Sulfhydryl Groups in the Reconstitution of Ceruloplasmin[1] (34741)

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(Introduced by R. J. Winzler)
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Ceruloplasmin is the blue, copper-containing metalloprotein found in the blood of most vertebrates. It has a probable role in copper transport (1, 2) and iron utilization (3, 4). This protein contains 6 to 8 copper atoms/molecule; the precise number being still subject to some controversy (5). Magnetic susceptibility measurements confirm that the bound copper is present in both the +2 and the +1 valence states (6, 7). The intense blue color (λ_{max} 610 nm) of ceruloplasmin has been attributed to the bound Cu(II) atoms (8, 9).

ERP studies suggest that there are two distinctly different Cu(II) binding sites (10, 11) implying that there are at least three types of copper binding sites in ceruloplasmin. The nature of the ligands and the symmetry of the copper sites are unknown. It was hoped that physical and chemical studies of apoceruloplasmin and its reconstitution product particularly with reference to sulfhydryl groups, might aid in the elucidation of the copper binding sites of ceruloplasmin.

Materials and Methods. Ceruloplasmin was prepared by a modification of the method of Deutsch *et al.* (12) (using Cohn IV-1 fraction of human plasma provided by Mass. Public Health Biol. Labs., Jamaica Plains, Mass., and by the American Red Cross, Washington, D.C.), except that DEAE-Sephadex A-50 was used for chromatography. The final crystallized product had an R value of 22.5 ± 0.5 [$R = \epsilon_{280}/\epsilon_{610} = 21.9$ for pure ceruloplasmin (5)] and exhibited a single component on immunoelectrophoresis and in the ultracentrifuge (13).

Apoceruloplasmin was prepared by several methods. One method was adapted after that of Morell *et al.* (14). Reduced ceruloplasmin (reductant, cysteine) was dialyzed 24 hr against 0.20 M acetate buffer, pH 5.5, containing 2–3 mg/ml of Na–diethyldithiocarbamate (DTC). The reaction mixture was then treated according to Aisen and Morell (15). Apoceruloplasmin was also prepared by removal of copper from native ceruloplasmin with Na_2–ethylenediaminetetraacetate (EDTA). Ceruloplasmin in 0.20 M acetate buffer, pH 5.5, 1% NaCl was dialyzed at 3° against 50 vol of 0.20 M PO_4 buffer, pH 5.75, containing 3 mg/ml of EDTA. The dialysis was continued for 5–6 days with several changes until the characteristic blue color of ceruloplasmin was no longer visible. The mixture was then dialyzed against buffer to remove EDTA and stored at 3°. A third preparative method involved dialysis of ceruloplasmin against 40 vol of 0.20 M KCN in 0.20 M acetate buffer, pH 5.5, 1% NaCl at 3°. The mixture decolorized within 1 hr and was dialyzed against several changes of KCN and then buffer to remove KCN and stored at 3°. In one preparation ceruloplasmin was reduced with excess cysteine·HCl prior to the KCN treatment described above.

The extinction coefficient at 280 nm for ceruloplasmin and apoceruloplasmin was determined by the gravimetric method of Hoch and Vallee (16). The $E^{1\%}_{280}$ for ceruloplasmin was determined to be 14.9 as reported

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(12), whereas $E^{1\%}_{280}$ for apoceruloplasmin was 13.6. The copper content of ceruloplasmin, apoceruloplasmin, and other products was determined by the method of Felsenfeld (17). The sulfhydryl titer of the protein solution was determined by the spectrophotometric method of Boyer (18). Stock solutions of sodium *p*-hydroxymercuribenzoate (*p*-MB) (Sigma) were prepared by dissolving 9.0 mg in 1 ml of 0.010 *M* NaOH and dilution to 25 ml. Working solutions were prepared by dilution of the stock with 0.050 *M* phosphate buffer, pH 6.5, 1% NaCl. This solution was standardized spectrophotometrically on the basis of $E_{233\,nm} = 1.69 \times 10^4$.

Results. Reconstitution was initiated by addition of Cu(II) to a solution of the apoprotein, which had been previously incubated with either cysteine or hydroxylamine · HCl, and the resultant increase in 610 nm absorbancy was measured.[5] DTC treatment of ceruloplasmin yielded a colorless apoprotein product containing less than 0.3 atoms of Cu/molecule of protein. The DTC–apoceruloplasmin preparations gave the highest reconstitution yields, 65 ± 10% for 15 preparations.

EDTA treatment of ceruloplasmin in a phosphate buffer removed Cu ion from the protein yielding a colorless protein product. The process was slow and was accompanied by the formation of a white precipitate and a 20–25% decrease in A_{280} during reaction. The percentage reconstitution for four preparations was 50 ± 5%.

Dialysis of ceruloplasmin against a KCN solution rapidly eliminated the characteristic blue color and yielded a product with >97% of the original Cu atoms removed. However, less than 15% reconstitution was obtained. Apoprotein prepared by KCN treatment of reduced ceruloplasmin did not recombine with Cu any more effectively.

