EVIDENCE FOR PROTEOLYTIC FRAGMENTS IN COMMERCIAL SAMPLES OF HUMAN CERULOPLASMIN

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Received 9 August 1971

1. Introduction

In studies of the subunit structure of human ceruloplasmin, polypeptide chains of molecular weight around 17,000 [1–3] and 59,000 [3] have been reported. Other investigators have confirmed the presence of subunits by gel electrophoresis, but not determined their molecular weight [4, 5]. In contrast to these findings procine ceruloplasmin has been reported to contain only one polypeptide chain of molecular weight 80,000 [6]. As this situation, if correct, has far-reaching implications for the evolution of the ceruloplasmin molecule, a closer study of the polypeptide chains of the human protein was undertaken. The results were, however, different for different samples investigated, and therefore the possibility of fragmentation of the protein in course of its preparation was considered. Indeed, ceruloplasmin prepared directly from fresh blood in a three-step procedure did not contain any of the above components, but rather a single heavy polypeptide chain. Furthermore, short time incubation of the so prepared ceruloplasmin with trypsin produced fragments similar to the earlier observed subunits. These results and the new preparation scheme are reported below.

2. Materials and methods

2.1. *Commercial samples of ceruloplasmin*

Ceruloplasmin prepared from Cohn fractions IV-a or III, as described by Björling [7] was obtained from AB Kabi, Stockholm. The protein was delivered and stored as a sterile filtered 5% solution in 0.3 M NaCl at 4°. It was further fractionated by gradient chromatography on hydroxyapatite [8].

2.2. *Preparation of ceruloplasmin from fresh serum*

The fresh ceruloplasmin was prepared in a three step procedure essentially as described by Broman and Kjellin [9]. The preparation was completed in about 7 days. All operations except clot formation were performed at 4°. Ceruloplasmin was located by its absorption at 610 nm. Its purity was estimated from the $A_{610\,nm}/A_{280\,nm}$ ratio, assuming this to be 0.045 for the pure protein (the value for the protein purified by this procedure). The buffers were always potassium phosphate pH 6.8 (FB), containing 0.02 M ε-aminocaproic acid to inhibit plasminogen activation [10].

Serum. Blood was withdrawn directly into glass centrifuge tubes, containing ε-aminocaproic acid to get a final concentration of 0.02 M. After clot formation (1 hr room temp.) the tubes were centrifuged and the serum collected. The serum was further run at 44,000 *g* for 2 hr to flotate the lipids. This was essential for obtaining a clear solution and avoiding precipitation in the ensuing gel filtration.

DEAE-chromatography. 300–400 ml of defatted serum was diluted five-fold with buffers to final concentrations 0.03 M FB, 0.1 M NaCl and passed through a column (3.2 × 7.0 cm) of DEAE-Sephadex (Pharmacia Fine Chemicals) equilibrated with the same buffer. A blue band developed on the column. The sample, followed by 500 ml of starting buffer, was eluted by a linear gradient of NaCl (0.1 M → 0.45 M, total volume 1600 ml) the other salts being unchanged. The flow rate was about 100 ml/hr. Fractions with $A^{1\,cm}_{610\,nm}$ not less than 0.003 were pooled and concentrated by ultrafiltration in Diaflo equipment (Amicon). 20–25 ml of about 20% pure ceruloplasmin was obtained per 100 ml of defatted serum.

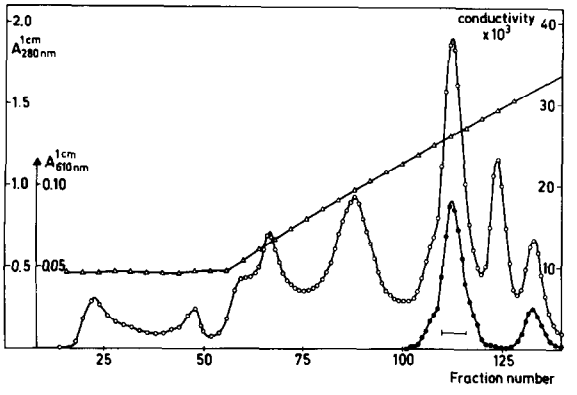

Fig. 1. Chromatography on a column (2.0 × 75 cm) of hydroxyapatite of a ceruloplasmin-enriched fraction obtained by DEAE-chromatography of human serum. The fractions indicated by the horizontal bar were pooled and further purified.

