Reinvestigation of Some Physicochemical and Chemical Properties of Human Ceruloplasmin (Ferroxidase)†

Lars Rydén* and Ingemar Björk

ABSTRACT: The original molecular weight determinations and hydrodynamic data on ceruloplasmin have later been contradicted by crystallographic and other investigations. In order to arrive at the correct copper stoichiometry of the protein we have undertaken a careful reinvestigation of some aspects of the physical chemistry and chemistry of ceruloplasmin with particular attention directed towards molecular weight and copper content. Human ceruloplasmin form I (the major form) was isolated from fresh normal or retroplacental serum by a procedure designed to avoid proteolysis. The molecular weight of the protein, determined by meniscus depletion sedimentation equilibrium, was found to be 134 000 ± 3 000. The sedimentation coefficient (7.25 S) and diffusion coefficient (4.46 10^{-7} cm^2/s) are consistent with this molecular weight. The frictional ratio calculated from these data, 1.41, and the observed intrinsic viscosity of 4.5 ml/g indicate that ceruloplasmin has a slightly more extended shape than a typical globular protein. This might at least partly be ascribed to its carbohydrate moiety. The amino acid composition, carbohydrate composition, and copper content of ceruloplasmin were determined. The copper content of several preparations varied between 6.0 and 6.6 atoms per mol. The copper stoichiometry of ceruloplasmin is discussed.

Ceruloplasmin (ferroxidase), first described by Holmberg and Laurell (1948), is the blue copper-containing oxidase present in mammalian serum. Their preliminary characterization of the enzyme indicated a molecular weight around 150 000 and a copper content of 8 atoms/mol of protein. A later and more detailed investigation by Kasper and Deutsch (1963) essentially confirmed this view, although a slightly higher molecular weight, 160 000, was reported. However, repeated studies of the copper content of the protein gave variable results. Finally, Huber and Frieden (1970) demonstrated that one copper could be taken away by Chelex treatment without affecting the visible spectrum or the enzymatic activity. This indicated that only 7 of the 8 copper atoms were an integral part of the enzyme and was in perfect agreement with electron paramagnetic resonance measurements. Forty-three percent of the copper in ceruloplasmin is detectable by this method (Deinum and Vänngård, 1973) requiring a multiple of 7 coppers to be present. Quantitative potentiometric titrations (Carrico et al., 1971) show that the enzyme can accept as many electrons as there are copper ions. Current theories (Malkin and Malmström, 1970) attribute the absence of an EPR signal from the remaining 57% of the coppers to spin pairing of cupric copper ions. This theory requires that there is an even number, in this case 4, of EPR-invisible coppers.

The results from a crystallographic investigation reported by Magdhoff-Fairchild et al. (1969), giving a molecular weight of 132 000 and a copper content of 6 atoms/mole, debated this model of the enzyme. The lower molecular weight was later confirmed by investigations on the polypeptide-chain structure of the protein (Rydén, 1972). Nevertheless, this molecular weight has been repeatedly questioned (see e.g., Deinum and Vänngård, 1973; Byers et al., 1973) most certainly due to the difficulties of reconciling the spectroscopic and potentiometric data described above with a model where ceruloplasmin contains 6 copper atoms.

It is obvious that a careful determination of the copper stoichiometry in ceruloplasmin is essential for future investigations of the enzyme copper. The physicochemical properties and chemical composition of the protein has therefore been reinvestigated with particular attention directed towards molecular weight and copper content. The investigation has been complicated by the instability of the enzyme, which involves proteolysis during preparation (Rydén, 1971a), a slow loss of copper and precipitation during handling, and possibly also a loss of blue color not due to dissociation of copper. A further circumstance that has to be considered is the heterogeneity of the carbohydrate part of the protein (Rydén, 1975), which is most easily seen in gradient chromatography on hydroxylapatite, where a minor and a major form separates (Broman, 1958). In this investigation only the major form (richest in carbohydrate) was used. Earlier investigations of the hydrodynamic properties of ceruloplasmin did not take into account all of the factors mentioned, which thus makes the results questionable.

Experimental Procedures

Materials

Blood from normal donors was collected as described earlier (Rydén, 1971a) and the serum used directly for the preparation of protein. Retroplacental blood was collected at the obstetric ward, stored at 4 °C, and centrifuged after not more than 24 h. 6-Aminocaproic acid (final concentration 0.02 M) was added to the serum, which was stored frozen until used (but not longer than 1 week).

Hydroxylapatite was prepared as described by Tiselius et al. (1956) with the following modifications. The brushite was left overnight and converted to apatite by boiling with an equilimolar amount of NaOH (40 g in the standard procedure). The apatite was treated with phosphate buffer only once,

† From the Institute of Biochemistry, University of Uppsala, and Department of Medical Chemistry, Royal Veterinary College, Uppsala, Sweden. *Received February 12, 1976.* Supported by Grant K-3568-2 from the Swedish Natural Science Research Council and Grant 4212 from the Swedish Medical Research Council.

* Address correspondence to Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706.

[1] Abbreviations used are: EPR, electron paramagnetic resonance; DEAE, diethylaminoethyl.

i.e., it was boiled with 0.15 M buffer for 15 min. The adsorbent was used after careful removal of fines by settling.

Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) was elutriated (Fischer, 1969) and the finest fraction was wet filtered on a 100 μm mesh sieve.

