Purification and properties of bovine caeruloplasmin

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A novel method is reported for isolation of bovine caeruloplasmin from plasma; it involves a rapid and mild procedure, namely two column chromatographies with stepwise elution and one $(\text{NH}_4)_2\text{SO}_4$ precipitation, and results in a proteolytically undegraded homogeneous protein. The general structure of the protein, as evaluated by molecular-weight determination and amino acid composition, is very similar to that established for human and rat caeruloplasmin. Copper determination and e.p.r. spectral analysis on the native and NO-treated protein gave a metal-to-protein stoichiometry of six atoms of copper per molecule. Three copper atoms were detectable by e.p.r., with Type 2/Type 1 ratio = 1:3 in most samples. The protein is very sensitive to storage and/or handling. A component was isolated from aged samples, which was found to contain approximately four copper atoms per 125 000 daltons, two of which were detectable by e.p.r. with the characters of Type 2 copper. However, the same component was found to be present, although to a lesser extent, in the fresh preparation and does not seem to be related to proteolytic degradation. This component has no oxidase activity. On the basis of these results it is suggested that caeruloplasmin molecules are intrinsically heterogeneous with respect to both copper content and copper type, and this can explain the intriguing stoichiometry regarding the different types of copper centres.

Caeruloplasmin is a protein of mammalian blood plasma that contains intensely blue copper centres and has oxidase activity towards a number of substrates, including ferrous iron (Curzon, 1961) and aromatic diamines (McDermott *et al.*, 1968). The biological role of this protein is still a matter of considerable debate (Frieden & Hsieh, 1976). Also the study of its molecular structure resulted in conflicting reports about molecular weight, number of protein subunits and number and type of copper atoms bound per molecule [Rydén & Björk (1976), Fee (1975) and references therein].

The most contradictory point concerns the stoichiometry of copper atoms, reported to be between six and seven per molecule (Fee, 1975). This uncertainty obviously affects the relative number of the various types of copper typically present in blue copper oxidases (Malkin & Malm-

ström, 1970): three or four copper atoms undetectable by e.p.r. (Type 3 Cu) and variable (1–3:1) ratios between the two types of copper that appear in the e.p.r. spectrum [the blue Type 1 and the non-blue Type 2 Cu; Andréasson & Vänngård (1970); Veldsema & Van Gelder (1972); Deinum & Vänngård (1973)].

Caeruloplasmins from man, pig, rabbit, horse and rat serum have been studied. They apparently have an identical molecular architecture, i.e. a single-chain structure, and a very similar molecular size, approx. 130 000 daltons (Rydén, 1972*b*). In spite of this similarity they exhibit different sensitivities towards denaturation and degradation processes (Rydén, 1972*b*; Gunnarsson *et al.*, 1973).

The aim of the present work was to study bovine caeruloplasmin, which still requires detailed investigation. In particular, a very rapid and mild procedure of purification has been developed, in view of the peculiar sensitivity of the protein to manipu-

Abbreviation used: SDS, sodium dodecyl sulphate.

lation and storage, which is in turn mostly responsible for the disparate nature of the published results.

Experimental

All products used were reagent grade.

Polyacrylamide-gel electrophoresis was performed by the method of Davis (1964); protein was stained with 1% (w/v) Amido Black in 7% (v/v) acetic acid. To detect oxidase activity, gels were incubated with an *o*-dianisidine reagent (Schosinsky *et al.*, 1974); the reaction was stopped by adding solid NaN_3 (final concn. 6 mM).

SDS/polyacrylamide-gel electrophoresis was performed by the method of Fairbanks *et al.* (1971). The following proteins were used as standards: β -galactosidase, mol.wt. 116000; bovine serum albumin, 69000; catalase, 58000; ovalbumin, 43000; myoglobin, 17000. Densitometric analysis of the electrophoretograms was made with a CGA Cellomatic densitometer equipped with a CGA AT3 recorder. Protein was determined by the methods of Lowry *et al.* (1951) and Goa (1953).

