Limited Trypsin-Catalyzed Hydrolysis of Crystalline Human Ceruloplasmin. Partial Characterization of the Digest*

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ABSTRACT: Crystalline human ceruloplasmin has been subjected to limited trypsin-catalyzed hydrolysis under carefully controlled conditions of pH, trypsin concentration, temperature, and time of hydrolysis. The enzymic modification resulted in (1) a marked alteration of native electrophoretic properties, (2) maintenance of most of the sedimentation behavior of native ceruloplasmin, (3) cleavage of 10–23 of a possible 114 susceptible peptide bonds, (4) retention of 91–95% of the copper chromophore absorption, (5) no detectable loss of protein-bound copper, (6) retention of 82–100% of the original oxidase activity, and (7) an increase in the amount of the wider hyperfine component of the electron paramagnetic resonance spectrum. The 7S component comprising 86–91% of the tryptic digest appeared to be composed of at least two dissimilar catalytic units which were distinguished by their differential susceptibility to reduction by mercaptans. Anion-exchange chromatography of the digest led to the loss of 69% of the total activity and permitted the isolation of colorless copper-containing products with sedimentation coefficients near 3 S. Components with intermediate or lower sedimentation coefficients were not noted. Starch gel electrophoresis of the digest produced a multicomponent pattern of which only one component exhibited oxidase activity. The results support the view that the catalytically active 7S component remaining after tryptic hydrolysis is, in part, a complex of predominantly 3S fragments held together as a 7S unit by noncovalent interactions which are disrupted by chromatography or electrophoresis; reduction of this complex with mercaptans also produces 3S fragments. The hydrolysis product comprising the remainder of the 7S component does not experience a decrease in molecular weight on reduction and is stable to chromatography and electrophoresis, not being converted to enzymically inactive lower molecular weight species.

A variety of proteolytic enzymes have been successfully employed to investigate structure–function relationships for a number of biologically active proteins. Examination of the composition, catalytic activity, structure, and other properties of the hydrolysis products has yielded valuable insight into this general area. The enzymic hydrolysis of native proteins has been recently surveyed by Hill (1965).

Laurell (1960), Malmström and Neilands (1964), and Scheinberg (1966) have reviewed the physicochemical and biological properties of ceruloplasmin. Relatively few studies appear in the literature dealing with the effect of enzymic modifications on the physical and biological properties of this copper-containing protein. Curzon (1958) reported that chymotryptic digestion of ceruloplasmin resulted in a rapid parallel decrease in oxidase activity and absorption at 610 mμ; however, only one-half of the total copper in the chymotryptic digest could be removed by dialysis. Marriott and Perkins (1966) have reinvestigated the chymotryptic degradation of ceruloplasmin and observed that all protein-bound copper was ultimately converted to a dialyzable form although 45% of the copper was more resistant to removal than the rest.

Morell *et al.* (1966) sequentially treated ceruloplasmin with neuraminidase, galactose oxidase, and tritiated borohydride to produce an asialoceruloplasmin containing four residues of tritium-labeled galactose. Asialoceruloplasmin maintained many of the properties of native ceruloplasmin.

The purpose of this study was to enzymically degrade native ceruloplasmin under conditions which would permit maximum retention of chromophore absorption and oxidase activity. These two properties are closely coupled since protein-bound cupric copper is intimately associated with both the blue color and the catalytic process. Examination of hydrolysis products which still possess certain properties of the native molecule might provide information relevant to the understanding of certain structure–function relationships of this copper-containing α_2-globulin. Preliminary investigations with three proteolytic enzymes (trypsin, chymotrypsin, and subtilisin BPN′) indicated that trypsin was the enzyme of choice based on the above criteria. This report deals with the characterization of the products formed from limited trypsin-catalyzed hydrolysis of native human ceruloplasmin.

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Materials and Methods

Preparation of Crystalline Human Ceruloplasmin. Ceruloplasmin was prepared from Cohn fraction IV 1[1] by a modification of the method of Deutsch *et al.* (1962). The step involving chromatography on DEAE-cellulose at pH 5.5 which immediately follows the batch adsorption process was omitted. Instead, the material obtained by batch adsorption was dialyzed directly against 0.05 M pH 7.2 ± 0.2 sodium acetate and chromatographed on DEAE-cellulose (DE-11, Whatman) equilibrated with the same salt solution. The absorbancy ratio ($E_{280\,m\mu}:E_{610\,m\mu}$) for ceruloplasmin solutions immediately prior to this chromatographic fractionation ranged from 50 to 60 for different preparations. One-time-crystallized ceruloplasmin dissolved in 0.05 M sodium acetate containing 0.15 M sodium chloride gave the usual absorbancy ratio of near 21.6.

Ultracentrifugation. All measurements were made with the Spinco Model E analytical ultracentrifuge equipped with the RTIC temperature-control system. The buffers employed were either 0.2 ionic strength sodium acetate buffer of pH 5.2 or 0.2 ionic strength potassium phosphate buffer of pH 7.4. Protein solutions were dialyzed against the respective buffer prior to analysis. Protein concentrations were determined spectrophotometrically using the $E^{1\%}_{1\,cm}$ value at 280 mμ of 14.9 (Kasper and Deutsch, 1963).

Oxidase Activity. The catalytic activity of ceruloplasmin preparations was determined by measuring the rate of oxidation of *p*-phenylenediamine at 530 mμ. *p*-Phenylenediamine dihydrochloride (Eastman) was recrystallized by the procedure of Henry *et al.* (1960) and stored in a dark bottle at room temperature. The 1.1-ml assay system contained 0.50 ml of 0.15 M NaCl, 0.30 ml of 1% *p*-phenylenediamine dihydrochloride in a 1.2 M buffer (pH 5.2), 0.05 M sodium acetate buffer (pH 5.2), and enzyme to give a volume of 1.10 ml. All measurements were made at 25° in a thermostated cuvet chamber of a Beckman DB-G spectrophotometer equipped with a Sargent SRL recorder.

Electrophoresis. Vertical starch gel electrophoresis was performed according to the method of Smithies (1959). A Tris–boric acid buffer of pH 8.5 having the following composition was used to prepare 13.5% w/v starch gels: 0.045 M Tris–0.025 M boric acid. The electrode vessel buffer contained 0.54 M Tris and 0.3 M boric acid. Electrophoresis was conducted at room temperature at a measured field strength of 69 v/cm for 7 hr. Gels were stained for protein with 3% Amido Black in 5% acetic acid in the conventional manner. Destaining was accomplished electrophoretically. Oxidase activity was detected by overlaying the gel with filter paper saturated with a solution of 0.1% benzidine in 0.1 M sodium acetate buffer of pH 4.7.

Amino Acid Analyses. Analyses were performed on a Spinco Model 120C automatic amino acid analyzer as described by Spackman *et al.* (1958). All samples were rendered salt free by dialysis against deionized-distilled water prior to hydrolysis with 6 N HCl at 110° for the indicated periods of time.