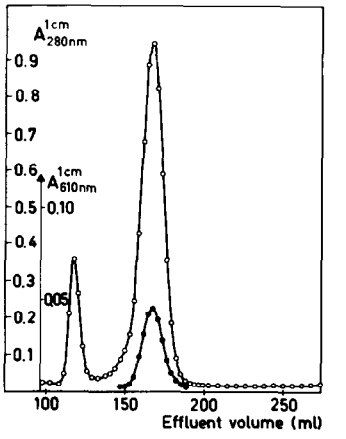

Fig. 2. Chromatography on a column (2.0 × 109 cm) of Sephadex G-200 of the concentrated first ceruloplasmin peak in fig. 1.

Hydroxyapatite chromatography. Hydroxyapatite was prepared as described by Tiselius et al. [11]. The sample was dialyzed against starting buffer (0.075 M FB) and applied on the column, which was developed by a linear potassium phosphate gradient (0.075 M → 0.5 M constant pH 6.8, total volume 1000 ml) at a flow rate of 12 ml/hr (fig. 1). Forms I and II of the protein [12] were well separated. The purity of ceruloplasmin was about 85% in the best fractions. 85% of the applied amount was recovered, but only the central part of the first peak (about 50% of the applied amount) was pooled.

Sephadex G-200 chromatography. The sample, concentrated to 2.0 ml in a Diaflo apparatus, was applied to a column of Sephadex G-200 equilibrated with 0.1 M NaCl, 0.03 M FB and run at 6.0 ml/hr (fig. 2). Some high molecular weight impurities were removed and the best fractions in the ceruloplasmin peak had a A_{610nm}/A_{280nm} ratio of 0.045. This material was completely pure as tested by polyacrylamide gel gradient electrophoresis (fig. 3). The protein could be stored at −20° for several months without any noticeable change.

2.3. *Analytical procedures*

Gel filtration in 6 M guanidine HCl. Was carried out essentially as described by Fish et al. [13] on a column of Sepharose 6B (Pharmacia Fine Chemicals) in 6.0 M guanidine HCl, 0.1 M Tris HCl, pH 8.5. Guanidine HCl was prepared from guanidine carbonate (BDH) and recrystallized in ethanol. Samples were prepared by dissolving 5 mg of protein in 200 μl of the above buffer and incubating under N_2 with 10 μl of β-mercaptoethanol for 4 hr and 10 μl of acrylonitrile for another 2 hr. The reaction mixture was layered under the solvent onto the top of the column, which was run at 3.5 ml/hr. Molecular weights were determined from a calibration curve (fig. 4) constructed essentially as described by Hjertén [14].

Starch gel electrophoresis. Was run in horizontal trays [15] in 8 M urea, 0.05 M formic acid, 0.01 M NaOH, pH 3.0 [16]. Samples were applied on filter papers inserted in slots in the gel. Electrophoresis was carried out at 3.5 V/cm for 20–22 hr at 20°. The gels were stained with 0.015% nigrosin in water:methanol: acetic acid 4:5:1, v/v, for 6 hr.

Polyacrylamide gel gradient electrophoresis. Was performed as described by Margolis and Kenrick [17] in a 0.09 M. Tris-borate-EDTA buffer pH 8.28 [18] at 100 V for 24 hr in a Gradipore equipment (Universal Scientific Ltd, London). The gel gradient was 4–26%. Gels were stained in 0.1% AmidoBlack in 7% acetic acid.

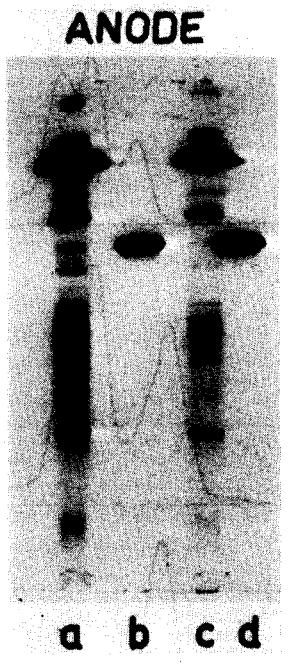

Fig. 3. Electrophoresis in a polyacrylamide gel gradient. Samples *a* and *c* are human sera and samples *b* and *d* are 30 and 75 μg, respectively, of the freshly prepared ceruloplasmin.

