Studies of Human Ceruloplasmin Fractions Separated by Chromatography on Hydroxylapatite*

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Recent physicochemical (1, 2) and immunochemical (3) studies from our laboratory have shown that human ceruloplasmin is a labile protein the properties of which are readily modified by a variety of experimental conditions. Our previous data, however, indicated that crystalline ceruloplasmin was a single protein. This is at variance with results from other laboratories which have shown that chromatography on hydroxylapatite columns resolves two discrete ceruloplasmins (4, 5). Curzon and Vallet (6) have also noticed small amounts of ceruloplasmin eluting from diethylaminoethyl cellulose after the main peak. Through use of a combination of diethylaminoethyl cellulose and hydroxylapatite chromatographic procedures, Morel1 and Scheinberg (7) have indicated that at least four different ceruloplasmins could be resolved. When crystalline ceruloplasmin (8, 9) is chromatographed on hydroxylapatite columns, a series of components are separated. Two of these, which appear to be analogous to the oxidase-active components first recognized by Broman (4), comprise the major portion of the protein. They have been subjected to various physicochemical and immunological studies, and the results are reported here.

EXPERIMENTAL PROCEDURE

Crystalline human ceruloplasmin was isolated from Cohn Fraction IV-1 (10) by methods previously described (9). For convenience, this will be referred to as native ceruloplasmin. Goat, rabbit, and guinea pig antisera were prepared from ceruloplasmin, from which the highly antigenic impurity has been removed by absorption with an antibody developed against it in each of the above species (3). The $\gamma$-globulins of the goat and rabbit antisera were isolated by ethanol fractionation (11).

Crystalline ceruloplasmin was further fractionated by chromatography on hydroxylapatite columns (12). The various apo-proteins were prepared by the cyanide method (11).

Amino acid analyses were performed by conventional methods with automatic recording apparatus (13). Sulphydryl determinations were performed by the spectrophotometric method of Boycr (14).

Total cysteine-cystine was determined as cysteic acid by the method of Moore (15). Copper analyses were performed by the bis-cyclohexanone-oxalylhydrazone method (16). The orcinol method (17) was used for hexose analyses, mannose being employed as the standard. The oxidase activity of ceruloplasmin was measured by the method of Scheinberg and Morell (18).

Moving boundary electrophoresis and velocity sedimentation experiments employed the Spinco instruments. Vertical starch gel electrophoresis was performed by the method of Smithies (19). Immunological studies included the standard agar gel double diffusion Ouchterlony technique, immunoelectrophoresis in both agar (20) and starch (21) gels, and the quantitative precipitin reaction.

RESULTS

Chromatography of crystalline ceruloplasmin on hydroxylapatite columns led to the separation of a number of components as shown in Fig. 1. The small amount of material eluting before P-1 and designated as F was chromatographically heterogeneous. The F, P-1, and P-2 hydroxylapatite fractions constituted near 3, 85, and 12%, respectively, of the eluted protein. Rechromatography of Fraction hydroxylapatite P-1 or of hydroxylapatite P-2 on hydroxylapatite again resulted in the formation of a similar amount of the F component, but neither hydroxylapatite P-1 or hydroxylapatite P-2 were formed from each other. Crude native ceruloplasmin or aged crystalline protein that has shown a considerable decrease in its absorption at 610 nm gives rise to increased amounts of the F component on chromatography on hydroxylapatite.

A small amount of protein was sometimes eluted after component hydroxylapatite P-2 but the presence of this material was variable. The hydroxylapatite P-1 and P-2 components were characterized. The small amounts of hydroxylapatite P-2 and hydroxylapatite $\varphi$ precluded their extensive study. Some of the analytical data for these hydroxylapatite fractions are presented in Table I and may be compared with data for the crystalline ceruloplasmin used as source material. These analyses indicated no striking differences between the P-1 and P-2 components and their parent source. The amino acid content of each fraction was essentially the same as the native ceruloplasmin. Each of the fractions contained 1 mol of sulphydryl per 160,000 g of protein. Their hexose levels were essentially the same. An average of 7.7 and 7.2 g atoms of copper per 160,000 g of protein were found for P-1 and P-2, respectively. The F component had from 4 to 15 copper residues per 160,000 g of protein. Since the sedimentation constant of this fraction suggests a molecular weight in the 30,000 range, a maximum of

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1 We are grateful to the Cutter Laboratories for generous supplies of the Fraction IV-1.
2 We wish to thank Miss Shirley Miekka for performing various amino acid analyses.

* The abbreviations used are: F (for fast), the first component eluting from the hydroxylapatite columns; P-1 and P-2, major components in order of elution from hydroxylapatite columns; DEAE-cellulose, diethylaminoethyl cellulose.
3 atoms of copper per molecule of protein is indicated. The copper of the F fraction appeared to be loosely bound, and much of it could be removed by dialysis against distilled water.