Amino-Terminal Analyses. The cyanate method (Stark and Smyth, 1963) was used to measure the appearance of new amino termini in trypsin-modified ceruloplasmin. Analyses for amino-terminal tryptophan, cystine, and cysteine were not performed. Fraction A was hydrolyzed a second time with 3 N HCl for 30 min at 100° in order to convert pyrrolidonecarboxylic acid and pyrrolidonecarboxyl peptides to glutamic acid and glutamyl peptides which could be removed by refractionation on AG 50W-X2. If this step were omitted, variable amounts of glutamic acid and other neutral amino acids were obtained. Prior to carbamylation, trypsin-modified ceruloplasmin was desalted by passage over a Sephadex G-10 column equilibrated with 1 M acetic acid. Urea was omitted from the carbamylation reaction mixture since ceruloplasmin carbamylated in 8 M urea was not readily soluble in aqueous nonurea solutions, *e.g.*, 50% acetic acid. This prevented the removal of salts and urea from the carbamylation mixture by gel filtration. Since a two-dimensional analysis employing electrophoresis followed by chromatography of trypsin-modified ceruloplasmin revealed the presence of peptide material, dialysis was not an acceptable procedure for desalting the carbamylated digest. Experiments designed to determine the extent of carbamylation in the absence of urea were performed. Native ceruloplasmin was carbamylated in the presence and absence of urea and the recovery of homocitrulline and lysine in 22-hr acid hydrolysates was determined. The amount of lysine recovered was 23 ± 2% of the sum of lysine and homocitrulline in both the presence and absence of 8 M urea. Since this is close to the yield of lysine from free homocitrulline under the hydrolysis conditions employed (Stark and Smyth, 1963), it was assumed that carbamylation was essentially complete in both cases.

The dansylation procedure as described by Gray (1966) was used as a qualitative confirmation of the results obtained by the cyanate method.

Digestion of Native Ceruloplasmin with Trypsin. Large-scale digests (80 mg or more) were performed in a water-jacketed reaction vessel under the following conditions: ceruloplasmin concentration, 8–12 mg/ml; trypsin concentration, 2% by weight of substrate; pH 7.3 at 25° for 2.5 hr. Crystalline ceruloplasmin was equilibrated against 0.05 M NaCl by dialysis at 5°. A 1% stock solution of trypsin (Worthington, lyophilized, two-times crystallized) was prepared by dissolving the enzyme in 0.001 N HCl. The rate of digestion was followed titrimetrically using the radiometer Model TTT-1 pH-Stat equipped with a Model SBR2 recorder. The titrant was 0.01 N NaOH. The system was thoroughly flushed with nitrogen before the addition of trypsin and a nitrogen atmosphere was maintained throughout the digestion. Aliquots were removed at various periods of time and their absorption at 610 mμ was measured. The digestion was terminated

[1] We are indebted to Cutter Laboratory, Berkeley, Calif., for a generous supply of Cohn fraction IV-1.

by the addition of 0.01 M diisopropylphosphorofluoridate (DFP) in anhydrous 2-propanol so that the molar ratio of inhibitor to trypsin was approximately 20:1. After 15 min acetic acid was added to pH 5.0. Aliquots were taken for oxidase assays, electrophoretic and ultracentrifugal analyses, and electron paramagnetic resonance measurements at this time.

Small-scale digests were performed in a quartz cuvet contained in a thermostated cuvet chamber of a DB-G spectrophotometer equipped with a SRL recorder. The conditions were identical with those described for the large-scale digests except that the reaction was conducted in a 0.05 M potassium phosphate buffer of pH 7.3 in the absence of a nitrogen atmosphere. This procedure permitted the continual monitoring of the 610-mμ absorption during proteolysis.

Copper Analyses. Copper was measured by the method of Peterson and Bollier (1955) which employs biscyclohexanone oxalyldihydrazone as the chelating agent. Standard copper solutions were prepared from electrolytic copper foil. Direct reacting copper was determined as previously described (Kasper *et al.*, 1963).

Estimation of Molecular Weights by Gel Filtration (Andrews, 1965). Protein samples (1 mg in 1 ml) were applied to a 150 × 0.9 cm Sephadex G-200 column equilibrated with 0.15 M KCl saturated with $CHCl_3$ at room temperature (24 ± 2°). Fractions of 1.5 ml were collected at a flow rate of approximately 4.4 ml/hr. Protein was detected in the effluent by measuring the absorbancy at 230 mμ. Blue Dextran (Pharmacia, Uppsala, Sweden), with an average molecular weight of 2×10^6, was used to determine the void volume. Proteins used to calibrate the gel column were crystalline ceruloplasmin prepared in this study, mol wt 160,000; bovine serum albumin (Pentex), mol wt 68,000; four-times-crystallized-ovalbumin prepared by the method of Woodworth and Schade (1959), mol wt 44,000; and chymotrypsinogen A (Worthington), mol wt 25,000.

Column Chromatography. DEAE-cellulose (DE-11 or DE-32, Whatman) was regenerated and equilibrated as described by the manufacturers. Buffers were prepared from reagent grade chemicals and deionized-distilled water. A constant-volume gradient elution technique (Bock and Ling, 1954) was used to develop the chromatograms.

Carbohydrate Analyses. Neutral hexose was measured by the anthrone method (Spiro, 1966) using a standard solution of mannose and galactose in a molar ratio of 3:2.

Electrophoresis and Chromatography. The tryptic digest and the dialysate of the digest were examined by the two-dimensional separation technique described by Ingram (1958). Electrophoresis was carried out at pH 4.7 employing a pyridine–acetic acid buffer (Michl, 1958) for a period of 30 min at 62 v/cm. Descending chromatography utilized the solvent system of 1-butanol–glacial acetic acid–water (200:30:75, v/v). After drying, the papers were sprayed with ninhydrin and the color was allowed to develop at 50°.

Electron Paramagnetic Resonance Measurements. Electron paramagnetic resonance spectra were obtained with a modified Varian X-band instrument employing 100-kcycle modulation, gas-flow low-temperature accessory, and a microwave circulator with bypass arm for crystal bias (Palmer, 1967). The amount of cupric copper in each preparation was estimated by double integration of the electron paramagnetic resonance spectrum and comparison with a Cu^{2+}-EDTA standard.

Results

In this study, four different preparations of crystalline ceruloplasmin were examined. Routinely each tryptic digest was characterized with respect to oxidase activity, absorption at 610 mμ, and starch gel electrophoresis behavior. The recovery of oxidase activity ranged from 82 to 100% while the recovery of chromophore absorption varied from 91 to 95% (Table I). The electrophoretic patterns of different digests were qualitatively very similar and varied within a narrow range only in the relative amounts of the different electrophoretic components. A typical electrophoretic pattern of the digest appears in Figure 1. In describing the properties of trypsin-modified ceruloplasmin, the particular experiment will be designated as in Table I.

Properties of Crystalline Ceruloplasmin. Freshly prepared crystalline preparations of ceruloplasmin gave a single symmetrical boundary in the analytical ultracentrifuge with a sedimentation coefficient in agreement with earlier studies (Deutsch, 1960). Electrophoretic examination of fresh crystalline ceruloplasmin in a Tris–borate starch gel at pH 8.5 revealed a single protein component which possessed oxidase activity. Identical results were obtained when fresh ceruloplasmin was examined in polyacrylamide gel in the pH range of 5.5–8.5. However, different results were obtained with ceruloplasmin that had been frozen and thawed or had been stored for 2–3 weeks at 3° either in the presence or absence of thimerosal (1:5000) or a crystal of thymol. In these cases, one or two new protein components of lower anodic mobility than the native protein were noted; however, all bands observed in the gel exhibited oxidase activity. Aged preparations frequently showed a slight decrease in absorbancy at 610 mμ as well as a decline in enzymic activity. These data suggest that the ceruloplasmin polymers which form are enzymically less active and have reduced extinctions at 610 mμ since both a reduction in oxidase activity and chromophore absorption are associated with aggregate formation. The tendency for ceruloplasmin to form discrete aggregates has been noted previously (Kasper and Deutsch, 1963; Poulik, 1963). The amino acid composition of native ceruloplasmin prepared in this study was in excellent agreement with earlier results (Kasper and Deutsch, 1963), except for finding 43 arginyl residues instead of 46 and 71 lysyl residues in place of 68.

