Dissociation and Reconstitution of Human Ceruloplasmin†

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ABSTRACT: The molecular weight and subunit structure of an ultracentrifugally and electrophoretically pure preparation of human ceruloplasmin were studied. A molecular weight of 124,000 was determined for the native protein by sedimentation equilibrium. It was found that the protein molecule dissociates into three smaller species L, H, and H′ by (a) sodium dodecyl sulfate in the presence of 2-mercaptoethanol or *p*-hydroxymercuribenzoate or (b) urea or Gdn·HCl in the presence of 2-mercaptoethanol. The molecular weights of the L, H, and H′ species, determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, are 16,000, 53,000, and 69,000 respectively. Removal by dialysis of the dissociating agent sodium dodecyl sulfate and 2-mercaptoethanol brings about reassociation, and recombination with copper leads to reconstitution of ceruloplasmin as evidenced by the restoration—up to 80%—of the original oxidase activity. The dissociation and reconstitution experiments unequivocally show that human ceruloplasmin is a multichain protein. It is proposed that ceruloplasmin is an L_2H_2 tetramer, where L and H are light and heavy chains of molecular weights 16,000 and 53,000, respectively.

Ceruloplasmin is a blue copper-containing serum protein with oxidase activity. It has been proposed (Osaki *et al.*, 1966) that the physiological function of ceruloplasmin involves the oxidation of ferrous iron and its incorporation into apotransferrin. In spite of the considerable effort invested in the last decade to solve the molecular structure of ceruloplasmin, there has not emerged a unique picture that has won unanimous acceptance. Early work had placed the molecular weight at about 160,000 (Holmberg and Laurell, 1948; Kasper and Deutsch, 1963). More recent determinations have tended toward a lower value of about 130,000 (Magdoff-Fairchild *et al.*, 1969; Rydén, 1972). As regards the subunit structure of ceruloplasmin, the situation is even more confused. One of the first proposals was that of Poillon and Bearn (1966) according to which the molecule would consist of eight subunits of identical molecular weight. Subsequently, their results were questioned by Simons and Bearn (1969), who found that the molecule could be dissociated into two polypeptide chains differing greatly in molecular weight, with the heavier chain showing electrophoretic heterogeneity. An octameric structure containing four nonidentical subunits has been proposed by Poulik (1968). More recently, however, Rydén (1972) has questioned the multichain structure of ceruloplasmin, arguing that the dissociation observed by previous workers could be attributed to cleavage of labile bonds in the protein by enzymatic contaminants present in the commercial preparations of the protein. Rydén was thus led to propose a single-chain structure for ceruloplasmin.

In view of the uncertainty regarding the molecular weight of native ceruloplasmin and the nature of its subunit structure, the present study was undertaken. A clear-cut answer was sought to the question: is ceruloplasmin a single chain protein as Rydén has proposed, or is it a multichain protein as the previous studies have tended to indicate? A critical evaluation of the results presented in this study unequivocally shows that human ceruloplasmin is a multichain protein and raises again the question of its correct subunit structure.

Experimental Section

Materials. Human ceruloplasmin was obtained from Miles-Yeda. The protein was prepared by affinity chromatography and showed the following characteristics: (a) one band was obtained in immunoelectrophoresis against antihuman whole serum; (b) the ratio of its absorbance at 280 to 610 nm was 22; (c) the oxidase activity (see below) was 54 units/mg of protein. Stock solutions, 35–40 mg/ml of protein in 0.1 M NaCl, were kept at −20° until use. Ceruloplasmin concentrations were determined spectrophotometrically at 280 nm, using $E^{1\%}_{1\text{ cm}} = 14.9$ (Kasper and Deutsch, 1963). Sodium dodecyl sulfate, of 95% purity, was obtained from Sigma. The proteins which served as markers in sodium dodecyl sulfate gel electrophoresis were obtained from Mann, Sigma, Worthington, and Miles-Seravac. Reagent grade chemicals and double-distilled water were used throughout.

Methods. POLYACRYLAMIDE GEL ELECTROPHORESIS. Analytical gel electrophoresis was performed according to Davis (1964) in gels having a total acrylamide concentration of 7% in Tris–glycine buffer (pH 8.9). A constant current of 5 mA/gel for 1 hr was applied. Staining and destaining were done according to Weber and Osborn (1969).

