Cytochrome b_5 from Microsomal Membranes of Equine, Bovine, and Porcine Livers. Isolation and Properties of Preparations Containing the Membranous Segment†

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ABSTRACT: Cytochrome b_5 was isolated from microsomal membranes of equine, bovine, and porcine livers. A general procedure for preparing homogeneous preparations of these hemoproteins from mammalian liver, in quantities sufficient for amino acid sequence analysis, is described. The isolation procedure consisted of solubilizing microsomes by means of the detergent action of Triton X-100 and deoxycholate. The hemoproteins were purified by the combination of gel filtration on Sephadex G-100 and chromatography on DEAE-cellulose in the presence of detergents and chaotropic ions. Amino acid analyses indicated that all three proteins contained approximately 150 amino acid residues. No NH_2-terminal residue was detected in any of the preparations. Hydrazinolysis of the proteins released aspartic acid. The terminal peptides were isolated from tryptic or chymotryptic digests of these preparations. Their partial characterization led to the following NH_2-terminal sequences: (Asx,Glx,Glx,Glx,Ala,-Ser)Ser-Lys-Ala-Val-Lys-Tyr for the bovine protein and (Asx,Glx,Glx,Ala)Ser-Lys for the equine and porcine preparations. The COOH-terminus of the equine preparation was established as Ile-Tyr-Thr-Ala-Glu-Asp and for bovine protein as Thr-Ser-Glu-Asp.

The structural features which impart membrane proteins with their unique physicochemical character are not understood at the present time. To elucidate the character of the amino acid arrangement of protein segments which penetrate and interact with membrane components, we have undertaken the isolation and determination of the entire covalent structure of cytochrome b_5 from various mammalian livers.

Cytochrome b_5 is a membrane protein present in the endoplasmic reticulum of mammalian cells (Strittmatter, 1967). In liver microsomes, cytochrome b_5 is an integral part of a system responsible for oxidative conversion of stearoyl-CoA to oleoyl-CoA (Halloway and Wakil, 1970; Oshina *et al.*, 1970) and has been implicated as a participant in the cytochrome P-450 dependent hydroxylation reactions (Estabrook *et al.*, 1971); a similar system in erythrocytes catalyzes the reduction of methemoglobin (Hultquist and Passon, 1971).

Cytochrome b_5 appears to be partially submerged in the endoplasmic membrane, since a hemoprotein preparation of about 11,000 daltons is released from endoplasmic membranes upon mild proteolysis of the intact microsomes, either by trypsin or cathepsins. The latter preparation, consisting of some 80–90 residues, is water soluble and contains the catalytic site but not the area responsible for membrane attachment. When microsomes are extracted with detergents, an oligomer is obtained (Ito and Sato, 1968), each monomer of which has a molecular weight of about 17,000 daltons (Spatz and Strittmatter, 1971). This preparation presumably contains both the catalytic site and the membranous segment (Spatz and Strittmatter, 1971; Ito and Sato, 1968; Strittmatter *et al.*, 1972). The structure of the water-soluble moiety of calf cytochrome b_5 at 2.0-Å resolution has been reported by Mathews *et al.* (1972).

In previous studies, we report the amino acid sequence of the water-soluble fragment of cytochrome b_5 from six mammalian species (Nobrega and Ozols, 1971). A preparation of cytochrome b_5 from human liver which contains the segment presumably involved in the strong association between the hemoprotein and the membrane has also been reported (Ozols, 1972).

Studies on the amino acid sequence of the catalytic site of cytochrome b_5 reveal an unexpected sequence similarity between this protein and hemoglobin (Ozols and Strittmatter, 1967). Very recently Guiard and Lederer (1973) reported that the heme-binding, 80 residue polypeptide segment of bakers' yeast L-lactate dehydrogenase (cytochrome b_2) also shows striking homology with that of cytochrome b_5.

This paper reports the isolation and partial characterization of intact cytochrome b_5 from microsomal membranes of bovine, porcine, and equine liver. This report is the first portion of the determination of the entire amino acid sequence of these proteins.