Copper analyses performed on several crystalline

TABLE I: Chromophore Absorption and Oxidase Activity of Crystalline Ceruloplasmin before and after Exposure to Trypsin.

Expt	$E_{610\ m\mu}$ Before Trypsin	$E_{610\ m\mu}$ After Trypsin[a]	% $E_{610\ m\mu}$ Remaining	Activity[b] Before Trypsin	Activity[b] After Trypsin	% Act. Remaining
I	0.740	0.705	95.4	6.20	6.18	99.7
II-1	0.880	0.818	93	8.60	8.75	102
II-2	0.825	0.760	92	8.65	7.60	88
III	0.815	0.762	93	5.95	5.23	88
IV	0.795	0.720	91	6.30	5.15	82

[a] Optical densities are corrected for the volume of enzyme, titrant, and DFP added. [b] $\Delta E_{530\ m\mu/(min/\mu g\ of\ Cu)} \times 10^2$.

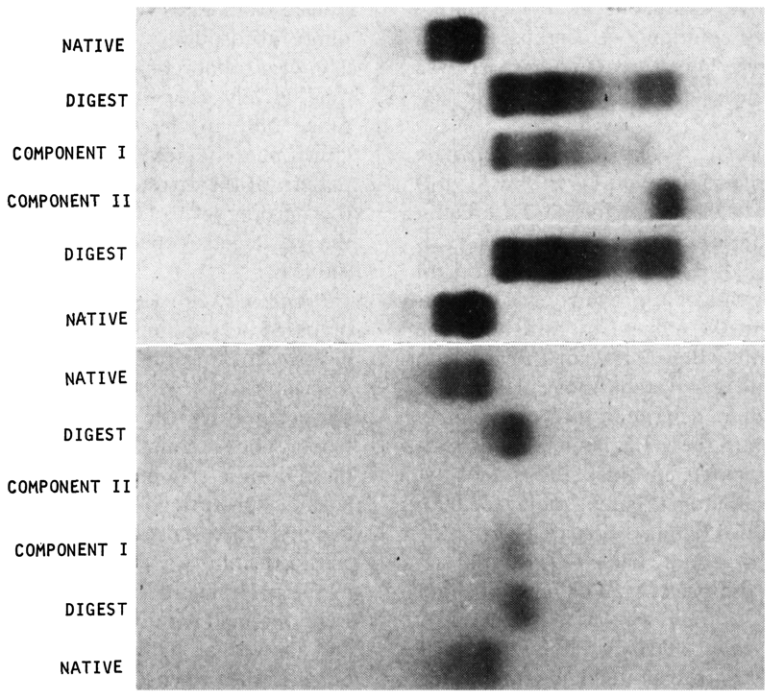

FIGURE 1. Starch gel electrophoretogram of native ceruloplasmin, tryptic digest, and the components obtained by fractionation of the digest on Sephadex G-200. The upper photograph is the protein stain; the lower photograph is the corresponding oxidase stain. The anode is to the right.

protein preparations yielded values ranging from 0.270 to 0.288% (w/w). These values correspond to 6.8–7.2 g-atoms of copper/160,000 g of protein.

Hydrolysis of Native Ceruloplasmin with Trypsin. A typical time course for the tryptic digestion of crystalline ceruloplasmin (expt IV, Table I) is shown in Figure 2. The addition of trypsin to the level of 1% total weight of substrate resulted in a rapid release of protons over the first 30-min period. At 40 min, the trypsin concentration was increased to a final value of 2% by weight of substrate. It is evident that after 1 hr no measurable proteolysis occurred. From the amino acid composition of ceruloplasmin, a total of 114 peptide bonds/mole of protein should be susceptible to the action of trypsin (71 lysyl and 43 arginyl residues) providing no sequences resistant to trypsin hydrolysis such as Lys-Pro or Arg-Pro exist. Since it is the native protein that is being acted upon by trypsin, only those susceptible bonds on the surface of the ceruloplasmin molecule should be cleaved in addition to those bonds made available by the action of trypsin. If it is assumed that the average pK' of the liberated amino groups is approximately 7.8, then a total of 23 out of the 114 theoretically susceptible peptide bonds has been

hydrolyzed.

During the course of the digestion, the absorbancy ratio ($E_{280\,m\mu}$:$E_{610\,m\mu}$) of the ceruloplasmin solution increased from 22.0 to 26.0 while the absorbancy at 610 mμ dropped from 0.795 to 0.720 (Figure 2). This measurement should detect changes in the ligand field of the copper chromophore since the magnitude of the absorption of the copper–protein complex is a quantitative estimate of the integrity of the chromophore (Broman *et al.*, 1962).

Amino-Terminal Analysis of Tryptic Digest. Trypsin-modified ceruloplasmin obtained from the experiment described in Figure 2 was examined by the cyanate method for new amino-terminal residues. Native ceruloplasmin and the tryptic digest were analyzed under identical conditions (carbamylation in the absence of urea) and the results are presented in Table II. Between 10 and 11 new α-NH_2 groups appear in trypsin-modified ceruloplasmin. Lysine was present at the level of 2.22 moles/mole of protein followed by decreasing amounts of aspartic acid, serine, glycine, and arginine, all of which were above the 1 mole of amino acid/mole of protein level. Residues present at lower levels were glutamic acid, leucine, valine, methionine, isoleucine, and alanine. Evidence for free lysine and arginine was obtained by a two-dimensional analysis of the digest. The native protein yielded only 0.13 residue of amino-terminal valine when the carbamylation was carried out in the absence of a denaturing agent such as urea; carbamylation in the presence of 8 M urea yielded close to 1 mole of amino-terminal valine/mole of ceruloplasmin in agreement with earlier studies (Kasper and Deutsch, 1963). Both in the presence and absence of urea, native ceruloplasmin yielded 0.8 mole of amino-terminal lysine/160,000 g of protein.

Dansylation of trypsin-modified ceruloplasmin permitted the identification of DNS[2]-amino acids which were in qualitative agreement with those amino acids determined by the cyanate method; however, the DNS derivatives of methionine, alanine, and isoleucine were not detected. Since the protein was not completely soluble during the reaction with DNS-Cl, incomplete labeling with DNS-Cl may be responsible for not detecting DNS-Met, DNS-Ala, and DNS-Ile; the low level at which these amino acids occurred at the amino-terminal position also makes their detection difficult.