The ratio of the optical densities at 280 and 610 m\(\mu\) of the P-1 and P-2 components was 30. This may be compared with the value of 22 to 24 for the crystalline starting material and is indicative of a change in the copper binding as the result of the chromatographic procedure. A similar increase in absorption ratio is obtained if the crystalline ceruloplasmin is chromatographed on DEAE-cellulose (1).

The oxidase activities of the hydroxylapatite fractions were compared with crystalline ceruloplasmin. The P-1, and P-2, and F fractions showed 76, 66, and 22\%\textsuperscript{,} respectively, of the activity of the native protein. This is indicative of modification of the protein and is in keeping with the decrease in absorption at 610 m\(\mu\) resulting from the chromatographic procedure. The oxidase activity of a given preparation shows decreases on aging that may be correlated with loss of blue color, i.e. decreased absorption at 610 m\(\mu\).

The P-1 and P-2 fractions can be crystallized by the method previously described (3) in yields ranging from 20 to 80\%. These crystalline proteins possess the absorbances at 280 and 610 m\(\mu\) of the starting crystalline material.

Sedimentation diagrams of various ceruloplasmin preparations including those obtained by chromatography on hydroxylapatite columns during their preparation are shown in Fig. 2. The concentration dependency of the sedimentation coefficient for the P-1 and P-2 fractions are given in Fig. 3. Extrapolation to infinite dilution yields a value near 7.1 S (1). Variable amounts of material sedimenting in the 3 to 5 S range are seen in both the P-1 and P-2 fractions that have been aged near neutral pH for a week or more at near 2\°. The diagram for an aged P-2 fraction is included with the data of Fig. 2. The formation of this slow sedimenting fraction is accompanied by a marked decrease in the intensity of the blue color of the fraction. A portion of the F fraction sediments at about the same rate as this component (see Fig. 2).

Further attempts to find chemical differences in the major hydroxylapatite fractions were directed to studies of their apoproteins. Sedimentation diagrams of protein from which the copper had been removed by the cyanide method (1) are included in Fig. 2 and show that apoproteins from both the P-1 and P-2 fractions contain a series of protein components in contrast to the apoprotein of native ceruloplasmin. About 25\% of the P-1 apoprotein sediments at the rate of the apoprotein prepared directly from crystalline ceruloplasmin. The remaining material sediments in the 3 to 5 S range.

The apoproteins of native ceruloplasmin and P-1 were chromatographed on hydroxylapatite and gave the results shown in Fig. 4. The major component of the apoprotein of native ceruloplasmin constituted near 90\% of the protein and was eluted at a salt concentration at which the native protein is held on the column. This is surprising, for the loss of copper gives a molecule of greater anionic charge, and it would be expected to be held more strongly by the hydroxylapatite at pH 7.4. The heterogeneous nature of the apoprotein of the P-1 fraction is

### Table I

<table>
<thead>
<tr>
<th></th>
<th>Native</th>
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<tr>
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<td>(s_{24, w} \times 10^3)</td>
<td>7.08</td>
<td>7.10</td>
<td>7.10</td>
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</table>

* Corrected for 20-hour destruction in 6 n HCl at 110\°. Threonine, 6\%; serine, 10\%; tyrosine, 8\% (1). The amino acid levels of the P-1 and P-2 fractions are adjusted to the aspartic acid level of the native protein.
† As cysteic acid. One residue deducted for cysteine.
‡ Cysteine was determined as the \(p\)-mercuribenzoate derivative (14); the amino-terminal amino acids\textsuperscript{,} were determined by the method of Stark and Smyth (22).
FIG. 2. Velocity sedimentation diagrams of various ceruloplasmin preparations. The numbers in parentheses indicate the duration of sedimentation in minutes at 59,780 r.p.m. Apo., apoprotein; HA, hydroxylapatite.

FIG. 3. Dependency of sedimentation rate on protein concentration for two hydroxylapatite (HA)-separated ceruloplasmin fractions. Shown by the resolution of a series of components and is further indication that ceruloplasmin has been extensively modified by passage over hydroxylapatite.

The results of starch gel electrophoretic experiments are shown in Fig. 5 and indicate the complexity of the various ceruloplasmin fractions. The slower band of native ceruloplasmin is likely a dimer as has been previously indicated (1). The F component is quite complex and a sharply defined component is seen in the region of the ceruloplasmin dimer. The apoprotein of crystalline ceruloplasmin is comprised largely of a protein with a slightly higher anodic mobility than its native precursor. Small amounts of three other components are also seen. In contrast, the apoproteins of P-1 and P-2 show considerable amounts of faster migrating material which appear to be slower sedimenting compounds noted in the ultracentrifuge diagrams of Fig. 2.