Copper was measured by atomic absorption (Hilger and Watts Atomспек instrument equipped with a Varian Techtron heated-graphite atomizer model 63) and by the method of Brumby & Massey (1967). In the latter case the determination was made either by diluting small samples of caeruloplasmin with the copper reagent solution, or by incubating samples, dried to constant weight after exhaustive dialysis against water, with the copper reagent solution for 12 h. Copper content was then calculated as a function of either protein or dry weight.

For amino acid composition analysis, protein samples were hydrolysed in 6 M-HCl containing 0.1 M-phenol, at 110°C for 24, 48 or 72 h. Norleucine was added to the sample as an internal standard. Tubes were flushed with nitrogen and evacuated before sealing. Hydrolysates were analysed on an LKB 4400 amino acid analyser equipped with a Spectra-Physics System I computing integrator. Cysteine + cystine content was determined as cysteic acid after hydrolysis in the presence of dimethyl sulphoxide (Spencer & Wold, 1969). Tryptophan was determined either by a chromatographic method after hydrolysis by 4 M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)-indole (Simpson *et al.*, 1976) or spectrophotometrically (Edelhoch, 1967). Values obtained by the chromatographic method were approx. 20% lower than those obtained spectrophotometrically, probably because of the interference of carbohydrate with the method (Simpson *et al.*, 1976).

E.p.r. spectra were recorded at 110 K on a Varian E9 spectrometer operating at 9 or 35 GHz.

Cu(II)-EDTA was used as a standard for double integration. The *g* values were calculated by comparison with an internal free-radical [DPPH (diphenylpicrylhydrazyl)] standard.

Sedimentation-velocity experiments were carried out at approx. 12°C at 48000 rev./min in a Spinco model E ultracentrifuge equipped with a temperature-control unit. The sedimentation coefficient was calculated by a least-squares procedure and corrected for the viscosity and density of water at 20°C in the conventional way. The molecular weight was calculated from the equation of Svedberg & Pedersen (1940) by using the partial specific volume reported by Magdoff-Fairchild *et al.* (1969). The value for $\bar{v}$ calculated from compositional data was in good agreement.

Oxidase activity was measured spectrophotometrically as described by Osaki *et al.* (1966), in 0.1 M-phosphate buffer, pH 6, containing 50 μM -EDTA, with chloride-free *p*-phenylenediamine as the substrate. Substrate solutions were prepared by passing a solution of the commercial dihydrochloride compound through a DEAE-cellulose (DE22) column, previously equilibrated with water. *p*-Phenylenediamine dihydroxide was eluted just after the void volume and the solution was immediately adjusted to pH 5 with 1 M-sodium acetate buffer. Substrate concentration was determined from the A_{286} at pH 5, by using a *p*-phenylenediamine dihydrochloride solution as a standard. Specific activity ($\mu\text{mol/min}$ per mg of protein) was calculated as described by Rice (1962).

Monoamine oxidase activity was assayed as described by Tabor *et al.* (1954).

Buffers in the first two steps of the preparation procedure contained 20 mM-6-aminoheptanoic acid, an inhibitor of plasminogen activation (Ryden & Björk, 1976).

Results

Preparation of bovine caeruloplasmin

Step 1. Plasma (2 litres) was adjusted to pH 7.4 by addition of small amounts of 1 M- H_3PO_4 , and 200 ml of a thick suspension of QAE-Sephadex (quaternary aminoethyl-Sephadex) A50 previously equilibrated with 0.1 M-potassium phosphate buffer, pH 7.4, was added. The resultant slurry was stirred for 2 h, and then transferred into a 6 cm $\times$ 15 cm column, which was exhaustively washed with the same buffer. Potassium phosphate buffer (0.2 M) was then passed through the column until the colour of the resin turned blue. Elution was performed with 0.3 M buffer. Fractions with A_{610}/A_{280} of at least 0.02 were pooled.