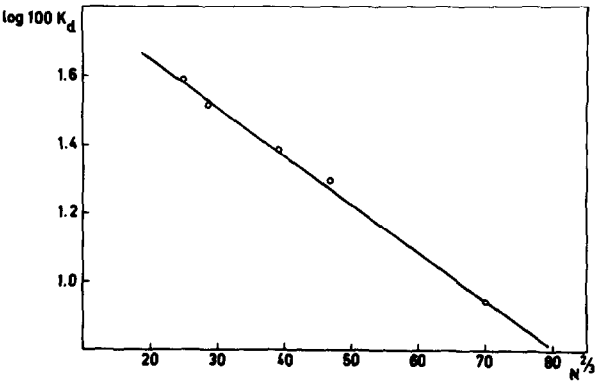

Fig. 4. Plot of log 100 K_d-*vs*-$N^{\frac{2}{3}}$ for ribonuclease, myoglobin, chymotrypsinogen, pepsin and bovine serum albumin in order of decreasing value for N. $K_d = V_e - V_0 / V_{re} - V_0$ where V_e, V_0 and V_{re} denote, respectively, the elution volumes of the protein, Blue dextran 2000 (Pharmacia Fine Chemicals) and excess reagent from the reduction and cyanoethylation reaction. N is the number of amino acids in the protein polypeptide chain.

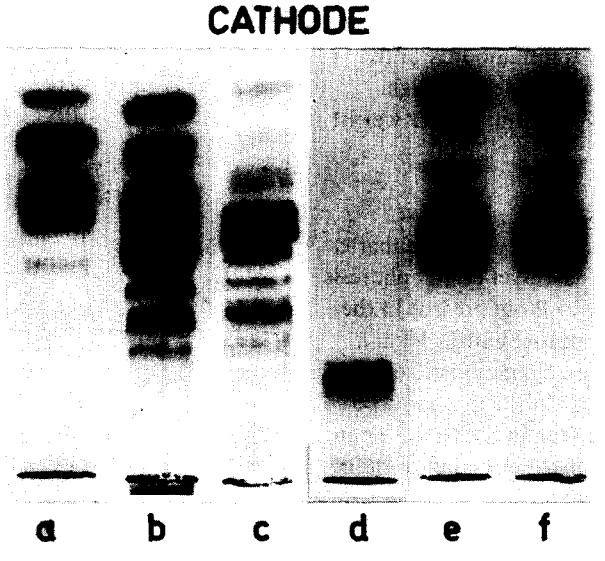

Fig. 5. Starch gel electrophoresis in 8 M urea at pH 3 of ceruloplasmin preparations (non-reduced proteins). Samples *a–c* are three different Kabi preparations. Sample *d* is the fresh ceruloplasmin preparation. Samples *e–f* is the fresh ceruloplasmin preparation after digestion with trypsin for 10 and 90 min (digestion conditions: enzyme: substrate 1:100 (w/w), 0.03 M Tris-HCl pH 8.0, 0.01 M $CaCl_2$, room temp.). The runs were done on different occasions and are not exactly comparable.

3. Results

3.1. *Comparison between commercial samples of ceruloplasmin and ceruloplasmin prepared from fresh serum*

Starch gel electrophoresis of three different batches of Kabi ceruloplasmin are shown in fig. 5 a–c. They all contain similar components, but their proportions differ considerably in the three samples. Heavier components are seen in two of them. The fresh ceruloplasmin preparation (fig. 5d) does not contain any of these peptides but only one slow main component and a weak band just in front of it.

Gel filtration in 6 M guanidine HCl of three different reduced and cyanoethylated Kabi ceruloplasmin preparations are seen in fig. 6 a–c. Two smaller peptides (peak fractions 60 and 66) were present in all three samples, though their relative amounts varied. Their molecular weights, as measured from a calibration curve (fig. 4) were 22,000 and 16,000. The heavier component in sample *a* (peak fraction 52), molecular weight 38,800, is absent in samples *b* and *c*, where peptide chains of molecular weight 58,100 (peak fraction 46, sample *b*) and 55,000 (peak fraction 47, sample *c*) are present. The fresh ceruloplasmin preparation (fig. 6d) does not contain any of the discussed components except for trace amounts of the 16,000 molecular weight peptide. Instead a single polypeptide chain with an estimated molecular weight of 110,000 is present.

3.2. *Limited proteolytic digestion*

The fresh ceruloplasmin preparation was incubated with trypsin and analyzed by starch gel electrophoresis (fig. 5 e–f). Fragments similar to those present in the Kabi ceruloplasmin batches appeared within 10 min and the heavy polypeptide chain characteristic of fresh ceruloplasmin disappeared. In the non-reduced protein no other changes were seen in the first 90 min, while some changes were seen in gel electrophoresis of the reduced protein (not shown).