Preparation of Ceruloplasmin

The preparation starts with normal or retroplacental serum and requires no delipidation. Time-consuming dialysis and concentration steps are avoided so that the preparation can be completed in a few days. Potassium phosphate buffer (made up from equal molar amounts of monobasic and dibasic salt), having a pH of 6.8 at a concentration of 0.05 M, is used throughout up to the final gel filtration step. The buffers contained 0.02 M 6-aminocaproic acid (an inhibitor of plasminogen activation) and 1% 1-butanol (as a bacteriostatic agent) except in the final gel filtration procedure. The temperature always was 4 °C. Ceruloplasmin was located by its absorbance at 610 nm. The ratio A_{610nm}/A_{280nm} was used as a measure of purity. The columns are sized for a preparation from 1 l. of retroplacental serum or 2 l. of normal serum.

Step 1: Concentration on DEAE-Sephadex. The serum is diluted threefold with buffer and salt to a final concentration of 0.03 M phosphate buffer and 0.1 M NaCl. An amount of 5 g (retroplacental serum) or 2.5 g (normal serum) of dry DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) is added for each liter of serum. The suspension is stirred and washed with the buffer until A_{280} is less than 0.1. The gel is finally packed in a column and the ceruloplasmin eluted by raising the NaCl concentration to 0.2 M. The A_{610}/A_{280} ratio of the pooled fractions is 0.013–0.015.

Step 2: Gradient Chromatography on DEAE-Cellulose. The pooled fractions are diluted fourfold and applied to a column (3.2 × 18 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with 0.015 M phosphate buffer, containing 0.05 M NaCl. Ceruloplasmin is eluted with a linear gradient of 0.05–0.25 M NaCl (600 + 600 ml). Fractions with A_{610}/A_{280} of at least 0.020 are pooled. The yield is about 70% of the amount applied, and the A_{610}/A_{280} ratio is 0.026–0.028.

Step 3: Gradient Chromatography on Hydroxylapatite. An appropriate volume of a 1 M stock solution of buffer is added to the pooled fractions to a final concentration of 0.075 M phosphate buffer and the sample is directly applied to a column (3.2 × 70 cm) of hydroxylapatite equilibrated with phosphate buffer of the same molarity. Ceruloplasmin is eluted with a linear gradient of 0.075–0.5 M phosphate buffer (900 + 900 ml). The two forms of the protein are separated in this step. Fractions with A_{610}/A_{280} ratio of at least 0.035 are pooled. The yield of the major form (form I) is about 50% of the amount applied and the A_{610}/A_{280} ratio of the pooled fractions is about 0.038.

Step 4: Gel Filtration on Sephadex G-150. The pooled fractions are diluted threefold with water and adsorbed to a small column (2 × 8 cm) of DEAE-cellulose, equilibrated with 0.025 M sodium acetate buffer, pH 5.5, containing 0.05 M NaCl. Ceruloplasmin is eluted in a small volume by raising the sodium chloride concentration to 0.3 M and is then applied to a column (3.2 × 142 cm) of Sephadex G-150 equilibrated with 0.05 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl (without 6-aminocaproic acid or 1-butanol). Ceruloplasmin elutes as a single peak, often with some front tailing. In the center and rear half of the blue peak, 50% or more of the amount applied is recovered. The A_{610}/A_{280} ratio is 0.048–0.049. In this step aggregates and apoprotein, eluting ahead of the native enzyme, are removed.

Storage and Handling of Ceruloplasmin

No satisfactory way was found to store the purified enzyme of high activity for longer periods of time. The experiments to be described have thus been carried out with freshly prepared enzyme. No noticeable loss of blue color occurred during the first week.

When concentration of the purified enzyme by binding to DEAE-cellulose and elution with 0.3 M sodium chloride is attempted the A_{610}/A_{280} ratio typically decreases to 0.046–0.048, i.e., slightly lower than for the best fractions in the last gel filtration. When such a concentrated enzyme solution was dialyzed against a buffer with an ionic strength of 0.1 some white precipitate invariably formed. Samples for hydrodynamic experiments were therefore taken from the peak fractions of the gel-filtration eluate, and then dialyzed against the same buffer as used in the gel-filtration step (0.05 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl). For those viscosity measurements where a higher protein concentration than could be reached in this way was necessary, the sample was concentrated in a collodium bag by negative pressure.

Chemical Analyses

Amino Acids. Samples were hydrolyzed in sealed evacuated tubes for 24 or 72 h at 110 °C in 1 ml of 6 M HCl containing 10 mg/ml of phenol and an appropriate amount of norleucine as an internal standard. The hydrolysates were analyzed on a Durrum D-500 amino acid analyzer. Methanol (2%, v/v) was included in the first two buffers that were also adjusted to pH 3.34 and 4.20, respectively, to avoid overlap between glucosamine and phenylalanine. The recovery of glucosamine was 69% after hydrolysis as described above in the presence of phenol.

Tryptophan was determined after hydrolysis in 3 M *p*-toluenesulfonic acid at 115 °C for 24 h (Liu and Chang, 1971). As the recovery of tryptophan is not quantitative in the presence of carbohydrates, the actual recovery was determined by standard addition and found to be 64%.