Experimental Section

Materials. Horse livers were purchased from Pel-Freez Biologicals Inc. (Rogers, Ark.). Beef livers were obtained from Snyder Brothers, Inc. (Wilkinsonville, Mass.), and pig livers were obtained from Corsair Packing Co. (Pawtucket, R. I.). The nonionic detergents Triton X-100 and Triton N-101 were obtained from Rohm and Haas. Sodium deoxycholate was purchased from Matheson Coleman and Bell. All other chemicals were of analytical grade. The water used was distilled in an all-glass apparatus from deionized water. Sephadex gels were obtained from Pharmacia. Diethylaminoethyl (DEAE) type DE 52 was a product of Whatman. Trypsin, chymotrypsin, and carboxypeptidases were obtained from Worthington.

Methods. Spectrophotometric measurements were made using a Cary Model 14 recording spectrophotometer at approximately 23°. The amount of cytochrome b_5 was determined by one of several possible methods: (a) the absorbance of the oxidized form at 413 nm using a millimolar extinction

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coefficient (ϵ mM) of 117 cm^{-1} mM^{-1} (Strittmatter and Velick, 1956); (b) the absorbance of the reduced form at 423 nm (ϵ mM 177) and 556 (ϵ mM 26.5); or (c) by the change in absorbance at 423 nm ($A^{423}_{red} - A^{423}_{ox}$) ($\epsilon$ mM 118) which occurs upon reduction effected by addition of solid dithionite. Cytochrome P-450 was determined from the difference spectrum, reduced CO complex minus the reduced form ($A^{450} - A^{500}$) (ϵ mM 91 cm^{-1} mM^{-1}) (Omura and Sato, 1964); cytochrome b_5 reductase was assayed at 25° by measuring NADH–ferricyanide reductase activity (Rodgers and Strittmatter, 1973).

Protein concentration in partially solubilized preparations was determined by methods of Lowry *et al.* (1951) using bovine serum albumin as standard of reference. Protein content in effluent fractions was determined by a ninhydrin method following alkaline hydrolysis. In a typical experiment 25-μl (0.1 mg/ml) samples were pipetted into the bottom of (18 × 120 mm) polypropylene test tubes, followed by 1 ml of 2.5 N NaOH. The tubes were placed in an autoclave and heated with steam under pressure of 15 psi for a period of 20 min. The pressure was released gradually, and the tubes were cooled. Following this, each tube received 1 ml of 30% acetic acid and 1 ml of ninhydrin reagent (obtained from the reservoir of the amino acid analyzer). Tubes were shaken gently to mix the contents, and heated for 15 min in a covered boiling water bath. The color of each tube was then measured at 570 nm. The concentration of trypsin and chymotrypsin solutions was determined from amino acid analysis of appropriate aliquot.

Phospholipid was measured directly from the eluent fractions according to Bartlett (1959), and the amount of phospholipid calculated by multiplying the amount of phosphorus by 25.

Cytochrome solutions were concentrated routinely by ultrafiltration. A 400-ml Amicon ultrafiltration vessel was used in these procedures, fitted with a PM-30 or PM-10 membrane and maintained under 8 psi of nitrogen.

Amino acid analyses were performed with the Beckman Model 120C automatic amino acid analyzer. Protein samples (10–15 nM) were hydrolyzed at 108° in constant-boiling HCl, containing 1% (v/v) β-mercaptoethanol, in evacuated and sealed ampoules (Ozols, 1970). Duplicate analyses were performed on 22- and 92-hr hydrolysates. Tryptophan was determined on the amino acid analyzer from a 20-hr hydrolysate containing 3% (v/v) thioglycolic acid (mercaptoacetic acid, Eastman) (Nobrega and Ozols, 1971). Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1967).

The NH_2-terminal residues of the cytochromes were determined after reaction with dansyl chloride according to the method of Gray (1972). The proteins were dissolved in a 1% aqueous solution of sodium dodecyl sulfate and the solution was heated in a boiling water bath for 5 min. The labeling was carried out in 50% *N*-ethylmorpholine, and dansyl amino acids were identified by means of thin-layer chromatography systems of Woods and Wang (1969).