Examination of the Tryptic Digest by Gel Filtration. Trypsin-modified ceruloplasmin (expt IV, Table I) (194 mg in 19 ml) was fractionated on a column of Sephadex G-200 (63 × 2.5 cm gel bed) equilibrated with 0.15 M KCl (Figure 3). One main component and two minor components were noted. By normalizing the curves and determining the areas by planimetry it was found that components I, II, and III represented 83, 12, and 5%, respectively, of the total area prescribed by the 280-mμ absorption of the effluent fractions. All of the protein possessing absorption at 610 mμ was found in fraction A. Of the total amount of ceruloplasmin copper applied to the column, 97% was recovered in fractions A, B, C, and D which contained 91, 5, <0.5, and 1%, respectively. The absorbancy ratio ($E_{280\,m\mu}$:$E_{610\,m\mu}$) for the ceruloplasmin solutions in tubes 30–34 inclusive ranged from a high of 22.0 to a low of 20.8. These values are significantly less than the ratio of 26.0 for the unfractionated digest.

In order to detect the presence of peptides lacking tyrosine and tryptophan, aliquots from alternate effluent fractions (tubes 1–170, Figure 3) were analyzed for ninhydrin-reactive material after alkaline hydrolysis

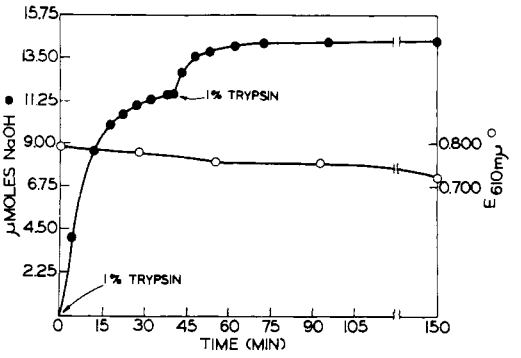

FIGURE 2: Alkali consumption and chromophore absorption during the course of digestion of native crystalline ceruloplasmin by trypsin at 25°. Absorbancy values are corrected for dilution by titrant and enzyme solution. The titrant was 9 × 10^{-3} N sodium hydroxide.

TABLE II: Amino-Terminal Residues in Trypsin-Modified Ceruloplasmin.

Amino Acid	Moles of Amino Acid/Mole of Protein		
	Trypsin-Modified Ceruloplasmin	Native Ceruloplasmin	New NH_2 Termini
Lysine	3.02	0.80	2.22
Arginine	1.04		1.04
Aspartic acid	1.58		1.58
Serine	1.34		1.34
Glutamic acid	0.92		0.92
Glycine	1.29		1.29
Alanine	0.14		0.14
Valine	0.73	0.13	0.60
Methionine	0.47		0.47
Isoleucine	0.27		0.27
Leucine	0.57		0.57
Total			10.44

[2] Abbreviation used: DNS, 1-dimethylaminonaphthalene-5-sulfonyl.

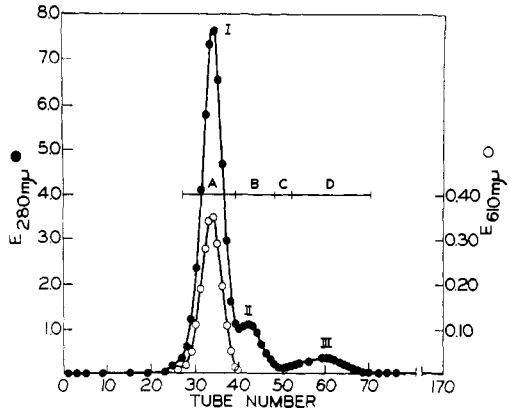

FIGURE 3: Elution pattern obtained when trypsin-modified ceruloplasmin was subjected to gel filtration on a column (63 × 2.5 cm) of Sephadex G-200 equilibrated with 0.15 M KCl saturated with chloroform. The sample was applied as a 1% solution in a volume of 19 ml and the eluting solvent was 0.15 M KCl. Fractions of 5 ml were collected at a flow rate of 23 ml/hr.

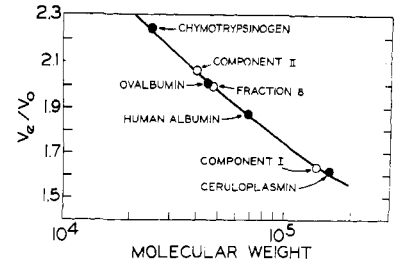

FIGURE 4: Plot of the ratio of elution volume (V_e) to void volume (V_o) against molecular weight. The values for proteins of known molecular weight appear in closed circles and represent an average of three determinations. The open circles (average of two determinations) represent protein samples obtained by the fractionation of trypsin-modified ceruloplasmin. Experimental details are presented in the Methods section.

(Hirs *et al.*, 1956). The results of the ninhydrin analysis gave a qualitatively similar elution curve as obtained by optical density measurements at 280 mμ; no additional components were detected.

Alternate effluent fractions were analyzed for hexose by the anthrone method in an attempt to find carbohydrate-rich fragments that could have been released by the action of trypsin on native ceruloplasmin. No anthrone-positive material was found beyond tube 66 (Figure 3).

Properties of Components I and II. The contents of tubes 33 and 34 of fraction A (Figure 3) were pooled as were the contents of tubes 44–46 of fraction B. Both pooled fractions were concentrated by dialysis against 20% Ficoll in 0.05 M sodium acetate; these concentrates were considered representative of components I and II, respectively, and were used for oxidase activity measurements, starch gel electrophoresis, molecular weight estimations, and amino acid analysis.

Component I contained 2.88 μg of copper/mg of protein and was found to yield a $\Delta E_{530\ m\mu/(min/\mu g\ of\ Cu)}$ value of 0.0521. This figure is in close agreement with the value of 0.0515 (expt IV, Table I) obtained for the unfractionated digest. Component II was devoid of enzymic activity.

Figure 1 illustrates the relationship of the starch gel electrophoresis patterns of components I and II to the unfractionated digest and native ceruloplasmin. The native protein yielded a major and minor component which were both catalytically active. It was presumed that the component of lowest anodic mobility was an aggregate of the native enzyme which was retarded in the starch gel. The pattern produced by the unfractionated digest was quite complex and was characterized by two major and three minor components. A striking feature of the digest was the apparent total absence of an electrophoretic species with a mobility of native ceruloplasmin. Component I contained all of the electrophoretic species present in the digest except for the component of highest anodic mobility. This rapidly moving component was detectable only in the fraction representing component II. Of the multiple bands produced by the digest and component I, only a singular species with an anodic mobility greater than that of native ceruloplasmin was observed to possess catalytic activity; no oxidase-positive bands were detected which corresponded to the native protein. From results of starch gel electrophoresis experiments comparing the intensity of the oxidase stain for native and trypsin-modified ceruloplasmin at identical levels of enzyme activity, it became evident that the single oxidase-positive component noted in the digest did not account for all of the activity applied to the gel. Protein in fraction D migrated toward the cathode and possibly represents in part the diisopropylphosphoryltrypsin present in the digest. The pattern of fraction D is not shown in Figure 1.

The molecular weight of components I and II were estimated by gel filtration on Sephadex G-200 and found to be near 140,000 and 40,000, respectively (Figure 4). These values indicate that the blue species (component I) had a molecular weight close to that of native ceruloplasmin while the colorless species (component II) represented one-quarter of the native molecule.