Cysteine was determined as *S*-carboxymethylcysteine after alkylation with radioactive iodoacetic acid as described in detail earlier (Rydén and Eaker, 1975). Cysteine plus cystine was determined by the same method after reduction for 4 h under nitrogen with a tenfold molar excess of dithioerythritol in 0.7 M Tris-HCl, pH 8.6, containing 6 M guanidine hydrochloride. Total cysteine content was also determined as cysteic acid after performic acid oxidation.

Carbohydrate. Neutral sugars and amino sugars were analyzed by gas chromatography, essentially as described by Clamp et al. (1972). Lyophylized samples were dried in a desiccator over P_2O_5 for 24 h and were then treated with water-free methanol containing 1.25 M HCl for 24 h at 80 °C together with an appropriate amount of mannitol as an internal standard. After neutralization with $AgCO_3$ and treatment with acetic anhydride, the samples were dried and derivatized with trimethylsilylating reagent and then analyzed on a Pye Unicam Model 104 gas chromatograph equipped with Chromosorb WHP-SE 30 (3.8%).

Values for amino sugars were also obtained from the amino acid analyses as described above.

Sialic acid was analyzed by the thiobarbituric acid method according to Warren (1959) after hydrolysis in 0.05 M sulfuric acid at 80 °C for 1 h.

Copper. Samples containing about 30 μg of copper were analyzed by atomic absorption on a Techtron atomic absorp-

tion spectrophotometer (Techtron, Ltd, Melbourne). As the presence of protein and buffer was found to disturb the analysis, the samples were wet washed in a 4:1 mixture of HNO_3:$HClO_4$ in a quartz flask. The solutions were boiled in the acid mixture until all color had disappeared and fumes of perchloric acid were seen. Buffer blanks with and without copper standards were carried through the same procedure. The precision of the measurements was estimated to be 2%. The amount of protein was determined from simultaneous amino acid analysis of the same sample. The values from a 24-h hydrolysate was normalized to be consistent with complete compositional data.

Physicochemical Analyses

Sedimentation velocity determinations were carried out with a Beckman Model E ultracentrifuge (Beckman Instruments, Palo Alto, Calif.). Two 12-mm 2.5° double-sector cells (with Kel-F centerpieces and normal and wedge windows, respectively) were used in an AnD rotor at 44 000 rpm for experiments at different protein concentrations. The schlieren optical system was employed and the temperature was regulated at 23 °C during the runs. The slopes of the plots of ln r vs. time (where r is the distance from the center of rotation to the peak) were determined by a linear-regression analysis and converted to $s_{20,w}$ in the usual manner.

Sedimentation Equilibrium. Molecular weights were determined by sedimentation equilibrium using the meniscus depletion method. Experiments at lower speeds were performed by the long-column layering technique described by Chervenka (1970), while the run at 18 000 rpm was a short column experiment as originally developed by Yphantis (1974). The AnJ rotor was used for all runs below 18 000 rpm. No temperature regulation by heating was employed but instead the rotor was allowed to reach thermal equilibrium during constant refrigeration. Thus, the final temperature varied between 17 and 20 °C depending on the speed. Each run lasted about 16 h in order to ascertain that equilibrium had been reached. After the run, the cell was emptied and rinsed without disassembly and a water blank was then run to account for optical aberrations.

The interference optical system was used to analyze the concentration distribution in the cell. For each experiment and its corresponding water blank, five consecutive fringes were read in a Nikon microcomparator, and the difference between the means of these two sets of data was computed. The slopes of the plots of the logarithm of fringe displacement vs. the square of the distance to the center of rotation were determined by regression analyses, using all points with displacements larger than 100 μm.

Diffusion was measured in an apparatus described by Clasesson et al. (1974), where the boundary is formed in a convection-free manner in a 1-cm spectrophotometric cuvette. The concentration distribution was analyzed by the interference optical system using a helium–neon laser with an emission wavelength of 630 nm as light source. As ceruloplasmin absorbs light of this wavelength, half of the photographic plate was lighter than the other half, but this did not disturb the measurements significantly. Measurements were performed at 22.8 °C in a constant-temperature room, thus avoiding special thermostating.

The plates were analyzed according to a simplified method described in detail by Sundelöf (1974). The fringes were paired symmetrically around the center of the boundary and the square of the distance between the fringes were plotted vs. time for each pair. The slopes of these lines are proportional to the diffusion coefficient, the proportionality coefficient being a function of the difference in fringe number (δj). The apparent diffusion coefficient obtained for each fringe pair was plotted vs $(\delta j)^2$. The slope of the lines was evaluated by a weighted least square analysis, using $(\delta j)^2$ as weights, since slopes corresponding to small distances are less precise. The intercept at $(\delta j)^2 = 0$ is taken as the experimentally determined diffusion coefficient.

Viscosity was measured in a Hewlett Packard auto viscometer equipped with a thermostated bath regulated at 25.00 ± 0.01 °C. A Cannon-Ubbelhode capillary viscometer with an outflow time for water of about 200 s was used. All solutions were passed through a 0.22-μm sterile filter (Millipore Co.) prior to measurements. A plot of η_{red}/C (where the symbols have their usual meaning) vs. concentration in g/ml was treated by a linear regression analysis. Protein concentration was measured spectrophotometrically. The data were corrected for differences in density between solvent and solution by adding the term $1 - \bar{v}\rho_0/\rho_0$ as described by Tanford (1955).