SODIUM DODECYL SULFATE GEL ELECTROPHORESIS. Except for modifications explicitly indicated in each case, sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (1969), using half-concentration of the cross-linker—in the connotation of the authors—in the preparation of the gels. Gels were scanned at 580 nm on a Gilford 2400 spectrophotometer.

ULTRACENTRIFUGATION. Ultracentrifugations were performed with a Beckman Model E analytical ultracentrifuge equipped with schlieren and Rayleigh interference optics, and a split-beam photoelectric scanning absorption optical system. Schlieren optics were generally used in sedimentation velocity runs; interference and absorption optics were used to determine the concentration distribution in sedimentation equilibrium experiments. In the sedimentation equilibrium experiments, 3-mm liquid columns were employed. Interference patterns were read using a Nikon Model 6C microcomparator.

Low-speed sedimentation equilibrium experiments were carried out at initial protein concentrations of 0.2–4.0 mg/ml.

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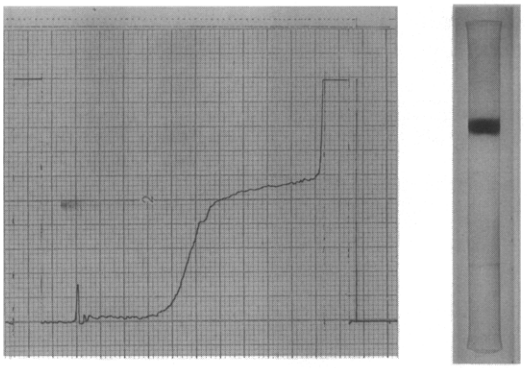

FIGURE 1: Purity tests for the human ceruloplasmin used in the present study. Left: Absorption scan at 280 nm of solution of 0.37 mg/ml of the protein in 0.1 M phosphate (pH 7.2) at 23°, centrifuged at 56,000 rpm, 55 min after reaching speed. Right: Analytical polyacrylamide gel electrophoresis of ceruloplasmin in Tris–glycine buffer (pH 8.9).

Absorption scans were carried out at 280 nm. When interference optics were used, the initial protein concentration, in fringes, was determined from an auxiliary layering experiment in a synthetic boundary cell. The position of the zero-order fringe in the equilibrium pattern was determined from the location of the white light fringe with the appropriate amount of 1,3-butanediol added to the solvent compartment and confirmed by calculations based on the conservation of mass within the cell (Richards *et al.*, 1968).

High-speed sedimentation equilibrium (Yphantis, 1964) was used for dilute protein solutions with initial concentrations of 0.20–0.85 mg/ml. Occasionally, the temperature was controlled by the use of the refrigeration unit alone, to eliminate any possibility of convection (Cassman and Schachman, 1971).

Calculation of the molecular weight from sedimentation equilibrium experiments was carried out from the equation $M = \{2RT/[(1 - \bar{v}\rho)\omega^2]\}\mathrm{d} \ln c/\mathrm{d}r^2$, where R is the gas constant, T is the absolute temperature, ω is the angular velocity of the rotor, $\bar{v}$ is the partial specific volume, ρ is the density of the solution, c is the protein concentration, and r is the distance from the axis of rotation. A value of $\bar{v} = 0.714$ ml/g (Magdoff-Fairchild *et al.*, 1969) was used.

OXIDASE ACTIVITY. Oxidase activity was measured according to the method of Curzon and Vallet (1960); 10 min was chosen for color development. One unit of oxidase activity was defined as the activity that causes, at 550 nm, an increase in absorbance of 0.01 per min at pH 5.5 and 37°, in a 7-ml reaction volume using *N*-dimethyl-*p*-phenylenediamine as the substrate.

Results

Purity of Ceruloplasmin. Human ceruloplasmin used in this study gave a single band in analytical gel electrophoresis (Figure 1). In sedimentation velocity experiments, the protein sedimented as a single symmetrical peak, (Figure 6C) with $s^0_{20,w} = 7.07$ S. Absorption scans at 280 nm showed that no absorbing material remained behind the sedimenting boundary (Figure 1). Linearity of the log c *vs.* r^2 plots in sedimentation equilibrium points to the presence of a single sedimenting component. These findings, together with the high copper content as shown by the 280:610 nm absorbance ratio (see Experimental Section), may be taken as a good indication of the purity of the ceruloplasmin preparation used here.