The amino acid composition of components I and II is presented in Table III. Only slight differences in composition can be noted between native ceruloplasmin and component I. Specifically, component I contains 4.7 and 4.8% less of both arginine and isoleucine, respectively, while showing a 4.8% increase in the half-cystine content. For the remaining amino acids the composition of component I is in excellent agreement with the native protein. Component II which appears as a homogeneous species in starch gel electrophoresis (Figure 1) has an amino acid composition markedly different from native ceruloplasmin. All

TABLE III: Summary of Amino Acid Analyses.[a]

	Native	Component I[b]		Component II[b]		Fraction 1[c]		Fraction 5[c]		Fraction 8[c]		Dialyzed Digest[d]	
	μmoles %	μmoles %	% Change	μmoles %	% Change	μmoles %	% Change	μmoles %	% Change	μmoles %	% Change	μmoles %	% Change
Lysine	6.23	6.14		5.59	−10	5.99	−3.8	6.14		5.81	−6.7	5.65	−9.3
Histidine	3.92	4.03		3.79		3.87		4.32	+10	3.69	−5.9	3.94	
Arginine	3.83	3.65	−4.7	3.10	−19	3.56	−7.0	3.54	−7.6	3.61	−5.7	3.51	−8.4
Aspartic acid	12.02	12.17		11.64		11.98		11.85		11.89		12.43	
Threonine	7.12	7.16		8.14	+14	6.98		6.82	−4.2	7.89	+10.8	7.18	
Serine	5.43	5.43		5.80	+6.8	5.38		5.58		5.35		5.36	
Glutamic acid	11.40	11.41		10.67	−6.3	11.30		11.58		11.01		11.60	
Proline	4.90	4.84		5.12	+4.5	5.61	+14.5	4.53	−7.6	5.38	+9.8	4.99	
Glycine	7.57	7.68		8.31	+9.8	7.66		7.81	+3.2	8.01	+5.8	7.83	+3.4
Alanine	4.99	4.94		5.34	+7.0	5.08		5.03		5.00		5.09	
Half-cystine	1.25	1.31	+4.8	1.22		1.06	−15	1.02	−18	1.00	−20	1.24	
Valine	5.97	5.85		6.43	+7.7	5.91		5.91		6.35	+6.4	5.84	
Methionine	2.32	2.35		2.83	+22	2.65	+14.2	2.63	+13.4	2.66	+14.6	2.35	
Isoleucine	4.99	4.75	−4.8	4.61	−7.6	4.78	−4.2	4.89		4.70	−5.8	4.90	
Leucine	7.03	7.05		6.65	−5.3	7.05		7.21		6.51	−7.4	6.99	
Tyrosine	6.06	6.27		5.50	−9.1	6.06		6.17		5.89		6.14	
Phenylalanine	4.99	4.96		5.26	+5.4	5.08		4.97		5.24	+5.0	4.96	

[a] Composition is expressed as micromoles per cent, *i.e.*, μmoles of each amino acid/100 μmoles of total amino acids. Tryptophan and cysteine were not determined and are omitted from this table; literature values for these residues are 27 and 1, respectively (Kasper and Deutsch, 1963). No correction has been made for the hydrolytic destruction of threonine or serine. The per cent change with respect to native ceruloplasmin is also included. Deviations of ±3% or less were omitted. [b] Components obtained by fractionation of digest on Sephadex G-200 (Figure 3). [c] Fractions obtained by chromatography of trypsin-modified ceruloplasmin on DEAE-cellulose (Figure 5). [d] Tryptic digest of ceruloplasmin which was dialyzed against 5×10^{-3} M pH 5.2 ammonium acetate after inactivation of trypsin with DFP.

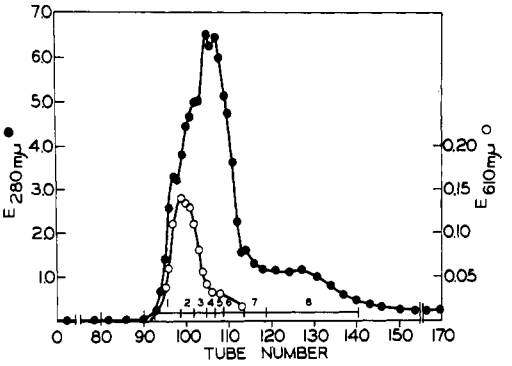

FIGURE 5: Chromatographic fractionation of trypsin-modified ceruloplasmin on DEAE-cellulose (DE-32). The resin bed (66 × 2.5 cm) was equilibrated with 0.05 M sodium acetate (pH 6.8) and the chromatogram was developed by a constant-volume gradient elution scheme. The mixing flask contained 200 ml of 0.05 M sodium acetate while the second flask contained 0.3 M sodium chloride in 0.05 M sodium acetate. Fractions of 6.3 ml were collected at a flow rate of 51 ml/hr. The effluent fractions which were pooled and examined are numbered 1–8.

residues show a change of greater than ±3% except for histidine, aspartic acid, and half-cystine. Methionine and threonine are increased by 22 and 14%, respectively, while arginine and lysine exhibit respective decreases of 19 and 10%. The remaining differences are noted in Table III.

Effect of Dialysis on the Properties of the Tryptic Digest. A tryptic digest of ceruloplasmin (expt III, Table I) was dialyzed exhaustively against pH 5.3, 5×10^{-3} M ammonium acetate at 3°. The outer solution was concentrated by rotary evaporation at 40° and examined for dialyzable ninhydrin-positive peptides by a two-dimensional separation technique. The most distinguishing feature of the "fingerprint" was the presence of relatively large amounts of free lysine and arginine; low levels of neutral and basic peptides were also noted. The dialyzed digest had lost only 12% of the original oxidase activity and retained 93% of the original chromophore absorption. Based on absorption measurements at 280 mμ, the recovery of protein was 97%. Copper analyses indicated that essentially all of the protein-bound copper present in the tryptic digest was nondialyzable; a recovery of 95% was obtained. The amino acid composition of the dialyzed digest was quite similar to that of native ceruloplasmin except for a decrease of 9.3 and 8.4% in the lysine and arginine content, respectively (Table III).

Chromatography of the Tryptic Digest on DEAE-cellulose and Some Properties of Isolated Fractions. Trypsin-modified ceruloplasmin (expt II-2) when chromatographed on DEAE-cellulose (Figure 5) behaved as a heterogeneous population of protein molecules. Of the total protein applied to the column (229 mg), 89.8% was recovered in fractions 1–8 based on absorption measurements at 280 mμ (Table IV). These eight fractions also accounted for 83.1% of the total protein-bound copper (Table IV). The unfractionated digest contained 53% cupric copper as determined by a combination of electron paramagnetic resonance spectroscopy and chemical measurements; this value is in good agreement with the 50% Cu^{2+} found in fraction 1. The cupric copper content of fractions 5 and 8 was 85 and 76%, respectively.

A recovery of 31% of the total units of enzyme activity applied to the column was obtained along with a recovery of 35% of the total absorbancy at 610 mμ. Examination of various fractions by starch gel electrophoresis revealed that the material eluting early

TABLE IV: Summary of Analytical Data for DEAE-Cellulose Fractions of Trypsin-Modified Ceruloplasmin.

Fraction	Protein Recov[a]	Cu Recov[b]	[Cu] (mM) Epr[c]	[Cu] (mM) Chemical	% Cu^{2+}	Act.[d]
Unfractionated digest			0.53	1.0	53	7.60
1	10.8	11.9	0.33	0.67	50	6.10
2	11.2	12.2				6.80
3	12.8	13.5				4.66
4	8.2	7.9				1.96
5	8.1	7.3	0.40	0.47	85	1.23
6	12.6	10.8				1.03
7	5.3	4.2				1.00
8	20.8	15.3	0.38	0.50	76	0.48
Total	89.8	83.1				

[a] Per cent of total protein (229 mg) applied to chromatographic column. [b] Per cent of total copper (680 μg) applied to chromatographic column. [c] Determined by double integration and comparison to a standard Cu^{2+}-EDTA solution; electron paramagnetic resonance (epr) conditions as in Figure 8. [d] $\Delta E_{530\ m\mu/(min/\mu g\ of\ Cu)} \times 10^2$.