Step 2. The pooled fractions from the previous step were precipitated with 28%-satd. $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was discarded by centrifugation

(27 000 g for 15 min). The blue supernatant was again precipitated with 50%-satd. $(\text{NH}_4)_2\text{SO}_4$, and the precipitate was collected by centrifugation, dissolved in a small volume of 0.05 M-potassium phosphate buffer and dialysed overnight against the same buffer.

Step 3. The dialysis residue was applied to a column (3 cm $\times$ 40 cm) of DE-52 DEAE-cellulose equilibrated with 0.05 M-phosphate buffer. The column was eluted with 0.1 M-phosphate buffer. Fractions with A_{610}/A_{280} of at least 0.035 were pooled.

Step 4. The pooled fractions from the previous step were dialysed against 0.1 M-sodium acetate buffer, pH 5.4, until a massive precipitate was formed (1–4 h). The precipitate was collected by centrifugation, resuspended in the same buffer and centrifuged again: five to seven such cycles were found to give optimal yields of pure protein. The final precipitate was dissolved in 0.1 M-phosphate buffer and dialysed against the same buffer. An $A_{610}/A_{280} = 0.046$ was measured for the pure protein.

Molecular properties of bovine caeruloplasmin

Purified bovine caeruloplasmin showed a single band on polyacrylamide-gel electrophoresis in both protein and activity staining (Fig. 1). Specific activity of freshly prepared samples was 0.35 ± 0.04 . No monoamine oxidase activity could be detected in purified samples.

Analytical SDS/polyacrylamide-gel electrophoresis showed a principal band corresponding to an apparent mol.wt. of 126 000 and minor components corresponding to mol.wts. 115 000, 90 000 and 50 000. The content of minor components was about 7%, as determined by quantitative densitometry of the gel after Coomassie Blue staining.

Sedimentation-velocity experiments gave a molecular-weight value of $123\,000 \pm 2000$, in good agreement with SDS/polyacrylamide-gel electrophoresis.

The amino acid composition is shown in Table 1. The values obtained are based on a mol.wt. of 125 000. Automatic analysis reveals an amino acid content of 86% by weight in the hydrolysed protein samples. The difference can be reasonably accounted for by the carbohydrate moiety of the protein. It is in fact close to the value reported for the human protein (Van Leeuwen, 1979).

Copper content and spectroscopic properties

Copper analyses by various methods gave, for very fresh pure samples, an average value of $0.29 \pm 0.01\%$, which means 5.7 copper atoms per 125 000 daltons; 41–45% of total copper was e.p.r.-detectable.

Fig. 1. Polyacrylamide-gel electrophoresis of bovine caeruloplasmin

The gel on the left was stained for protein (20 μ g applied to the gel) with Amido Black; that on the right was stained for oxidase activity with *o*-dianisidine (2 μ g of protein was applied to the gel).

Fig. 2 shows the low-temperature e.p.r. spectra of bovine caeruloplasmin at 9 and 35 GHz. The essential features of both spectra agree with published spectra of the human and pig proteins (Gunnarsson *et al.*, 1973), for which the line shape was interpreted in terms of two Type 1 copper components, beside the single Type 2 copper. The intensity of the Type 2 copper component, as evaluated from the intensity of the low hyperfine line as described by Vännegård (1967), varied somewhat from sample to sample. Higher values were always obtained in samples having higher percentage of e.p.r.-detectable copper. Attempts to remove possibly adventitious copper by Chelex 100 treatment at pH 6 or 7, in 0.1 M-phosphate buffer, always resulted in marked increase in the amount of Type 2 Cu^{2+} and decrease of A_{610}/A_{280} , suggesting protein denaturation. The relative proportions of the three Types of copper in two fresh preparations with slightly different contents of Type 2 copper are reported in Table 2.