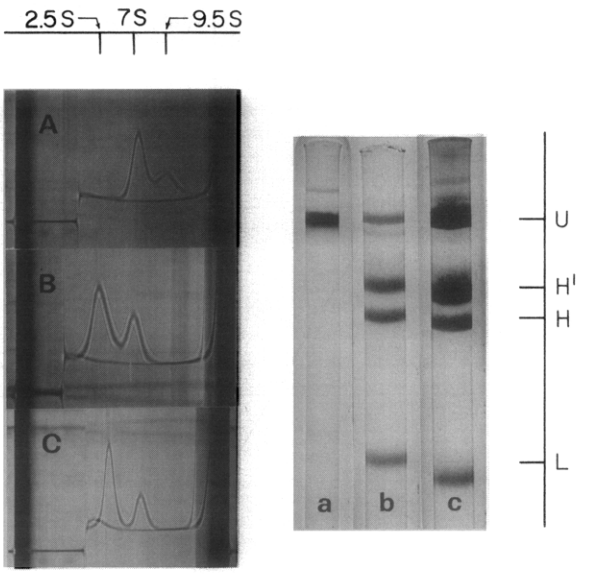

FIGURE 2: Sedimentation velocity photographs (left) and sodium dodecyl sulfate gel electrophoresis patterns (right) of ceruloplasmin incubated with 0.2% sodium dodecyl sulfate in 0.1 M phosphate (pH 7.0) (A,a); 0.2% sodium dodecyl sulfate and 0.2% HSEtOH in 0.1 M Tris (pH 8.0) (B,b); and 0.2% sodium dodecyl sulfate and 10^{-4} M HgOHBzO in 0.1 M Tris (pH 8.0) (C,c). The protein solution, about 10 mg/ml, was incubated for 48 hr at 4° with the reagent following which one aliquot was sedimented at 56,000 rpm and another subjected to sodium dodecyl sulfate gel electrophoresis. In c, HgOHBzO was substituted for HSEtOH in the preparation of the sample for electrophoresis; in a, HSEtOH was altogether excluded. Photographs show the sedimentation pattern 50 (A,C) and 80 min (B) after reaching maximum speed; approximate s_{obsd} values of the sedimenting peaks are indicated. In electrophoresis, component U is attributed to undissociated ceruloplasmin; H′, H, and L, in order of increasing mobility, are atrributed to dissociated species.

Molecular Weight of Native Ceruloplasmin. The molecular weight of native ceruloplasmin was determined from the slope of the log c *vs.* r^2 plots in conventional (low-speed) and meniscus depletion (high-speed) sedimentation equilibrium experiments. The results are: 126,000 (6800 rpm, interference optics), 122,000 (9000 rpm, absorption optics), and 123,000 (20,000 rpm, interference optics).

Dissociation of Ceruloplasmin. The effect on ceruloplasmin of denaturing agents, known to cause dissociation in multichain proteins, was studied. Whether a given denaturant was effective or not was determined from the sedimentation velocity picture in the ultracentrifuge and from the banding pattern in sodium dodecyl sulfate gel electrophoresis.

The effect of sodium dodecyl sulfate, alone and in combination with 2-mercaptoethanol (HSEtOH[1]) or *p*-hydroxymercuribenzoate (HgOHBzO), is shown in Figure 2. The sedimentation pattern in sodium dodecyl sulfate alone shows a 7S peak characteristic of native ceruloplasmin and, in addition, a faster moving peak, 9.5 S, presumably an aggregate of higher molecular weight. In the presence of HSEtOH or HgOHBzO, sodium dodecyl sulfate causes the appearance of a slow moving peak, ~2.5 S, in addition to the 7S peak of the native protein. The corresponding three electrophoresis patterns show a band, designated as U in Figure 2, moving with the same relative migration velocity. The molecular weight corresponding to this common band was estimated to be ~120,-

[1] Abbreviations used are: HSEtOH, 2-mercaptoethanol; HgOHBzO, *p*-hydroxymercuribenzoate; Gdn·HCl, guanidine hydrochloride.