Partial Specific Volume. The value reported by Magdhoff-Fairchild et al. (1969) of 0.7149 ml/g at 20 °C was used and recalculated to the actual temperature of the experiments with the aid of a temperature coefficient of 0.0004 ml/g deg. The value calculated from compositional data, 0.718, is in good agreement with the experimentally determined value.

Other Analyses

Enzymatic activity was measured as described by Broman (1958), with *N,N'*-dimethyl-*p*-phenylenediamine as substrate.

Optical spectra were read on a Beckman Acta III spectrophotometer. The spectrum of the reduced enzyme was taken after addition of a threefold molar excess of ascorbate over enzyme copper. For calculation of molar extinction coefficients protein concentrations were determined from amino acid analysis.

Results

Preparation and Stability of Enzyme. Ceruloplasmin prepared as described under Methods had an absorbancy ratio A_{610}/A_{280} of 0.049, which is slightly better than the best preparations reported so far (e.g., Deutsch et al., 1962, and Morell et al., 1969). The corresponding extinction coefficients are $A_{280}^{1\%} = 15.3$ ($\epsilon = 20.5 \times 10^4$ M^{-1} cm^{-1}) and $A_{610}^{1\%} = 0.750$ ($\epsilon = 1.00 \times 10^4$ M^{-1} cm^{-1}). The visible spectrum was not significantly different from spectra reported earlier. The peak around 450 nm was clearly resolved. The best preparations had an enzymatic activity of $\Delta A_{552}^{10min} = 18.0$ per mg of protein. The activities of different preparations were always found to be proportional to their absorbancies at 610 nm. The value of A_{610}/A_{280} has therefore been used synonymously with specific activity throughout.

The purified protein of high activity was found to be unstable. When frozen in dry ice, kept at −90 °C, and thawed, about 5% of the absorbance at 610 nm was lost, while no change in the absorbance at 280 nm was observed. When stored as a sterile filtered solution in 0.05 M sodium acetate buffer, pH 5.5, containing 0.3 M NaCl, a slow loss of blue color occurred (about 30% after 3 months). No way to restore full activity to a sample that had lost some of its blue color was found. Chromatography on DEAE-cellulose and subsequent gel filtration gave a preparation that typically had a A_{610}/A_{280} ratio of only 0.042–0.044 and appeared homogeneous in gel filtration, with constant specific activity over the entire peak. Its copper content was also the same as for the fully active

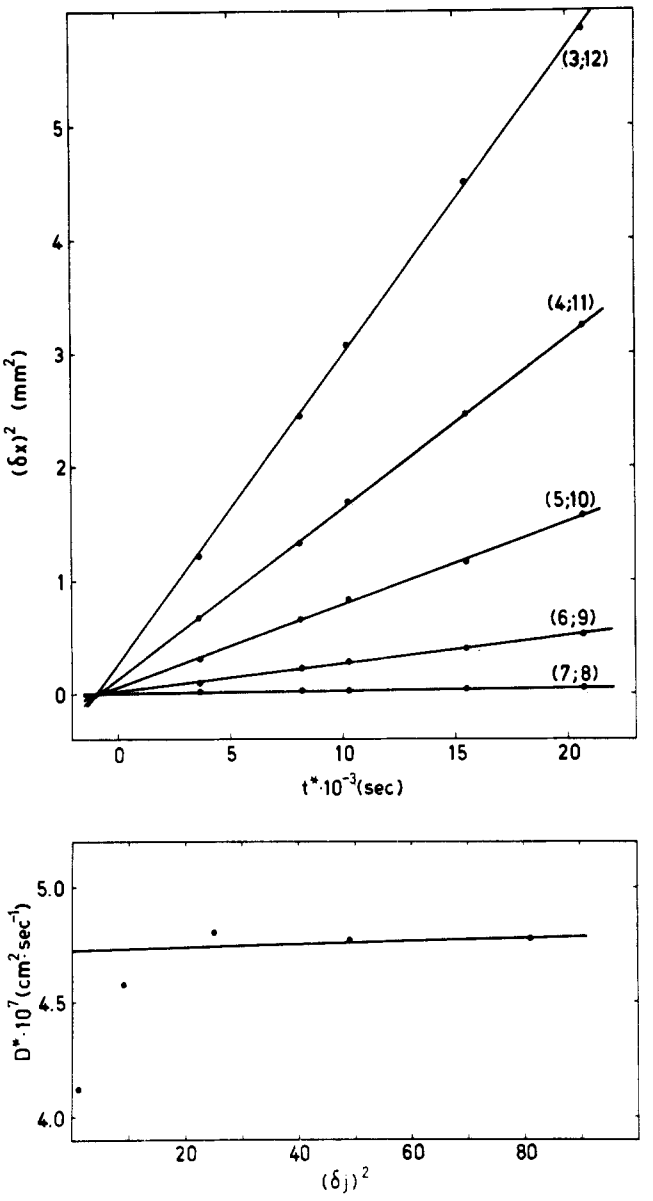

FIGURE 1: Determination of diffusion coefficient of human ceruloplasmin form I in 0.1 M sodium acetate, pH 5.5, containing 0.1 M NaCl at 22.8 °C and at a protein concentration of 5.1 mg/ml. The upper plot shows the square of distance between fringes paired symmetrically around the center of the boundary vs. the time of diffusion. The number of the fringes paired are indicated on the right side of the lines, which are linear-regression analyses of data points. The lower plot shows the diffusion coefficient calculated from each of the lines in the upper graph plotted vs. the square of the difference in fringe number ($(\delta j)^2$). The line represents a weighted least squares analysis with the weights equal to $(\delta j)^2$.

preparations. These experiments indicate that irreversible conformational changes resulting in an enzyme species with 10–20% lower absorbance at 610 nm occur easily.