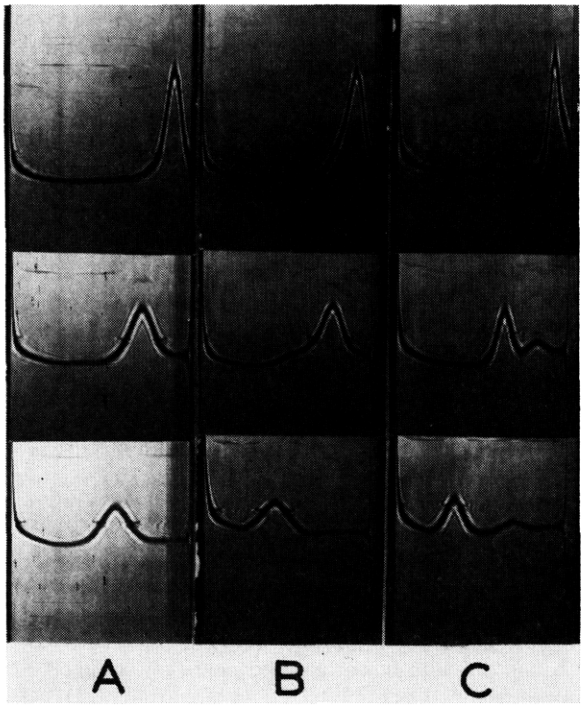

FIGURE 6: Sedimentation velocity patterns of fractions obtained from DEAE-cellulose chromatography of trypsin-modified ceruloplasmin (Figure 5). All measurements were made in pH 7.4, 0.2 ionic strength potassium phosphate buffer in a single-sector 12-mm cell. (A) Fraction 8 (16, 64, and 105 min); (B) fraction 5 (25, 57, and 139 min); (C) fraction 2 (7, 39, and 71 min). The times in parentheses indicate the time at 59,780 rpm for the upper, middle, and lower exposures, respectively.

from DEAE-cellulose (fractions 1 and 2) contained predominantly those components of lowest anodic mobility which were present in the unfractionated digest (Figure 1). This would be expected since those molecules with the lowest net negative charge would be retained least by the anion exchanger. Fractions 3–8 contained increasing amounts of the more acidic components.

Fractions obtained by DEAE-chromatography of trypsin-modified ceruloplasmin possessed sedimentation patterns quite different from those of the unfractionated digest (Figure 6 and Table V). Fraction 2 which represented the material with the maximum absorbancy at 610 mμ contained 21.2% of a 3.19S component and 78.8% of a 7.09S component. Fraction 5 contained approximately 75% of a 3.12S species in addition to some material sedimenting near 7 S. Fraction 8 appeared to be composed almost entirely of a 3.2S species that had an estimated molecular weight of 47,000 as determined by gel filtration on Sephadex G-200 (Figure 4).

The amino acid compositions of fractions 1, 5, and 8 are presented in Table III. All three fractions show marked quantitative differences in composition when compared to native ceruloplasmin and also when compared to each other.

Sedimentation Properties of the Tryptic Digest. Trypsin digestion of native ceruloplasmin resulted in a slight but reproducible change in the sedimentation properties of the crystalline protein. This treatment when applied to three different ceruloplasmin preparations produced between 8.8 and 14.1% of a second component with a sedimentation coefficient in the range of 3.12–3.27 S (Table V). The remainder of the trypsin-treated protein sedimented as a symmetrical boundary with a sedimentation coefficient indistinguishable from that of the native protein. A typical sedimentation pattern of trypsin-treated ceruloplasmin appears in Figure 7B. It is interesting to note that the oxidase activity retained by the trypsin-modified protein is closely related to the amount of 7S component present in the digest (Table V).

Incubation of native ceruloplasmin with 0.1 M 2-mercaptoethanol or 0.005 M dithiothreitol in pH 7.4, 0.2 ionic strength potassium phosphate buffer failed to alter the sedimentation properties of the native protein (Table V). This result is in agreement with earlier studies (Kasper and Deutsch, 1963). Reduction of trypsin-modified ceruloplasmin by either of the methods mentioned above produced a marked change in the sedimentation behavior (Figure 7A). Approximately 60% of the protein present in the reduced digest now sediments as a 3.0–3.2S component. Two different tryptic digests yielded similar proportions of the slow and fast components (Table V) reflecting the reproducibility of the proteolytic modification.

Electron Paramagnetic Resonance Spectra of the Tryptic Digest and Some Isolated Chromatographic Fractions. The spectra of both native ceruloplasmin and the tryptic digest contain two sets of hyperfine lines (Figure 8A,B). As reported by Vänngård (1967), the more narrow and intense set usually described is superimposed on a weaker set with a larger hyperfine splitting constant. Directly beneath each spectrum, the hyperfine pattern at $g_{||}$ is presented at a fivefold increase in amplification permitting a detailed comparison of the various spectra. The spectra of fractions 1, 5, and 8 obtained by chromatographic fractionation of the digest on DEAE-cellulose are presented in Figure 8C–E. The amount of Cu^{2+} in the colorless fractions was markedly increased over the level of Cu^{2+} in the unfractionated digest and fraction 1 (Table V). All three column fractions, however, exhibited substantial increases in the wider hyperfine component, particularly those with the higher cupric copper content.

Discussion

Data have been presented characterizing the product(s) formed when trypsin is allowed to act on native human ceruloplasmin in the pH range of 7.2–7.4 at 25°. Measurement of alkali consumption during trypsin digestion and chemical end-group analyses performed on the digest indicated that 10–23 of a possible 114

TABLE V: Summary of Sedimentation Studies.

Sample	Protein Concn (mg/ml)	$s_{20,w} \times 10^{13}$ (S) Slow Component	$s_{20,w} \times 10^{13}$ (S) Fast Component	Slow Component (%)	Fast Component (%)	Act. Remaining (%)[d]
Digest, expt II-2	9.1	3.27	6.80	8.8	91.2	88
Digest, expt III	7.0	3.16	6.96	10.7	89.3	88
Digest, expt IV	9.8	3.12	6.70	14.1	85.9	82
Reduced digest from expt IV[a]	9.8	3.19	6.64	60.6	39.4	
Reduced digest from expt III[b]	10	3.05	6.80	57.5	42.5	
Fraction 2[c]	5.2	3.19	7.09	21.2	78.8	
Fraction 5[c]	4.0	3.12	~7	~75	~25	
Fraction 8[c]	7.7	3.22		98	2	
Native ceruloplasmin	9.0		6.79		100	
Reduced native ceruloplasmin	8.5		6.75		100	

[a] Digest was reduced by dialysis against pH 7.4, 0.2 ionic strength potassium phosphate containing 0.1 M 2-mercaptoethanol for 16 hr at 3°. [b] Digest was dialyzed against pH 7.4, 0.2 ionic strength potassium phosphate containing 0.005 M dithiothreitol for 16 hr at 3°. [c] Fractions obtained by chromatography of trypsin-modified ceruloplasmin on DEAE-cellulose (see Figure 5). [d] Oxidase activity of the unfractionated tryptic digest.

susceptible peptide bonds were cleaved/molecule of protein. It is noteworthy that lysine and arginine were found at the level of 2.22 and 1.04 moles/mole of protein, respectively, indicating that a cluster(s) of bases must occur in the ceruloplasmin molecule. Lack of agreement between the titrimetric and cyanate method as to the total number of new amino termini is most likely due to the relative inaccessibility of amino-terminal residues to cyanate in the absence of urea. At best, the total bonds cleaved as measured by the cyanate method should be considered a minimum value.