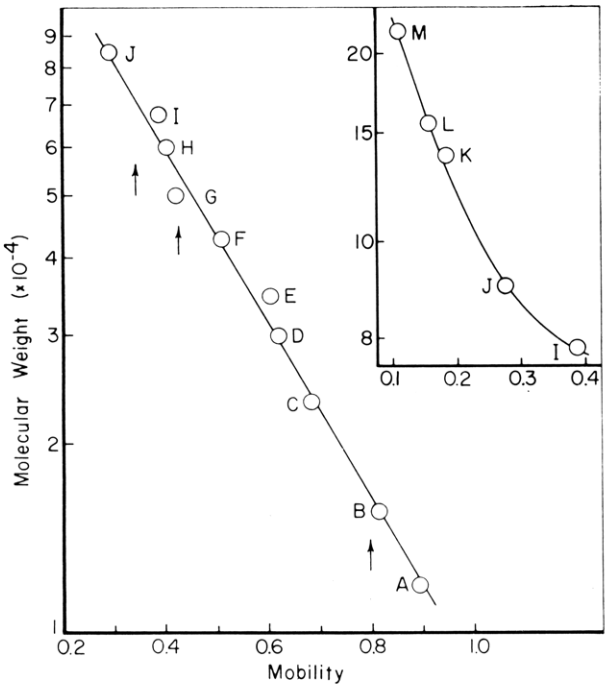

FIGURE 3: Semilogarithmic plot of molecular weight *vs.* relative band mobility in sodium dodecyl sulfate gel electrophoresis. The marker proteins used were (A) cytochrome *c*; (B) hemoglobin; (C) light chains of γ-globulin; (D) carbonic anhydrase; (E) carboxypeptidase A; (F) ovalbumin; (G) heavy chains of γ-globulin; (H) catalase; (I) bovine serum albumin; (J) ovalbumin dimer; (K) bovine serum albumin dimer; (L) γ-globulin; (M) myosin. The arrows from left to right indicate the mobilities of the H′, H, and L species in dissociated ceruloplasmin (see Figure 2); the molecular weight of the species corresponding to the U band was estimated by reference to the calibration curve shown in the inset.

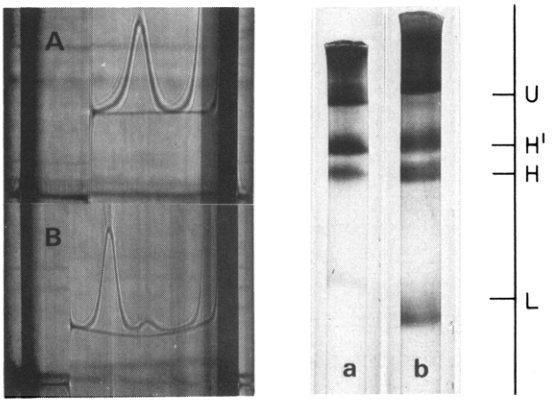

FIGURE 4: Left: Sedimentation velocity photographs of ceruloplasmin incubated with 6 M Gdn·HCl in 0.1 M Tris (pH 8.0) (A) and 6 M urea in 0.1 M phosphate (pH 7.2) (B), both containing 0.2% HSEtOH. Protein solutions, 8–12 mg/ml, were incubated for 48 hr at 4° with the reagents following which aliquots were sedimented. Photographs show the sedimentation patterns after 11 hr at 52,000 rpm (A) and 5 hr at 56,000 rpm (B). Sedimentation coefficients, corrected for solvent density and viscosity, are $s_{20,w}$ = 0.75 S (A) and $s_{20,w}$ = 1.4 and 2.8 S (B). Right: Ceruloplasmin treated with Gdn·HCl–HSEtOH or urea–HSEtOH (the remaining solutions from the sedimentation runs in A and B of this figure) was alkylated with iodoacetic acid, dialyzed against 0.1% sodium dodecyl sulfate in 0.1 M phosphate (pH 7.0), and subjected to sodium dodecyl sulfate gel electrophoresis. In the preparation of samples for electrophoresis, HSEtOH was excluded. The symbols designating the bands in the electrophoretic patterns are as in Figure 2.