E.p.r. parameters of the Type 2 copper [$g_{\parallel} = 2.26$ and $A_{\parallel} = 15$ mT (150 G)] were evaluated from the 35 GHz spectrum (Fig. 2b). The Type 1 copper hyperfine pattern was not resolved at this frequency (Andréasson & Vännegård, 1970). A Type 2 copper spectrum was also obtained by reaction of caeruloplasmin with NO (Wever *et al.*, 1973; Van Leeuwen & Van Gelder, 1978). On incubation with NO, the

signal intensity decreased to 28% of the control (Fig. 3b). However, two distinct Type 2 species were present and the reaction was not completely reversible.

Table 1. *Amino acid composition of bovine caeruloplasmin*

Values for the corresponding human and rat proteins are reported for comparison. Values are expressed as mol of residues/mol of protein.

	Bovine	Human		Rat
		(Rydén & Björk, 1976)	(Noyer <i>et al.</i> , 1980)	
Asp	102.6	127.1	118.4	124.7
Thr	57.9*	76.7	77.0	75.3
Ser	60.5*	62.0	68.0	74.5
Glu	105.3	119.5	120.9	135.1
Pro	47.4	46.3	49.6	52.0
Gly	78.9	76.6	78.4	84.1
Ala	55.3	49.4	49.5	62.5
Cys	13.2†	13.5	13.8	20.6
Val	57.9‡	61.9	60.0	71.1
Met	21.1	26.5	23.1	29.8
Ile	44.7	53.2	51.1	48.7
Leu	57.9	70.4	69.4	87.9
Tyr	52.3	63.5	63.3	55.3
Phe	44.7	49.0	48.2	58.2
His	34.2	39.4	45.2	35.6
Lys	68.2	65.7	74.4	36.2
Arg	34.2	41.1	40.3	42.0
Trp	17.1§	21.9	14.3	17.0

* Values are corrected to zero time of hydrolysis.

† Determined as cysteic acid.

‡ Values of 72 h hydrolysis.

§ Determined spectrophotometrically.

The optical spectrum of bovine caeruloplasmin was very similar to that reported for the human protein, with maximum visible absorption at 610 nm ($\epsilon = 8.4 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{\text{cm}}^{1\%} = 0.67$) and a less intense absorption band at 330 nm ($A_{610}/A_{330} = 2.1$). Bovine caeruloplasmin exhibited a pronounced pH-dependence of its spectroscopic properties. E.p.r. spectra at pH 6.3 (Fig. 4a) and pH 7.4 (Fig. 4b) showed changes in the positions of hyperfine lines and in particular of the well-resolved Type 2 component at lowest magnetic field. The e.p.r. changes were paralleled by a decrease in the 610 nm band (6–8%) and of the A_{610}/A_{330} ratio (from 2.1 to 1.9), on going from pH 7.5 to 6.3. At higher pH values a more marked and irreversible decrease in the A_{610} occurred, coincident with changes in the e.p.r. spectra of the Type 2 copper (Fig. 4c).

Stability of the protein

The purified protein was found to be unstable. Although samples kept in liquid nitrogen exhibited only minor modifications, storage at -20°C induced heterogeneity and decrease of the oxidase activity. In particular, electrophoretic analysis (Fig. 5b) revealed that a new component was present in addition to the original oxidase-positive band. Such component could be separated by precipitating the protein by dialysis against 0.1 M-sodium acetate buffer, pH 5.4. The soluble fraction contained 10% of the total protein content and clearly corresponded to the new component seen on gels of aged samples (Fig. 5d).

Aged samples showed a decrease in the 610 nm band and a concomitant increase (up to 10–15%) of the Type 2 copper fraction of e.p.r.-detectable copper (Fig. 6b). Again, this novel e.p.r. species could be separated from the native protein by

Fig. 2. *E.p.r. spectra of bovine caeruloplasmin*

Caeruloplasmin (1 mM) was in 0.1 M-phosphate buffer, pH 7. (a) Spectrum recorded at 9.14 GHz; (b) spectrum recorded at 35 GHz. The low-field part of the spectra is also shown with 10 times higher gain. The temperature was 110 K, the microwave power 20 mW and the modulation amplitude 1 mT for both spectra. The stick diagrams were drawn according to Gunnarsson *et al.* (1973)

Table 2. Copper content of two typical fresh preparations of purified bovine caeruloplasmin
In each column are reported both the absolute number of copper atoms per molecule of protein and the value as a percentage of the total.