Molecular Weight. The molecular weight of ceruloplasmin was calculated from a series of sedimentation equilibrium runs performed at the following rotor speeds and initial protein concentrations: 10 000 rpm at 1.0 and 2.0 mg/ml, 15 000 rpm at 0.5 and 1.0 mg/ml and 18 000 rpm at 0.2 mg/ml. The plots of the logarithm of fringe displacement vs. the square of the distance from the center of rotation[2] did not reveal any important heterogeneity, but when they were analyzed closely

[2] These plots were submitted for examination by the reviewers and are available as supplementary material.

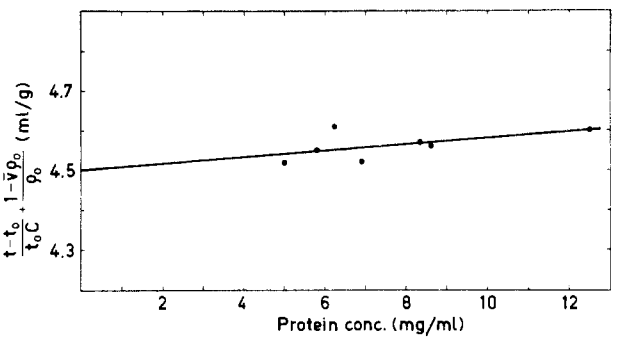

FIGURE 2: Determination of intrinsic viscosity of human ceruloplasmin form I at 25 °C in 0.1 M sodium acetate, pH 5.5, containing 0.1 M NaCl. The plot is a combination of data from two different preparations of the enzyme.

a difference in molecular weight of about 2000 was discovered between the upper and lower parts in some of them. This difference was statistically significant at the 5% level of confidence. It might reflect the presence of small amounts of aggregate or a difference in carbohydrate content of different molecules, which has been suggested by other data (Rydén, 1975). The samples used for sedimentation equilibrium were analyzed in parallel by polyacrylamide gel electrophoresis at pH 8.0 without any heterogeneity being detected.

The molecular weights computed from the slopes of the plots using all points down to the cell bottoms range from 131 000 to 138 000 with a mean of 134 000 and a standard deviation of 3000. If only the initial slopes are used in the runs where a slight curvature is found the average molecular weight reduces to 133 000.

Molecular Form. The sedimentation coefficient of ceruloplasmin was determined at several protein concentrations in the range 1–7 mg/ml using two different preparations.[2] The Schlieren patterns showed a single symmetric peak in all instances. The data extrapolate to a value of 7.25 S at zero protein concentration.

The diffusion coefficient was determined at a single protein concentration of 2.5 mg/ml (average concentration) (Figure 1). The slope of the plot of the apparent diffusion coefficient D* (Sundelöf, 1974) vs. the square of the difference in fringe number was small indicating neither gross heterogeneity nor considerable concentration dependence. The intersection at $(\delta j)^2 = 0$ gives a value of $D = 4.46 \times 10^{-7}$ cm^2 s^{-1}. An older sample of ceruloplasmin had a diffusion coefficient of 4.02×10^{-7} cm^2 s^{-1}. This value was obtained at two concentrations (6.0 and 3.0 mg/ml, average concentrations) indicating the absence of noticable concentration dependence.

The intrinsic viscosities of two different preparations of ceruloplasmin was measured as shown in Figure 2. The intercept at zero protein concentration gave an intrinsic viscosity of 4.5 ml/g.

The sedimentation and diffusion coefficients were each used in combination with the molecular weight of 134 000 to obtain frictional ratios, f/f_{min}. The values obtained, 1.39 and 1.43, respectively, as well as the intrinsic viscosity, 4.5 ml/g, indicates that the ceruloplasmin molecule is slightly asymmetric or more than normally solvated, or both. The corresponding ranges of values for typical globular proteins are 1.1–1.24 and 3–4 ml/g (Tanford, 1961), while they are 1.47 and 6.0 ml/g for the flexible immunoglobulin G molecule (Noelken et al., 1965).

Molecular weights were calculated by combining the sedimentation and diffusion coefficients in the Svedberg equation

TABLE I: Amino Acid and Carbohydrate Composition of Human Ceruloplasmin Form I.[a]

Amino Acid Residue	Value
Aspartic acid	127.1
Threonine[b]	76.7
Serine[b]	62.0
Glutamic acid	119.5
Proline[c]	46.3
Glycine	76.6
Alanine	49.4
Half-cysteine	13.5[d]
	14.7[e]
Cysteine[f]	3.0
Cystine[g]	6.0
Valine[h]	61.9
Methionine[i]	26.5
Isoleucine[h]	53.2
Leucine[h]	70.4
Tyrosine	63.5
Phenylalanine	49.0
Histidine	39.4
Lysine	65.7
Ammonia	113.1
Tryptophan[j]	21.9
Arginine	41.1
Sum of residues	1065.2
Glucosamine	15.7[k]
	15.9[j]
	19.2[l]
Mannose[l]	14.2
Galactose[l]	12.3
Fucose[l]	1.6
Sialic acid[m]	8.6
Sum of residues	52.5