000 from the calibration curve in Figure 3; it may be assumed, therefore, that this band, like the 7S peak in sedimentation, represents undissociated ceruloplasmin. In sodium dodecyl sulfate alone the slowest migrating band (Figure 2) corresponds to the 9.5S aggregate; the L, H, and H′ bands in sodium dodecyl sulfate–HSEtOH and sodium dodecyl sulfate–HgOHBzO correspond to dissociated ceruloplasmin. The molecular weights corresponding to the L, H, and H′ bands in sodium dodecyl sulfate–HSEtOH and sodium dodecyl sulfate–HgOHBzO, estimated from the calibration curve in Figure 3, are 16,000, 53,000, and 69,000. The relative amount of protein in each band was estimated by measuring the area under the scans of the stained gels. Use of our estimated molecular weights enabled us to make a rough estimate, on a molar basis, of the distribution of protein among the three bands. The results of 12 separate determinations gave an approximate molar ratio of 1–2:1:1 for the L, H, and H′ protein species, respectively.

In order to eliminate the possibility that some bands in sodium dodecyl sulfate gel electrophoresis might be due to proteolytic degradation of ceruloplasmin by contaminating enzymes, two variations of the experimental procedure were tried (Pringle, 1970). In one, the protein samples were boiled for 3 min at 100° prior to or immediately after dissolving in the dissociating solution. In the second, a proteolytic enzyme inhibitor (phenylmethylsulfonyl fluoride) was added. The electrophoretic banding pattern was not affected by any of these treatments.

The effect on ceruloplasmin of urea, or Gdn·HCl, in the presence of HSEtOH, was next examined. The sedimentation pattern is shown in Figure 4. In urea–HSEtOH two peaks are apparent while in Gdn·HCl–HSEtOH a single peak is seen. Whether the peak in Gdn·HCl–HSEtOH is due to a single sedimenting species or is rather attributable to two or more unresolved components cannot be decided from the sedimentation velocity experiment alone. The results suggest that, at least in the case of urea–HSEtOH, dissociation of ceruloplasmin takes place. In view of the inconclusive nature of the sedimentation result under these conditions, the following experiment was carried out. A ceruloplasmin solution was treated with urea (or Gdn·HCl) in the presence of HSEtOH, alkylated, and, following dialysis of urea (or Gdn·HCl) and HSEtOH, was run in sodium dodecyl sulfate gel electrophoresis. Since sodium dodecyl sulfate alone cannot effect dissociation (Figure 2), the presence of the L, H, and H′ bands in the electrophoretic pattern (Figure 4) definitely shows that urea–HSEtOH and Gdn·HCl–HSEtOH were both effective in bringing about dissociation of ceruloplasmin.

The time course of dissociation in sodium dodecyl sulfate–HSEtOH is presented in Figure 5. The sedimentation patterns show that the area under the 7S peak diminishes as the incubation time increases; concomitantly, the area under the 2.5S peak increases. The progress of dissociation can also be followed in the banding patterns in electrophoresis where increased incubation time causes an increase in the relative intensities of the L, H, and H′ bands at the expense of the U band corresponding to the residual undissociated protein. From the results presented in Figure 5, it is seen that virtually complete dissociation of native ceruloplasmin can be achieved.

It should be mentioned that, in addition to the three main electrophoretic bands into which ceruloplasmin dissociates, less intense minor bands, corresponding to molecular weights of 30,000 and 83,000, on occasion appear. The minor band of mol wt 30,000 may be seen in Figure 5b,c.

Reconstitution of Ceruloplasmin. The ability of dissociated ceruloplasmin to reassociate, once the dissociating agent is re-