Total copper	E.p.r.-undetectable copper	E.p.r.-detectable copper	Type 1 copper	Type 2 copper
5.70 (100%)	3.36 (59%)	2.34 (41%)	1.98 (35%)	0.36 (6%)
5.70 (100%)	3.36 (59%)	2.34 (41%)	1.76 (31%)	0.58 (10%)

Fig. 3. Effect of NO on the 9.14 GHz e.p.r. spectrum of bovine caeruloplasmin
(a) Before NO treatment; (b) equilibrated under approx. 100 kPa (1 atm) of NO (for 5 or 60 min); (c) after removal of NO by evacuation and reoxidation with air. The reaction medium contained 0.5 mM-caeruloplasmin in 0.1M-phosphate buffer, pH 7. E.p.r. conditions were as in Fig. 2. Relative receiver gains: (a) and (c) 1, (b) 6, (a') and (c') 10.

Fig. 4. Effect of pH on the e.p.r. spectrum of bovine caeruloplasmin
Caeruloplasmin (0.4 mM) in 0.1M-phosphate buffer, (a) pH 6.3 or (b) pH 7.4; (c) sample (b) adjusted to pH 9.8 by addition of a small amount of KOH. (a'), (b') and (c') represent the low-field part of spectra (a), (b) and (c), respectively, shown with 5 times higher gain. E.p.r. conditions were as in Fig. 2.

precipitation at pH 5.4. In fact, although the e.p.r. and optical spectra of the redissolved precipitate were identical with those of the fresh protein (Fig. 6c), the spectra of the supernatant showed exclusively Type 2 copper (Fig. 6d). The copper content of the protein soluble at pH 5.4 was approx. 4 copper atoms per 125 000 daltons, and its e.p.r.-detectable fraction was 52% of the total copper. The soluble component was oxidase-inactive.

Discussion

Perhaps the most intriguing fact in previous work on caeruloplasmin has been the pronounced lability

of the enzyme, such that many preparations contained proteolytically degraded products (Rydén, 1972a; Kingston *et al.*, 1977; Moshkov *et al.*, 1979). The preparation scheme proposed here employs very mild treatments, which can be accomplished within a short time (48–60 h); both conditions are essential to avoid proteolytic attack that may escape inhibition by 6-aminohexanoic acid. Moreover, the DE52 DEAE-cellulose step can be omitted if the pooled fractions from QAE-Sephadex exhibit $A_{610}:A_{280} > 0.025$. The last step, i.e. the acetate precipitation at pH 5.4, is crucial in that it removes decolorized and poorly active caeruloplasmin molecules. In fact the supernatant of the last step contained protein material whose properties were very similar to that of the colourless inactive

Fig. 5. Polyacrylamide-gel electrophoresis of aged caeruloplasmin samples

(a) Fresh protein; (b) stored for 5 months at -20° or 24 h at room temperature; (c) precipitate obtained after dialysis of sample (b) against 0.1M-acetate buffer, pH 5.4, and redissolved in 0.1M-phosphate buffer, pH 7; (d) supernatant fraction from the same dialysis. Approx. $30\mu\text{g}$ of protein was applied to gels (a), (c) and (d); $50\mu\text{g}$ was applied to gel (b) to demonstrate the faster component. The arrow indicates the oxidase-positive band.

Fig. 6. E.p.r. spectra (9.14 GHz) of aged bovine caeruloplasmin samples

The samples (a)–(d) correspond to the samples of the experiment described in Fig. 5. Protein concentration was: (a) and (b) 0.3 mM; (c) 0.2 mM; (d) 0.04 mM. E.p.r. conditions were as in Fig. 2. Relative receiver gains: (a) and (b) 1, (c) 1.28, (d) 5; (a'), (b'), (c') 6.

component isolated by the same procedure from aged samples.

