$ | 55
| 3.00 x 10$^{-4}$ | 7.00 x 10$^{-4}$ | 56

**Table III**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Treatment</th>
<th>Cu$^{2+}$</th>
<th>Cu$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>36.8</td>
<td>8.1</td>
</tr>
<tr>
<td>B</td>
<td>Filtration through Dowex A-1</td>
<td>39.5</td>
<td>7.6</td>
</tr>
<tr>
<td>C</td>
<td>CHD at pH 7.2, 1 hour followed by gel filtration through Sephadex G-25</td>
<td>36.6</td>
<td>6.75</td>
</tr>
<tr>
<td>D</td>
<td>Solution C passed over Dowex A-1</td>
<td>34.5</td>
<td>5.75</td>
</tr>
</tbody>
</table>

* CHD method.
* Independent determination of Cu$^{2+}$ by the 2,2'-biquinoline method yielded 36% Cu$^{2+}$.
* These components contain 3 atoms of direct reacting copper per molecule of protein; however, 3.5 atoms of copper per molecule of protein are found by total copper analysis.

* Based on 1.6 x 10$^{4}$ g per mole of protein.
* The change in optical density at 530 mp per minute per mmole of protein in 3 ml at 25°C.
* The initial 280:610 mp absorbancy ratio of this preparation was 25; however, on dialysis against 0.05 M sodium acetate, pH 7.2, for 3 days at 2-4°C, the ratio had increased to 36.8.
* Chelating resin with a high affinity for transition metal.
* This procedure removes the blue chelate complex and permits analysis of ceruloplasmin for total copper.
Approximately 40% of the copper found in ceruloplasmin by chemical analysis is accounted for in the EPR spectrum. There is also general agreement on this observation (13, 14). Analyses for copper with 2,2'-biquinoline indicate that approximately 60% of the total is cuprous. Thus, there is agreement between the EPR and chemical estimations of cupric copper.

The chemical and physical data indicating at least two forms of copper in ceruloplasmin are in agreement with earlier studies showing that all of the copper atoms in this molecule do not show similar reactivities (7, 16–18).

The results of the present studies show that some of the changes in the physicochemical properties of ceruloplasmin are paralleled by modifications of the EPR spectrum. The effect of reducing agents and of elevated pH on the electrophoretic behavior and on the absorption spectrum of ceruloplasmin is accompanied by changes in the EPR spectrum and the formation of new species of cupric complexes. Similarly, new and enzymatically inactive electrophoretic components with low absorbancy at 610 nm, which arise on treatment of ceruloplasmin with ascorbate and with Tris, show EPR spectra distinct from that of the native protein. These spectra indicate conversion of the rather uniquely bonded cupric copper of ceruloplasmin to a state very similar to that of cupric ions in most copper complexes. We may, therefore, conclude that rearrangements of the kind induced in our experiments which have been shown to lead to changes of charge or of shape or of both, also involve the immediate environment of the cupric copper of the enzyme. The generally observed lability of the molecule is therefore reflected in the state of its cupric copper. The recent studies by Broman et al. (13) have indicated that modifications in the structure of ceruloplasmin effected by high urea concentrations are accompanied by paralleled changes in oxidase activity, loss of blue color, and changes in the nature of the bonding of the copper.

**SUMMARY**

Chemical chelation and electron paramagnetic resonance studies indicate that 40% of the copper in crystalline ceruloplasmin is in the cupric state. Modifications of ceruloplasmin leading to changes in charge and shape are reflected by changes in the immediate environment of the copper atoms which are responsible for the electron paramagnetic resonance spectrum of this protein.

**REFERENCES**

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