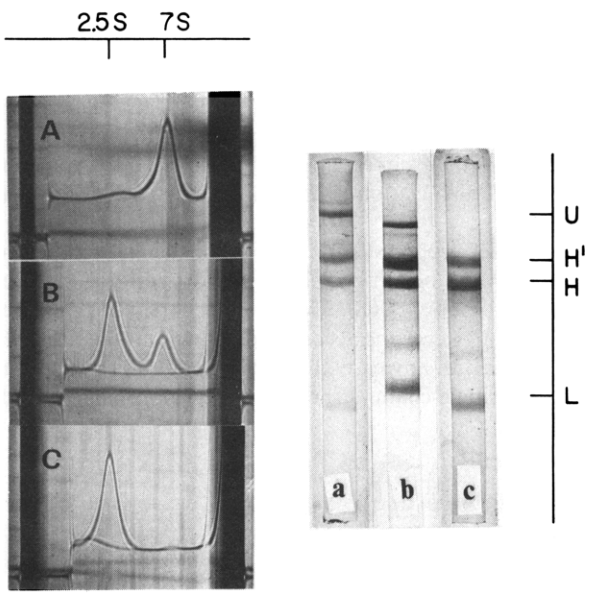

FIGURE 5: The effect of incubation time on the dissociation of human ceruloplasmin. Left: A protein solution, about 10 mg/ml, was incubated with 0.2% sodium dodecyl sulfate–0.2% HSEtOH in 0.1 M Tris (pH 8.0) at 4°. The photographs show the sedimentation pattern after incubation for 3 (A), 60 (B), and 90 hr (C). Sedimentation was carried out at 56,000 rpm and the photographs were taken about 90 min after reaching speed. Approximate sedimentation values are indicated. Right: A protein solution, about 1 mg/ml, was incubated with 1% sodium dodecyl sulfate–1% HSEtOH in 0.1 M phosphate (pH 7.0) at 37°. Aliquots were taken after 2 (a), 5 (b), and 8 hr (c) and subjected to sodium dodecyl sulfate gel electrophoresis. The symbols designating the bands in electrophoresis are as in Figure 2.

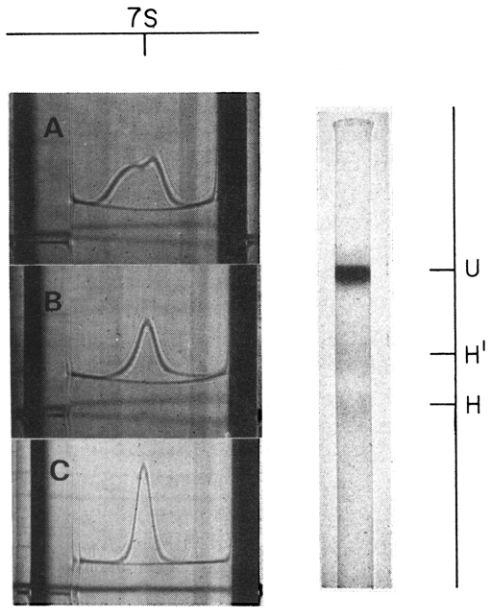

FIGURE 6: Reassociation of dissociated ceruloplasmin. Ceruloplasmin, completely dissociated by incubation with sodium dodecyl sulfate and HSEtOH for 90 hr (Figure 5C), was dialyzed against 0.1 M phosphate (pH 7.2). Left: Photographs showing the sedimentation pattern after dialyzing for 30 (A) and 70 hr (B). C shows the sedimentation pattern of native ceruloplasmin for comparison. Sedimentation was carried out at 56,000 rpm and the photographs were taken about 70 min after reaching speed. Approximate s_{obsd} value indicated. Right: Sodium dodecyl sulfate gel electrophoresis pattern of the same solution as in B, diluted (1:50) with 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer (pH 7.0). HSEtOH was excluded in the preparation of the sample for electrophoresis. The symbols designating the bands in electrophoresis are as in Figure 2.

moved, was studied. A solution of ceruloplasmin in sodium dodecyl sulfate–HSEtOH, incubated for a sufficiently long period to effect complete dissociation (Figure 5C), was dialyzed against phosphate buffer and the progress of reassociation was followed by observing the regeneration of the 7S peak in sedimentation and the appearance of the corresponding U band in electrophoresis (Figure 6). Following maximal attainable reassociation, divalent copper together with an appropriate amount of cysteine to bring about reconstitution were added. Ceruloplasmin reconstituted in this manner was found to regain 80% of the original oxidase activity (Table I).

Discussion

Human ceruloplasmin used in this study was shown to be pure as judged by the criteria of ultracentrifugation and gel electrophoresis. We have tried to resolve the controversy regarding the molecular weight of the native protein by employing the methods of low- and high-speed sedimentation equilibrium using interference and absorption optics, under a wide concentration range. From the molecular weights obtained we consider the value of 124,000 as the most reliable, since it was obtained by the low-speed method, known to be capable of higher accuracy than the high-speed one (Richards *et al.*, 1968). We do not consider the small difference between the high- and low-speed values significant enough to indicate a dissociation–association effect. The molecular weight determined here is in reasonable agreement with that of 132,000 determined by the independent method of X-ray crystallographic analysis (Magdoff-Fairchild *et al.*, 1969) and with that of 134,000 found from high-speed sedimentation equilibrium (Rydén, 1972). The higher molecular weights obtained in earlier studies (Holmberg and Laurell, 1948; Kasper and Deutsch, 1963) may in part at least be attributed in our opinion to errors in determining diffusion coefficients in the sedimentation–diffusion method and to difficulties in extracting the data necessary for the calculation of molecular weights in the Archibald method of approach-to-sedimentation equilibrium.