The protein purified after repeated precipitation cycles is free of such components and shows a very moderate degree of proteolysis. The amount of proteolytic fragments (less than 10%) is the same as reported for 'undegraded' human caeruloplasmin preparations (Noyer *et al.*, 1980).

The molecular weight of bovine caeruloplasmin is within the limit of lower values reported for human caeruloplasmin (Freeman & Daniel, 1973) and within the range reported for other mammalian proteins. Pig, horse and rabbit caeruloplasmin showed a slightly lower molecular weight than human caeruloplasmin, 120 000 (Rydén, 1972b); a value of 124 000 has been reported for the rat protein (Manolis & Cox, 1980).

The amino acid composition of bovine caeruloplasmin is very similar to that of the corresponding human protein (Noyer *et al.*, 1980; Rydén & Björk, 1976), but significantly different from that of rat caeruloplasmin (Manolis & Cox, 1980). When expressed as residues/1000, the most apparent differences (greater than 20%) in amino acid

composition are more lysine and less S-containing amino acid and leucine in the bovine protein.

The results of Fig. 4, compared with those of Gunnarsson *et al.* (1973), provide further evidence for the strong resemblance between bovine and human caeruloplasmin. Both proteins show pH-dependent changes in the e.p.r. spectrum. This behaviour has not been found in other caeruloplasmins, and seems to be associated with lower stability towards denaturation and degradation processes (Gunnarsson *et al.*, 1973). Pig caeruloplasmin, which is unaffected by pH variation in the range 5–7.4, is also unaffected by proteolytic attack during the isolation procedure (Rydén, 1972b).

The copper content of bovine caeruloplasmin was determined to be approximately six atoms per molecule, three of which can be detected by e.p.r. (Table 2). The Type 2 copper content in fresh preparations is at most 25% of the e.p.r.-detectable copper (10% of the total copper), which implies a Type 1/Type 2 ratio of 3. For the human protein, values of 33% for Type 2 copper (Type 1/Type 2 ratio = 2; Deinum & Vänngård, 1973) and of 25% (Wever *et al.*, 1973), have been reported. A total copper content of six atoms per molecule, with

approx. 50% of the copper detectable by e.p.r., has been repeatedly suggested for human caeruloplasmin (Rydén & Björk, 1976; Magdoff-Fairchild *et al.*, 1969). This result suggests that not all of the copper invisible by e.p.r. is in the state of cupric pairs, which is the situation generally proposed for the Type 3 copper (Malmström *et al.*, 1975). Obviously, heterogeneity of caeruloplasmin molecules with respect to the metal content and/or distribution of the three copper types would make this stoichiometry acceptable. The same explanation could apply for preparations where values lower than 25% (even 15% in some cases) of the e.p.r.-detectable copper were measured for the Type 2 copper. However, such heterogeneity is not shown by electrophoresis. No definitive conclusion is possible at this stage.

Heterogeneity of samples has been generally interpreted in terms of denaturation by different agents. On the other hand, Deutsch (1960) suggested that human caeruloplasmin was made up of a series of molecules of varying solubility and that the native molecules, which contained the maximum amount of copper, were preferentially recovered in the crystals, being the least soluble. The existence of molecules with different numbers of copper atoms has been proposed also by others (Rydén & Björk, 1976). Moreover, a colourless component (10–20% of the total immunologically reactive protein) has been detected by immunological techniques in fresh human serum also (Carrico *et al.*, 1969).

The present work shows that a similar component can be isolated from aged samples of bovine caeruloplasmin, but it can be also identified to a variable extent in samples of any age, including the final fractions of the preparation procedure. This component has modified physico-chemical features with respect to the native protein. In particular it shows different solubility and consists of molecules apparently deprived of Type 1 copper. Nevertheless, the percentage of e.p.r.-detectable copper is nearly the same as that of the native protein.

It can be reasonably suggested that the colourless component is primarily due to intrinsic heterogeneity of the 'native' protein population. Such heterogeneity may increase during manipulation and aging processes.

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