Trypsin-modified ceruloplasmin which had an altered primary structure due to the severance of covalent bonds still retained 91–95% of the original copper chromophore absorption and 82–100% of the original oxidase activity. Another characterizing feature was that the action of trypsin did not convert protein-bound copper to a dialyzable form or to a form which was direct reacting. A valid comparison, however, can not be made with the work of Curzon (1958) on the chymotryptic digest since a considerably higher enzyme to substrate ratio was used in the earlier study.

Examination of the electron paramagnetic resonance spectrum of the tryptic digest reveals an increase in the amount of wider hyperfine component over that present in the spectrum of the native protein. This wider hyperfine component has been ascribed to either denaturation (Vänngård, 1967) or to a naturally occurring copper species in ceruloplasmin (Peisach and Blumberg, 1967a). It should be pointed out that the colorless Tris and ascorbic acid derivatives of ceruloplasmin which contain approximately four atoms of direct reacting copper per molecule of protein have electron paramagnetic resonance spectra characterized by a high content of wide hyperfine structure (Kasper *et al.*, 1963). The absence of direct reacting copper in the tryptic digest suggests that loosely bound as well as firmly bound copper can produce this wide hyperfine structure. The spectra of fractions 5 and 8 contain the wide hyperfine lines characteristic of many copper complexes (Gould and Mason, 1966); this could be an indication that a significant amount of copper in these column fractions is bound nonspecifically to peptide chains and is in a coordination state quite different from that required for blue color. The spectrum of fraction 8 is associated with a molecule(s) which has a molecular weight of approximately 47,000. It is tempting to suggest that the coordination state of copper in native ceruloplasmin may be similar to the "strained complex" proposed for the chromophore of stellacyanin (Peisach and Blumberg, 1967b) and that trypsin acts on native ceruloplasmin to partially relieve this strain producing the spectra in Figure 8B.

The tryptic digest yielded a sedimentation pattern indistinguishable from that of native ceruloplasmin except for the presence of 9–14% of a 3S component (Figure 7B,C). Maintenance of most of the 7S character of the ceruloplasmin molecule after digestion with trypsin indicated that extensive proteolytic degradation to low molecular weight fragments had not occurred. It is of interest to note that the absorbancy ratio ($E_{280\,m\mu}:E_{610\,m\mu}$) of the 7S product (component I) was identical with that of native ceruloplasmin although

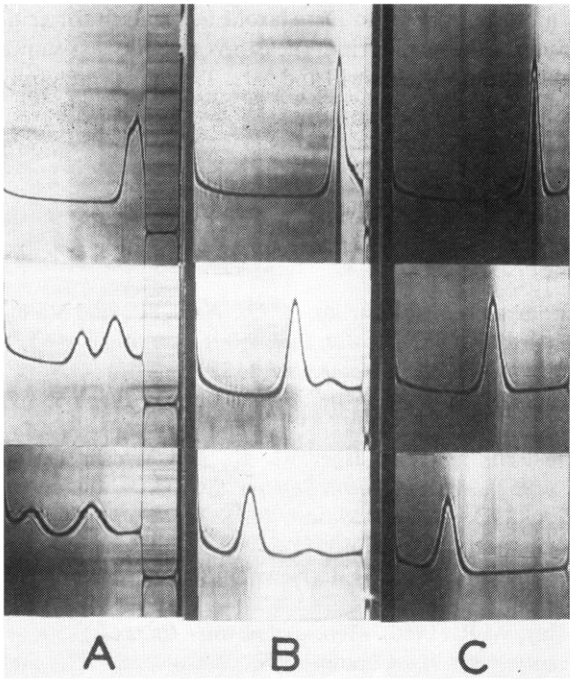

FIGURE 7: Sedimentation velocity patterns of (A) trypsin-modified ceruloplasmin in pH 7.4, 0.2 ionic strength potassium phosphate containing 0.1 M 2-mercaptoethanol (12, 44, and 76 min), (B) trypsin-modified ceruloplasmin in pH 7.4, 0.2 ionic strength potassium phosphate (16, 48, and 80 min), and (C) native ceruloplasmin in pH 7.4, 0.2 ionic strength potassium phosphate (21, 53, and 85 min). The times in parentheses indicate the time in minutes at 59,780 rpm for the upper, middle, and lower exposures, respectively.

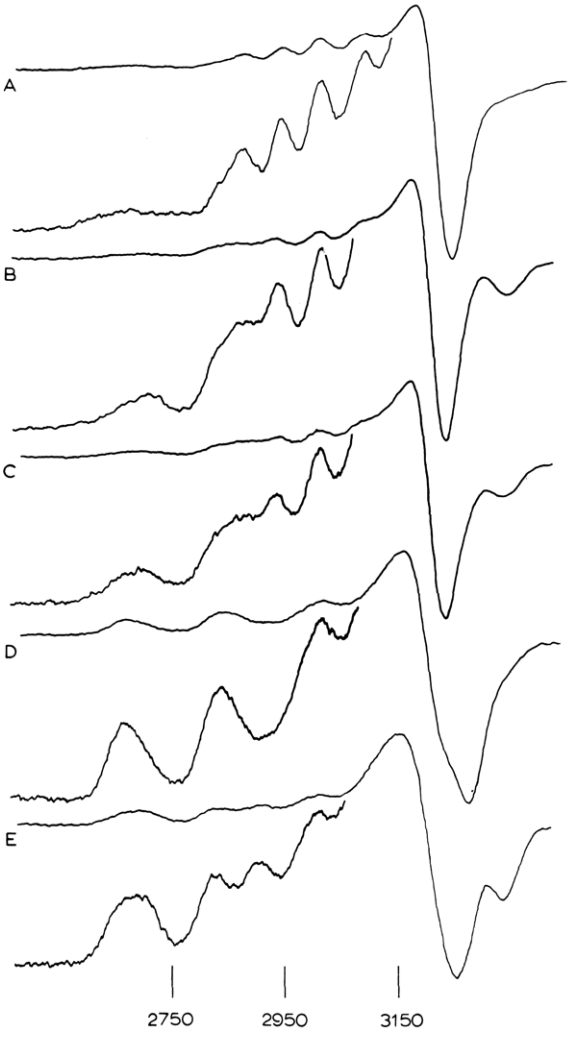

FIGURE 8: Electron paramagnetic resonance spectra of purified human ceruloplasmin (A), a tryptic digest of ceruloplasmin (B), and DEAE column fractions 1 (C), 5 (D), and 8 (E) as described in the text. Electron paramagnetic resonance conditions: incident microwave power, 23 mw; modulation amplitude, 6 gauss; sample temperature, 116°K; scanning rate, 200 gauss/min; time constant, 0.5 sec; microwave frequency, 9.254 kMHz. The gains for each spectrum were adjusted so that the main peak amplitudes were approximately equal. Directly beneath each spectrum the hyperfine pattern at $g_{\parallel}$ is displayed at a five-times increase in amplification. Field markers are given in gauss.

its specific activity was very similar to the specific activity of the unfractionated digest. This observation suggests the possibility that certain structural features of the ceruloplasmin molecule may be essential for enzymic activity while not being directly involved in maintaining the integrity of the copper chromophore. A disproportionate loss of activity and chromophore absorption has been observed when ceruloplasmin was exposed to alkaline pH (Kasper *et al.*, 1963). It is of interest to note that the amount of activity remaining in the digest closely paralleled the level of the 7S component in the digest.