[a] Values are normalized to a molecular weight of 134 000. [b] Corrected for loss during hydrolysis. [c] Performic acid oxidized sample, 24-h hydrolysis. [d] Determined as cysteic acid after performic acid oxidation. [e] Determined as labeled *S*-carboxymethylcysteine after reduction. [f] Determined as labeled *S*-carboxymethylcysteine (Rydén and Eaker, 1975). [g] From difference between half-cystine and cysteine. [h] Determined after 72-h hydrolysis. [i] Determined as methionine sulfone after performic acid oxidation. [j] Determined after hydrolysis in 3 M *p*-toluenesulfonic acid. [k] Determined by amino acid analysis. [l] Determined by gas–liquid chromatography. [m] Determined by thiobarbituric acid method.

and the intrinsic viscosity and the sedimentation coefficient, together with a value for the β-parameter of 2.12×10^6, in the Scheraga–Mandelkern equation (Scheraga and Mandelkern, 1953). The values obtained, 138 000 and 130 000, respectively, agree well with the value obtained by sedimentation equilibrium.

Chemical Composition. The amino acid and carbohydrate composition of ceruloplasmin form I is given in Table I. The data are normalized to 1065 amino acid residues and 55 carbohydrate residues to be consistent with a molecular weight of 134 000. The number of carbohydrate residues are in approximate agreement with the presence of three oligosaccharide units of 14–22 residues each, as has been found in isolated glyocpeptides (Rydén, 1971b).

Copper was analyzed in five different preparations obtained both from normal and retroplacental serum. The results varied from 6.0 to 6.6 atoms/mole of protein (values: 6.0, 6.2, 6.4, 6.4, 6.6), while the estimated error was 0.2 atom/mole of protein. This suggests that molecules with different numbers of copper atoms exist in the preparations. As there is no evidence that varying amounts of apoprotein are contained in them (apoprotein elutes ahead of native protein in the final Sephadex G-150 gel filtration), the most likely explanation is that molecules with either 6 or 7 atoms of copper are both present.

Discussion

In recent years the understanding of structure and enzymatic mechanisms of several copper-containing oxidases has been considerably increased. This knowledge is largely due to studies of well-defined preparations of laccases from lacquer tree and mushroom. In these enzymes (Malkin and Malmström, 1970) 4 copper ions are found. One of these is the blue or type 1 copper, a second one is a nonblue or type 2 cupric ion, while the last two are proposed to form an EPR-invisible pair of spin-coupled cupric ions (type 3 copper). Ceruloplasmin has been studied by the same techniques as the laccases (in particular, electron paramagnetic resonance, EPR) and all three types of copper have been observed. Nevertheless, the number of each of them, i.e., a well defined copper stoichiometry, has not been clearly established. The reason for this has been lack of agreement upon molecular weight of the protein, varying copper content, varying proportions of type 1 vs. type 2 copper, and finally, varying proportions of EPR-detectable copper vs. total copper.

This report is aimed at clarifying these inconsistencies. In particular, we have tried to devise a preparation scheme for the enzyme that gives a well defined homogeneous product. This has been much complicated by the pronounced lability of the protein, in that the high activity enzyme spontaneously looses activity and copper. No way has been found to store the enzyme without any losses in activity or to restore the activity to a sample that has become less active. As a consequence, we have made all analyses on freshly prepared enzyme. The drop in activity may be due to the production of enzyme species that have lost their blue color completely but are not separable from fully active protein, or it may be due to all enzyme molecules being less active by some 10–20%.

The preparation has been subject to a careful physicochemical investigation. The data are essentially self-consistent and reveal a molecule with a slightly extended shape and with a molecular weight close to 134 000. The diffusion coefficient we have determined, however, may be somewhat too low, since it is particularly dependent on handling and state of the protein. This is also indicated by the fact that the frictional coefficient obtained from diffusion experiments is 3% higher than the one obtained from sedimentation data.

The molecular weight that we obtained is in complete agreement with the value obtained from crystallographic data, i.e., 132 000 (Magdoff-Fairchild et al., 1969). It is also consistent with the value calculated from determinations of peptide chain length in 6 M guanidine hydrochloride, i.e., 129 000 (Rydén, 1972). Finally, it is also in complete accord with the minimum molecular weight derived from analysis of cysteine, i.e., 134 000 (Rydén and Eaker, 1975). The molecular weight values obtained from sedimentation equilibrium, hydrodynamic measurements, and crystallographic data are the more reliable ones, since they are independent of any assumptions about molecular form, while this is not the case for the value obtained by gel filtration in guanidine hydrochloride solutions. Also, cysteine determinations can be used for molecular weight estimations only if an integral number of cysteine residues are present, i.e., if no molecules with amino acid substitutions involving cysteine exist. However, the assumptions necessary for the last two molecular weight determinations are likely to be valid. As all values agree, we will use a molecular weight of

TABLE II: Suggested Stoichiometry of Ceruloplasmin Prosthetic Copper.[a]

Number	Designation	EPR Property	Other Property
1	Fast type 1 Cu^{2+}	Detectable	Blue, reoxidized fast[b,c]
1	Slow type 1 Cu^{2+}	Detectable	Blue, reoxidized very slowly[b,c]
1	Permanent type 2 Cu^{2+}	Detectable	Nonblue, binds anions[c,d]
2	Type 3 Cu	Nondetectable	Postulated to be a spin-coupled pair of cupric coppers in analogy with other blue oxidases. Possibly responsible for absorption at 310 nm[e]
1	Type 4 Cu	Nondetectable	Required by total copper content
0-1	Chelexable type 2 Cu^{2+}	Detectable	Not part of native enzyme but is easily picked up. Responsible for copper contents exceeding 6 atoms/mol[f]

[a] Based on a molecular weight of 134 000 and a copper content of 6 atoms/mole in the absence of the so-called Chelaxable copper. The postulation of a type 4 EPR-nondetectable copper ion is discussed in the text. [b] Carrico et al., 1971. [c] Deinum and Vänngård, 1973. [d] Andreasson and Vänngård, 1970. [e] Malkin and Malmström, 1970. [f] Huber and Frieden, 1970.