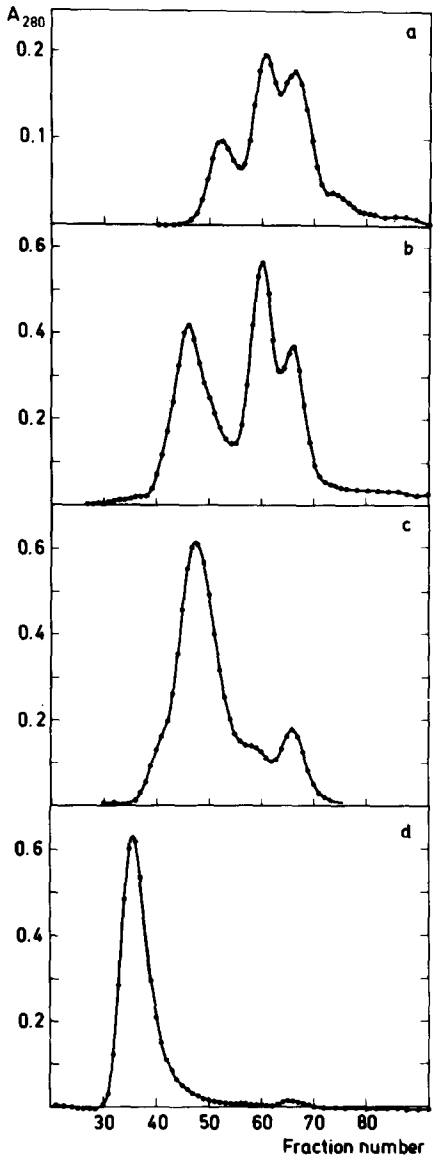

Fig. 6. Gel filtration on a column (1.5 × 85 cm) of Sepharose 6B in 6 M guanidine HCl, 0.1 M Tris-HCl pH 8.5, of reduced and cyanoethylated ceruloplasmin preparations. Samples *a–c* are three different Kabi ceruloplasmin batches. Sample *d* is the fresh ceruloplasmin preparation.

4. Discussion

The subunit strucure of human ceruloplasmin has been analyzed by starch gel electrophoresis in 8 M urea at pH 3 both in this laboratory and by others [1–5] but the outcome has differed considerably for different samples of the protein. As incomplete dissociation may occur in the conditions used, 6 M guanidine HCl, where most proteins are random coils [19], was tried. By gel filtration in this medium, three batches of ceruloplasmin was shown to still contain different polypeptides (fig. 6 a–c). These can then at least not all be true subunits of the protein. This is also indicated by the stoichiometry of the peptides, which not at all fit a simple molecular formula. An estimate of the stoichiometry is obtained by comparing peak areas in the chromatogram (fig. 6). For sample *a* molar absorptivities were determined from molecular weight and amino acid composition for the isolated and electrophoretically homogeneous components and the stoichiometry 1:3.3:4 was calculated. The peptide chains of molecular weight 16,000 and 58,000 are probably the same as those reported by Simons and Bearn [3].

The suspicion that the components observed might be proteolytic fragments was confirmed by the isolation of a ceruloplasmin that contained a single heavy polypeptide chain, homogeneous in gel filtration (fig. 6d) and gel electrophoresis (fig. 5d, the weak band just ahead of the main component might be a species with less sialic acid [8]) and the observation that this was particularly sensitive to proteolytic attack. (fig. 5 e–f). Porcine ceruloplasmin has not been reported to contain low molecular weight fragments [6] and might be less sensitive to proteolytic enzymes. In the preparation of ceruloplasmin from pig, horse and rabbit no special precautions were taken and still no low molecular weight fragments were found [20]. Exactly which precautions that have to be taken in the preparation of the human enzyme has not been worked out. However, in a preparation from 12-day-old freshly frozen serum (obtained from the hospital) considerably more of the 16,000 molecular weight peptide was found, so it may be essential to start from fresh blood. The A_{610nm}/A_{280nm} ratio was 0.045 for the new preparation and 0.042 for Kabi ceruloplasmin preparations after hydroxyapatite chromatography [8]. This means that the new preparation is only 5% more active than the best of the previous preparations.

The ceruloplasmin polypeptide chain eluted very close to the void volume on the Sepharose column (fig. 6). The estimation of the molecular weight is thus uncertain. A more rigorous study of its molecular weight is presently undertaken.

Acknowledgements

I am grateful for much advice, suggestions and practical help that was given by Dr. H. Bennich, Wallenberglaboratoriet, Uppsala and Dr. J.-C. Jansson, this institute. I am indebted to Mrs. Lena Bennich for running many of the starch gel electrophoreses, to Dr. H. Björling, AB Kabi, Stockholm, for ceruloplasmin preparations and to Prof. J. Porath for kind interest and valuable support. Financial aid has been received from the Faculty of Science of Uppsala University.

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