Various tryptic digests when examined by starch gel electrophoresis produced similar multicomponent patterns totally different from the pattern of native ceruloplasmin. No electrophoretic species with a mobility of native ceruloplasmin was detected by protein- or oxidase-staining techniques. It should be remembered, however, that this singular oxidase-positive band present in the digest did not account for all of the activity applied to the gel. Similar results were obtained when the tryptic digest was fractionated on DEAE-cellulose. This mild chromatographic procedure resulted in the loss of 69% of the total enzyme units applied to the column while the protein and copper recoveries were 90 and 83%, respectively (Table IV). Examination of the sedimentation patterns of fraction 1–8 (for representative patterns see Figure 6) revealed that a substantial amount (approximately 50%) of the total protein isolated from the anion exchanger sedimented as a 3S component (Table V). In this connection, it is important to remember that 91% of the protein applied to the chromatographic column sedimented

as a 7S component. The fractions richest in the 3S component were those eluting last from the chromatographic column. More than 80% of fraction 1 was composed of 7S material while 3S material composed 98% of fraction 8. Reduction of the digest with either 2-mercaptoethanol or dithiothreitol changed the relative proportions of 7 S:3 S from 89:11 to 40:60 (Table V). It appears that the 7S component present in the tryptic digest is composed of at least two different types of complexes which are distinguished by their differential susceptibility to reduction by mercaptans. Both complexes are catalytically active and are stable to ultracentrifugation and gel filtration. Component I (Figure 3) is a mixture of these two complexes. One complex has characteristics similar to those of native ceruloplasmin except for its altered electrophoretic properties and reduced specific activity. This complex is not detectably altered by anion-exchange chromatography or electrophoresis and is not converted to lower molecular weight products on reduction by mercaptans. The second complex which is also part of component I has undergone a more extensive enzymic alteration but yet maintains a significant portion of the oxidase activity. However, the integrity of this second catalytic complex is (1) disrupted by electrophoresis or chromatography resulting in a total loss of enzymic activity and (2) is susceptible to reduction by mercaptans being converted to components with sedimentation coefficients in the range of 3 S.

With the exception of finding free lysine and arginine plus a very low level of neutral and basic peptides in the dialysate of the tryptic digest, only molecules with sedimentation coefficients in the region of 7 and 3 S have been detected. It is possible that the 3S component is actually a complex of lower molecular weight fragments held together by a noncovalent type of bonding, but this remains to be established. The close agreement of the amino acid composition of the dialyzed digest (Table III) with that of native ceruloplasmin is strong support for the fact that trypsin is not producing substantial amounts of free low molecular weight fragments in the hydrolytic process. If trypsin were randomly cleaving amide bonds to produce a mixture of peptides, some of which were joined by disulfide bridges, a rather heterogeneous population of molecules with respect to molecular size and charge would be expected. In addition, it would be anticipated that reduction of such a mixture would lead to an even greater degree of heterogeneity. From sedimentation, chromatographic, and electrophoretic studies, this gross polydispersity is not observed. The constancy of the relative amounts of 7S and 3S products in the reduced digest suggests some degree of specificity for the limited trypsin-catalyzed hydrolysis of native ceruloplasmin.

Acknowledgments

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Diphosphopyridine Nucleotide Specific Isocitric Dehydrogenase of Mammalian Mitochondria. II. Kinetic Properties of the Enzyme of the Ehrlich Ascites Carcinoma*

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ABSTRACT: Interactions of substrates, activators, and inhibitors are examined in the reaction catalyzed by the diphosphopyridine nucleotide specific isocitric dehydrogenase of the mitochondria of the Ehrlich ascites carcinoma. Cooperative homotropic interactions in rate–concentration functions are found for isocitrate, magnesium ion, and the effectors, adenosine diphosphate (ADP) and reduced diphosphopyridine nucleotide (DPNH). Isocitrate homotropic cooperativity is pH dependent; ADP abolishes this effect, while DPNH, a negative effector, increases isocitrate cooperativity. Reduced triphosphopyridine nucleotide inhibits the enzyme and manifests somewhat smaller cooperative interactions. ADP antagonizes the inhibition by both reduced pyridine nucleotides. 2,4-Dinitrophenol inhibits the reaction and demonstrates marked homotropic cooperative effects in the presence of ADP or high isocitrate concentration. In the absence of ADP, inorganic phosphate is found to stabilize the enzyme and to activate the reaction. The effect of pH on effector and substrate cooperativity is interpreted as allosteric ligand binding of H^+. The data support a role for the diphosphopyridine nucleotide specific isocitric dehydrogenase in regulation of metabolism and are found to be consistent with the regulatory enzyme model proposed by Monod *et al.* (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88).

Solubilization and purification of the DPN^+-specific isocitric dehydrogenase (DICDH)[1] of mammalian mitochondria was first described by Plaut and Sung (1954). More recently, Chen and Plaut (1962, 1963) have purified further the beef heart enzyme and have demonstrated a specific activation of the enzyme by ADP manifested in an increased affinity for isocitrate; ADP was found to induce aggregation of the enzyme (Chen *et al.*, 1964), an effect prevented by DPNH, an inhibitor of enzyme activity. Chen and Plaut (1963) have drawn attention to the possibility of a role for DICDH in the control of respiration. The nonclassical, cooperative response of the rate of the DICDH reaction to concentration of substrates, modified by pH and adenine nucleotides, has been demonstrated in a number of laboratories (*Acetobacter peroxydans* (yeast), Hathaway and Atkinson, 1963; Atkinson *et al.*, 1965; *Neurospora*, Sanwal *et al.*, 1963; Sanwal and Stachow, 1965; Sanwal *et al.*, 1965; Sanwal and Cook, 1966; and rat heart and locust flight muscle mitochondria, Goebell and Klingenberg, 1964; Klingenberg *et al.*, 1965).

Monod *et al.* (1965) have proposed a model for the cooperative properties of regulatory enzymes based on changes in ligand affinities resulting from symmetrical cooperative changes in the conformation of protein subunits. The empirical Hill plot has been applied by these authors (Monod *et al.*, 1963) to quantitate cooperative effects in rate data obtained with L-threonine deaminase. This device has been used in the present study to evaluate the allosteric behavior of several substrates and effectors in the DICDH reaction catalyzed by extracts of acetone powders derived from mito-

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[1] Abbreviations used: DPN^+ and DPNH, diphosphopyridine nucleotides, oxidized and reduced, respectively; TPN^+ and TPNH, triphosphopyridine nucleotides, oxidized and reduced, respectively; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates, respectively; DCIP, 2,6-dichlorophenolindophenol; DICDH, DPN^+-specific isocitric dehydrogenase.