134 000 in calculations of copper content.

Hydrodynamic data for human ceruloplasmin have been reported by several investigators. The value for the sedimentation coefficient has usually been found to be 7.1–7.3 S (Kasper and Deutsch, 1964; Sanders et al., 1959; Poillon and Bearn, 1966; Vasiletz et al. 1972). Three reports agree on a diffusion coefficient of $4.4–4.6 \times 10^{-7}$ cm^2/s, (Sanders et al., 1959; Vasiletz et al., 1972; Andrews, 1966), one of these values being derived from gel filtration data (Andrews, 1966). The value given by Kasper and Deutsch (1964) of 3.8×10^{-7} cm^2 s^{-1} is obviously in error. Using a partial specific volume of 0.715 ml/g these literature values correspond to a molecular weight of 137 000 ± 5 000. Nevertheless, the molecular weights reported from sedimentation equilibrium runs consistently have been higher: 155 000 (Kasper and Deutsch, 1964), 140 000–150 000 (Vasiletz et al., 1972), 143 000 (Poillon and Bearn, 1966), and, for porcine ceruloplasmin, 150 000 (Mukasa et al., 1958). This might to some extent be due to an inappropriate technique, in that the approach-to-sedimentation equilibrium method was used. Another likely possibility is the presence of aggregates, which also was noted e.g., by Poillon and Bearn (1966). The presence of 10% dimer would increase the apparent monomer molecular weight by 10%, when determined by the approach-to-equilibrium method.

The frictional ratio calculated from the data in this work is about 1.40, suggesting that the ceruloplasmin molecule has a slightly extended shape. A recent study by low-angle x-ray scattering (Vasiletz et al., 1973) of freshly prepared ceruloplasmin from retroplacental serum indicates that the molecule can be approximated by a rotational ellipsoid with dimensions $65 \times 65 \times 130$ Å. If the data of that investigation are combined with a molecular weight of 134 000 a solvation of 50% can be calculated. The high solvation and extended shape found might at least partly be due to the carbohydrate moiety that makes up about 8% of the molecule.

The copper content of several preparations analyzed varied between 6.0 and 6.6 atoms/mole of protein. This range, which is outside experimental error, suggests that 6 copper atoms form an integral part of the enzyme, while an additional atom, not necessary for spectral properties or enzymatic activity, is easily picked up by some molecules during preparation. This conclusion is fully confirmed by investigations in other laboratories. The copper contents reported have varied between 0.27 and 0.34% corresponding to 5.7–7.2 atoms/mole. In several cases part of the copper could be taken away by Chelex treatment without effecting spectral properties or enzymatic activity. The reported values are, expressed as atoms/134 000 molecular weight: 6.8 (Kasper and Deutsch, 1964), 5.8 (Morell et al., 1969), 5.7–6.1 (Kasper, 1967), 7.2 (Vasiletz et al., 1972), 6.9 and after Chelex treatment 6.0 (Huber and Frieden, 1970), 6.9 and after Chelex treatment 5.9 (McKee and Frieden, 1971). The preparations studied all had an A_{610}/A_{280} ratio of 0.044–0.046, indicating that the variations are not due to varying amounts of apoprotein.

Our work, as well as the data available in the literature, thus seriously suggests that ceruloplasmin has a copper content of 6 atoms/mole. The potentiometric and spectroscopic data mentioned in the introduction, therefore, must be reconsidered.

A careful investigation of the EPR and redox characteristics of a preparation of ceruloplasmin similar to the one described here has been reported by Deinum and Vänngård (1973). In this investigation, type 2 copper was found to an extent of 33% of total EPR-detectable copper, and furthermore, two different type 1 copper atoms could be distinguished. It is thus clear that three EPR-detectable copper atoms exist. The total copper content of 6 atoms therefore requires that 3 EPR-nondetectable copper ions are present. Two of these may form a spin-coupled pair of cupric ions as have been postulated for the laccases (Malkin and Malmström, 1970) and as is suggested by the many fundamental similarities between all the blue copper-containing oxidases. In contrast, the third EPR-nondetectable copper ion cannot be included in such a model. It is thus necessary to postulate a further type of copper—type 4 copper. If, as redox titrations suggest, also this copper species is a cupric ion, its invisibility in EPR measurements must be due to a special binding condition, such as delocalization of the unpaired electron to the ligand groups. Low temperature magnetic-susceptibility measurements detected, on the other hand, only 44% of the copper in a sample with a A_{610}/A_{280} ratio of 0.040 (Aisen et al., 1967), indicating that the type 4 Cu might be cuprous.