The experiments carried out in this study show that ceruloplasmin may be dissociated into subunits in solutions con-

TABLE I: Reconstitution of Human Ceruloplasmin.

	Oxidase Activity as	
	Units/mg of Protein	% of Native Act.
Native ceruloplasmin	54	100
Reassociated protein[a]	5.7	10.5
Reconstituted ceruloplasmin[b]	43	79

[a] Reassociated protein was prepared from sodium dodecyl sulfate–HSEtOH dissociated ceruloplasmin by exhaustive dialysis of the dissociating agent as described in the legend to Figure 6B. [b] Reconstitution of ceruloplasmin was effected by addition of divalent copper (20 μl of 0.001 M $CuSO_4$) and cysteine (12 μl of 0.001 M solution) to reassociated protein (2 ml of 0.12-mg/ml solution).

taining: (1) detergent (sodium dodecyl sulfate) in the presence of reducing agent (HSEtOH) or mercurial (HgOHBzO) or (2) denaturant (urea or Gdn·HCl) in the presence of reducing agent (HSEtOH). In view of the relatively long periods of incubation needed to effect complete dissociation, it was found imperative to carry out additional tests to ensure that the dissociation observed is not artifactitious in nature. That the ceruloplasmin used was not degraded to begin with has been shown. The finding that samples which were heated, or treated with inhibitor, gave the same electrophoretic pattern shows that the dissociation is not due to proteolysis by enzymes that might have been present as a contamination. The ultimate proof that the observed dissociation is not an artifact of some kind or another is provided by the successful reassociation of the dissociated chains and their reconstitution to give enzymatically active ceruloplasmin. Our results confirm previous work by Kasper and Deutsch (1963), Poulik (1962), and Simons and Bearn (1969), who observed dissociation in the presence of urea–HSEtOH. Rydén (1972), however, did not observe dissociation in Gdn·HCl–HSEtOH. His inability to detect dissociation may be due, in our opinion, to the absence of a sufficiently long incubation period, the importance of which has now been shown by us.

The findings of the present study lead us to propose a model for ceruloplasmin according to which the native protein would be represented by L_2H_2, where L and H are polypeptide chains of molecular weights 16,000 and 53,000, respectively. In this model, the three major bands observed in sodium dodecyl sulfate gel electrophoresis would be L (16,000), H (53,000), and LH (69,000). The two minor bands would then correspond to L_2H (83,000) and L_2 (30,000). Comparison of the molecular weight of the native ceruloplasmin (124,000) with the value corresponding to the proposed L_2H_2 tetramer (138,000) shows an 11% discrepancy. Experimental uncertainties in the molecular weight determinations, ±3% for sedimentation equilibrium and ±10% for sodium dodecyl sulfate gel electrophoresis (Weber and Osborn, 1969), may account for this lack of agreement. A part of the discrepancy could be due to the 7% carbohydrate content of ceruloplasmin which may cause lowered electrophoretic mobilities (Weber *et al.*, 1972). In fact, the molecular weight of an LH_2 model would sum up to 122,000 in better agreement with the value found for native ceruloplasmin. However, the latter model is inconsistent with the relative abundance of L and H chains as determined in the densitometric analysis of the electrophoretic patterns and was therefore rejected. One difficulty with the proposed model needs, however, to be mentioned. Assuming the L_2H_2 structure to be correct, the H′ species would be none but an LH dimer and should, in principle, be capable of dissociation into L and H species. We have never been able to obtain complete dissociation of the H′ species. Incomplete dissociation by sodium dodecyl sulfate–HSEtOH is, however, by no means without precedent. For example, in the dissociation of γ-globulin, composed of two light and two heavy chains, a band in electrophoresis attributable to the light–heavy chain dimer is sometimes observed. Apparently, only partial dissociation is attainable under our experimental conditions.

The model proposed here for ceruloplasmin is in disagreement with the octameric structures of Poillon and Bearn (1966) and Poulik (1968). The findings of Simons and Bearn (1969) can, however, be accommodated within the framework of this model. In any case our findings contradict the recently proposed single-chain structure of ceruloplasmin (Rydén, 1972).

Acknowledgment

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