The percentage reconstitution depended not only on the method of preparation of the apoenzyme but also on the reaction mixture used for recombination of Cu with the apoprotein (Table I). The observation of Morell *et al.* (14) that the addition of Cu(II) to apoceruloplasmin gave ~25% yield of ceruloplasmin based on A_{610} absorbance was confirmed. The most effective Cu donor in these recombination studies proved to be Cu(II) added to apoprotein in the presence of a suitable reducing agent as reported earlier by Aisen and Morell (15). However, apoprotein prepared and reconstituted as reported by these authors (15) typically gave 50–60% yields, never as high as 85–90%. The reducing agent itself had some effect on the yield (Table I) with ascorbate, the reducing agent used by Aisen and Morell (15), being the least satisfactory. $Cu(CH_3CN)^+$ was also effective as a Cu donor yielding 40–45% reformation.

Sulfhydryl titrations. The –SH titers of native and apoceruloplasmins as determined by the method of Boyer (18) are summarized in Table II. "Native" ceruloplasmin as prepared in this laboratory demonstrated less than 0.5 –SH/molecule protein when titrated with *p*-MB. This value was less than a published value of one –SH/molecule (19). The –SH titer of apoceruloplasmin was dependent on the preparative method and varied from 2 to 4 –SH/molecule. The number of –SH/molecule of the apoprotein obtained from all preparations was consistently greater than the published value of one

TABLE I. Dependence of Reconstitution on Copper Reagent.

The data shown are for recombination of Cu(II) with a specific DTC prepared apoceruloplasmin at 30°. In each case, aliquots of the apoproteins were added to the sample cuvette of the spectrophotometer and A_{610} was recorded following addition of the Cu reagent ([Cu]:[protein] = 7). Reducing agents were incubated with the apoprotein 10–15 min prior to addition of Cu(II) ([reductant = 5 [Cu]).

Cu reagent	Percentage reconstitution
Cu(II)	22 ± 3
$Cu(CH_3CN)^+$	42 ± 3
Cu(II) + ascorbic acid	55 ± 5
+ cysteine	65 ± 5
+ hydroxylamine	75 ± 5

[5] R. Lovstad has found that the reconstituted enzyme had the same K_m but a slightly lower V_{max} using *N*, *N*-dimethyl-*p*-phenylenediamine as substrate.

TABLE II. Sulfhydryl Titer of Ceruloplasmin Preparations.

Sulfhydryls were determined in duplicate by the method of Boyer (18). Microliter portions of the protein solutions were added to 1.00 ml of standardized *p*-MB solution in the sample cuvette and to the same volume of buffer in the reference cuvette. ΔA_{250} was recorded after equilibration (20–30 min at 30°). The –SH titer was then determined graphically as in Fig. 1.

Sample	–SH/molecule	No. of preparations
Native ceruloplasmin	<0.5	4
DTC apoceruloplasmin	4.0 ± 0.5	15
EDTA ceruloplasmin	2.7 ± 0.3	4
KCN ceruloplasmin	2.2 ± 0.2	3
Reconstitution mixture[a]	0.8 ± 0.3	3

[a] DTC prepared apoprotein after reconstitution and after exhaustive dialysis to remove any excess Cu(II) and reducing agent. The fact that this value is larger than the number of –SH/molecule of native ceruloplasmin is due to the presence of about one-third of the unreconstituted apoenzyme, part of which may have been dimerized or had some of its –SH groups oxidized.

–SH/molecule (19). Figure 1 illustrates a typical titration curve for native and DTC-prepared apoceruloplasmin. In both cases, standardized *p*-MB solutions were titrated with the protein solutions Titrating the apoprotein solutions with standardized *p*-MB gave a less well-defined but identical inflection point. The –SH titer of apoceruloplasmin was unaffected by aging for 3 weeks when stored in 0.20 *M* acetate buffer, pH 5.5, 1% NaCl at 3°.

Addition of *p*-MB to apoceruloplasmin (DTC preparation) at a 4:1 molar ratio completely inhibited the formation of blue protein in reconstitution studies. This inhibition was not relieved by dialysis against cysteine in acetate buffer.

Discussion. In agreement with Aisen and Morell (15), no evidence for any intermediate species of ceruloplasmin containing less than 7–8 Cu atoms/molecule (160,000 mol wt) was observed. Apoproteins prepared from different batches of ceruloplasmin and different sources of Cohn IV-1 fractions showed considerable variation in percentage reconstitution but the value for a given preparation was constant to ±2%. Variations in % Cu and E_{280}/E_{610} ratios of the starting material seemed to affect the reconstitutability of apo-