Immunoechemical Studies—Immunoelectrophoresis of the same protein samples showed that the major components of native ceruloplasmin and of the P-1 and P-2 fractions seen in Fig. 5 react with the antibody. The normal human serum did not possess sufficient ceruloplasmin to give a visible reaction and the apoproteins of P-1 and P-2 gave only a faint precipitin reaction in the area of the protein components of lowest anionic charge. This indicates that the breakdown products of the apoprotein have lost much of their ability to precipitate with antibody. Further confirmation of this will be seen in the results of the quantitative precipitin reactions.

The results of the reactions of crystalline ceruloplasmin and of the P-1 and P-2 fractions with goat, rabbit, and guinea pig antibodies by double diffusion in agar gel are shown in Fig. 6.

Photographs A to C are experiments conducted with goat antibody, D to F with rabbit antibody, and G with guinea pig antisera. All of the fresh hydroxylapatite fractions show a heavy and a light precipitin band with all three antibodies. The latter band increases in intensity on aging of these fractions. Thus, these apoproteins have antigenic properties that resemble the apoprotein prepared from crystalline ceruloplasmin. The reaction of the P-1, P-2, and F fractions indicate that further changes in the ceruloplasmin molecule occurred during the chromatography on hydroxylapatite. In Fig. 6B, it can be seen that the supernatant to crystalline P-1 contains only the minor immunological reactive component. The supernatant proteins to P-2 crystals, however, reacts essentially the same as the crystalline protein and its P-2 parent fraction. This result probably reflects the low yield of crystalline P-2 obtained.

Fig. 6C shows that the apoproteins of P-1 and P-2 react essen-
FIG. 5. The results of vertical starch gel electrophoresis experiments with various ceruloplasmin fractions in pH 8.6 borate buffer. The gel was stained with Amido schwarz. NHS, normal human serum.

FIG. 6. The reactions of various ceruloplasmin preparations with rabbit (A, B, C), goat (D, E, F), and guinea pig (G) antibodies to native ceruloplasmin.
The two main components that may be resolved from crystalline ceruloplasmin by chromatography on hydroxylapatite appear to be the proteins with serum oxidase activity reported by Broman (4). They do not appear to undergo conversion into another during the chromatographic procedure since on rechromatography they resolve as single components. However, no distinctive difference in their chemical, physical, or immunochemical properties have thus far been found. The F fractions contain a higher percentage of copper than the native molecule and the hydroxylapatite fractions, suggesting that the copper of ceruloplasmin may be unequally distributed among the subunits of the native protein.

Richterich, Temperli, and Aebi (5) have indicated that their hydroxylapatite ceruloplasmin fraction analogous to our P-2 material has only 6 copper residues per mole of protein. This is calculated for a protein with a molecular weight of 125,000. However, the percentage of copper is near that of their major ceruloplasmin fraction. Their molecular weight of 125,000 is based on the diffusion coefficient of a ceruloplasmin of higher sedimentation (rate) and the sedimentation (coefficient) of the protein in question. However, an increase in molecular asymmetry of the protein during their extensive fractionation procedures would result in a lowered sedimentation constant (1). Thus the data of Richterich et al. (5) should not be interpreted as indicating two different size molecules of ceruloplasmin. Other reports of various molecular species of human ceruloplasmin that have used protein fractions subjected to various fractionation procedures (7) similarly must be viewed with caution in view of the marked changes observed in this protein in the current experiments.

The most marked evidence of change in ceruloplasmin as a result of its chromatography on hydroxylapatite is the presence of two precipitin bands characteristic of the apoprotein of native ceruloplasmin in immunodiffusion experiments.

The different antisera employed did not appear to be directed against one or the other of the two main antigenic apoceruloplasmin determinants. Since rabbit antibody to human ceruloplasmin has been found to cross-react strongly with various animal ceruloplasmins (3), it was hoped that one of the species of animals immunized might respond preferentially to one of the noted antigenic determinants. This result was apparently not obtained since the antisera of all three animal species give similar Ouchterlony results with native apoceruloplasmin. The guinea pig, however, produced little antibody even after prolonged immunization.

Pronounced changes are observed in the study of the apoproteins of hydroxylapatite fractions; a considerable conversion of the apoproteins of the P-1 and P-2 fractions to subunits apparently takes place in neutral solution. It has been previously shown that reduced-alkylated ceruloplasmin is converted into subunits in acid urea-formate systems (1, 25). The present experiments, however, show partial conversion to subunits which are quite soluble at neutral pH in the absence of urea. Studies are being continued on the apoproteins of hydroxylapatite-fractionated ceruloplasmin to determine conditions for the production of these subunits in larger amounts.

SUMMARY

Fractionation of crystalline ceruloplasmin on hydroxylapatite results in the separation of a series of components. Chemical, physical, and immunological studies of these fractions indicate that marked changes may occur during chromatographic procedures. The hydroxylapatite fractions and their apoproteins appear fairly liable to further breakdown into products of lower molecular weight.

REFERENCES

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