If the postulate of a type 4 copper is accepted all the copper ions in ceruloplasmin can be distinguished by their individual properties, and a copper stoichiometry can be formulated (Table II). Not all of the 6 copper ions, however, need to be part of an active site. The many similarities with the laccases again suggest that only 4 copper ions are included in the catalytic site and that the slow type 1 copper and the type 4 copper

are not part of this site, although it is possible to reduce them by various substrates.

Acknowledgments

The amino acid analyses were carried out by Dr. D. Eaker of the Institute of Biochemistry. Analyses of carbohydrates by gas-liquid chromatography were performed in the laboratory of Dr. Hans Bennich of the Uppsala University Biomedical Centre. Analysis of copper by atomic absorption was carried out by Dr. Lilly Gustafsson of the Analytical Department of the Institute of Chemistry of Uppsala University. We are indebted to Dr. L.-O. Sundelöf of the Institute of Physical Chemistry, University of Uppsala, for the use of his apparatus for diffusion measurements and for assistance in carrying out the measurements.

Supplementary Material Available

Plots of the logarithm of fringe displacement vs. the square of the distance from the center of rotation for five sedimentation equilibrium runs and of the sedimentation coefficient vs. protein concentration (3 pages). Ordering information is given on any current masthead page.

References

Aisen, P., Koening, S. H., and Lilienthal, H. R. (1967), *J. Mol. Biol. 28,* 225.

Andreasson, L. E., and Vänngård, T. (1970), *Biochim. Biophys. Acta 200,* 247.

Andrews, P. (1966), *Biochem. J. 96,* 595.

Broman, L. (1958), *Nature (London) 182,* 1655.

Byers, W., Curzon, G., Garbett, K., Speyer, B. F., Young, S. N., and Williams, R. J. P. (1973), *Biochim. Biophys. Acta 310,* 38.

Carrico, R. J., Malmström, B. G., and Vänngård, T. (1971), *Eur. J. Biochem. 20,* 518.

Chervenka, C. H. (1970), *Anal. Biochem. 34,* 24.

Claesson, S., Matsuda, H., and Sundelöf, L.-O. (1974), *Chem. Scr. 6,* 94.

Clamp, J. R., Bhatti, T., and Chambers, R. E. (1972), *Glycoproteins,* 300.

Deinum, J., and Vänngård, T. (1973), *Biochim. Biophys. Acta 310,* 321.

Deutsch, H. F., Kasper, C. B., and Walsh, D. A. (1962), *Arch. Biochem. Biophys. 99,* 132.

Fischer, L. (1969), in Laboratory Techniques in Biochemistry and Molecular Biology, Work, and Work, Ed., Amsterdam, North Holland Publishing Co., p 228.

Holmberg, C. G., and Laurell, C.-B. (1948), *Acta Chem. Scand. 2,* 550.

Huber, C. T., and Frieden, E. (1970), *J. Biol. Chem. 245,* 3973.

Kasper, C. B. (1967), *Biochemistry 6,* 3185.

Kasper, C. B., and Deutsch, H. F. (1963), *J. Biol. Chem., 238,* 2325.

Liu, T. Y., and Chang, Y. H. (1971), *J. Biol. Chem. 246,* 2842.

Magdoff-Fairchild, B., Lovell, F. M., and Low, B. W. (1969), *J. Biol. Chem. 244,* 3497.

Malkin, R., and Malmström, B. G. (1970), *Adv. Enzymol. 33,* 177.

McKee, D. J., and Frieden, E. (1971), *Biochemistry 10,* 3880.

Morell, A. G., van den Hamer, C. J. A., and Scheinberg, I. H. (1969), *J. Biol. Chem. 244,* 3494.

Mukasa, H., Nosoh, Y., and Sato, T. (1968), *Biochim. Biophys. Acta 168,* 483.

Noelken, M. E., Nelson, C. A., Buckley III, C. E., and Tanford, C. (1965), *J. Biol. Chem. 240,* 218.

Poillon, W. N., and Bearn, A. G. (1966), *Biochim. Biophys. Acta 127,* 407.

Rydén, L. (1971a), *FEBS Lett. 18,* 321.

Rydén, L. (1971b), *Int. J. Protein Res. 3,* 191.

Rydén, L. (1972), *Eur. J. Biochem. 26,* 380.

Rydén, L. (1975), *Protides Biol. Fluids, Proc. Colloq. 22,* 633.

Rydén, L., and Eaker, D. (1975), *FEBS Lett. 53,* 279.

Sanders, B. E., Miller, O. P., and Richard, M. N. (1959), *Arch. Biochem. Biophys. 84,* 60.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75,* 179.

Sundelöf, L.-O. (1974), *Chem. Scr. 6,* 55.

Tanford, C. (1955), *J. Phys. Chem. 59,* 798.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N.Y., Wiley.

Tiselius, A., Hjertén, S., and Levin, Ö. (1956), *Arch. Biochem. Biophys. 65,* 132.

Vasiletz, I. M., Kayushina, R. L., Kuranova, I. P., Moshkov, K. A., Shavlovsky, M. M., and Nevfakh, S. A. (1973), *Biochimika (Moscow) 18,* 972.

Vasiletz, I. M., Moshkov, K. A., and Kushner, V. P. (1972), *Mol. Biol. 6,* 246.

Warren, L. (1959), *J. Biol. Chem. 234,* 1971.

Yphantis, D. (1964), *Biochemistry 3,* 297.