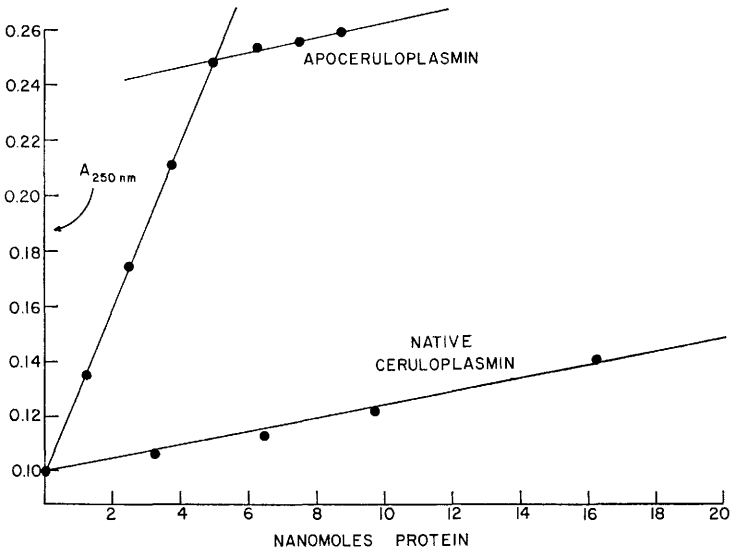

FIG. 1. Representative spectrophotometric titration of sulfhydryl groups in native and apoceruloplasmin. Experimental procedures are outlined in Table II. Apoprotein (DTC preparation) solution added to a solution containing 22.5 nmoles of *p*-hydroxymercuribenzoate in 0.050 *M* PO_4, pH 6.5. Native protein solution added to a similar solution containing 16.7 nmoles of *p*-hydroxymercuribenzoate. The A_{250} measurements were corrected for volume change.

ceruloplasmin. The –SH titer of the apoproteins (Table II) was also constant to ±5% for a given preparation. However, significant variations were observed for different apoprotein preparations.

The possibility of DTC binding to the apoprotein contributing at least partially to the –SH titer of the DTC preparations is unlikely. Kasper and Deutsch (19) reported one –SH/molecule of apoprotein prepared by essentially the same method, suggesting that DTC is not absorbed to the protein since they observed the same –SH titer for the native protein.

These studies, in contrast to Kasper and Deutsch (19), demonstrate a marked increase in –SH groups in the reconstitutable, and to a lesser extent, in the nonreconstitutable aproprotein preparations, *i.e.*, 4 –SH and 2 –SH groups/molecule, respectively. The increase in –SH titer suggests the involvement of the –SH residues in Cu binding. It was observed that for the apoproteins prepared by DTC treatment, an increase in –SH titer was directly proportional to an increase in the percentage reconstitution obtained from that preparation.

After this work was completed, the paper of Witwicki and Zakrezewski (20) appeared in which one –SH group was found in human ceruloplasmin by titration with β-hydroxyethyl-2,4-dinitrophenyl disulfide in 0.675 *M* Tris–Cl buffer, pH 7.5. However, under conditions more comparable to ours, only 0.35 –SH group was titrated in PO_4 buffer, pH 7.5. Two additional –SH groups were unmasked in 7 *M* urea and a third in EDTA–urea. It is possible that these –SH groups correspond to the 3–4 –SH groups exposed in apoceruloplasmin.

Gray (13) has studied the Cu(II) addition in more detail and has suggested a mechanism in which Cu(I) is generated through reduction of Cu(II) by the available –SH groups in the apoprotein. The unfractionated protein resulting from Cu(II) reconstitution was heterogeneous in the ultracentrifuge due to a small amount of a heavier component. Gray (13) proposed that this was a dimer of ceruloplasmin resulting from intermolecular disulfide formation.

Conformational changes in the apoprotein could account for the exposure of –SH groups which were buried in the native protein. The ultracentrifuge data of Kasper and Deutsch (19) was confirmed and suggested that minor unfolding does occur in the apoprotein of human ceruloplasmin. Hibino *et al.* (21) reported a similar behavior in the ultracentrifuge for porcine ceruloplasmin and apoceruloplasmin prepared by KCN treatment. Neither paper reported reconstitution data. However, the decrease in $S^0_{W,20}$ for the apoprotein relative to the holoprotein was $< 1S$, suggesting no large conformational change.

Morpurgo and Williams (22) have developed a theoretical reasoning for Cu binding in the "blue" copper proteins involving reducing ligands such as RS^-. While no direct evidence exists for the free cysteine residues being involved in Cu binding, it is an attractive hypothesis, consistent with these studies.

Summary. The involvement of sulfhydryl groups in the Cu binding and reconstitution of apoceruloplasmin was investigated. Recombination studies in which different Cu reagents were added to the apoprotein demonstrated a preference of the apoprotein for Cu(I) generated in solution by the action of a suitable reductant. The –SH titer for apoproteins prepared by different methods was correlated with the percentage reconstitution obtained in the Cu–protein recombination studies. It was found that reconstitution of the apoprotein was proportional to the average number of –SH groups/molecule in the protein preparation. The results of this study suggest that apoceruloplasmin contains 4 –SH groups/molecule and that these –SH groups are necessary for reconstitution. Under our conditions, native ceruloplasmin was found to have less than 0.5 –SH groups/molecule.

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