Carboxy-terminal residues were identified by hydrazinolysis of the cytochromes for 18 hr at 80° using the procedure of Schroeder (1972). Quantitative identification of individual amino acids was performed with the use of the amino acid analyzer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out as described by Neville (1971) with an 11% resolving gel and using the pH 9.18, 0.0308 N HCl–0.4244 M Tris system. Staining and destaining were done as described by Weber and Osborn (1969) using Coomassie Blue. All protein solubilization and fractionation procedures were performed at 2–5°. Other experimental procedures have been described in earlier publications (Ozols, 1970, 1972; Nobrega and Ozols, 1971).

Isolation of Microsomes.[1] Ten kilograms of partially thawed liver was freed of bile ducts, fat, and excess connective tissue, segmented, and homogenized for 1 min in the Gifford-Wood colloid mill with 2 volumes of buffer A (5 mM Tris-acetate (pH 8.3)–1 mM EDTA). The suspension was filtered through cheesecloth and centrifuged in the Sharples MD 16 rotor at 10,000 rpm and 20 l./hr. The debris was discarded and the supernatant fluid was recentrifuged at 14,000 rpm and 10 l./hr. The supernatant fraction was displaced with 5 l. of buffer A containing 5% sucrose and recentrifuged in the Electro-Nucleonics RK 6 rotor at 35,000 rpm and 6 l./hr. The microsomal pellet was resuspended in buffer A containing 0.25 M sucrose and the final volume of the preparation was adjusted to 1.6 l. (~30 mg of protein/ml). The suspended microsomes were then frozen and stored at −20°.

Solubilization of Microsomes. Four hundred milliliters of glycerol was added to the thawed microsomes prepared from 10 kg of liver, and the volume of the suspension was adjusted to 2 l. by the addition of a few milliliters of buffer A. After stirring the mixture for 30 min, 10 volumes of cold acetone was added at a rate of 1 l./min. During the addition of acetone, the temperature of the suspension was maintained between −5 and +5° by immersing the container in an acetone–Dry Ice bath. The suspension was allowed to settle for 30 min in the freezer (−20°). After standing, approximately 17 l. of the clear aqueous acetone solution could be removed by siphoning. The remaining suspension was centrifuged at 2400 rpm for 10 min in an International PR2 4-l. swinging rotor, and the supernatant was discarded. The precipitate was suspended in buffer A to a final volume of 4 l. The preparation was stirred for 15 hr after which it was centrifuged at 14,000 rpm for 20 min in the Sharples MD 16 rotor. The supernatant contained heme proteins other than cytochrome b_5. The sediment was further solubilized by the addition of 2.5 l. of buffer A containing 1.5% Triton X-100 and 0.1% sodium deoxycholate. The resulting suspension was stirred gently under nitrogen overnight and centrifuged at 10,000 rpm for 1 hr. The detergent extract contained cytochrome b_5, cytochrome b_5 reductase, as well as other hemoproteins.

Isolation of Cytochrome b_5. Step 1. Chromatography on DEAE-Cellulose. The entire detergent extract from 10 kg of liver was applied to a column (5 × 60 cm) of DEAE-cellulose that had been equilibrated with buffer A, and the column was washed with 2.5 l. of buffer A. Cytochrome b_5 was retained on the column whereas the cytochrome b_5 reductase was eluted in the wash. Cytochrome b_5 was eluted with 2 l. of 10 mM Tris-acetate (pH 8.3) containing 1 mM EDTA, 0.25 M sodium thiocyanate, and 0.25% sodium deoxycholate (buffer B) (Spatz and Strittmatter, 1971). Fractions containing the cytochrome b_5 were pooled and stored at −20° in three 400–450-ml aliquots.

Step 2. Gel Filtration on Sephadex G-100. A 400-ml aliquot of the preparation from step 1 was passed through a Sephadex G-100 column (21 × 100 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.15) containing 0.25 M sodium thiocyanate, 0.23% deoxycholate, and 1 mM EDTA. Elution was carried

[1] The isolation and solubilization of microsomes were designed and conducted in cooperation with Mr. Henry Blair and Dr. Stanley E. Charm of the New England Enzyme Center, Tufts University School of Medicine, Boston, Mass.

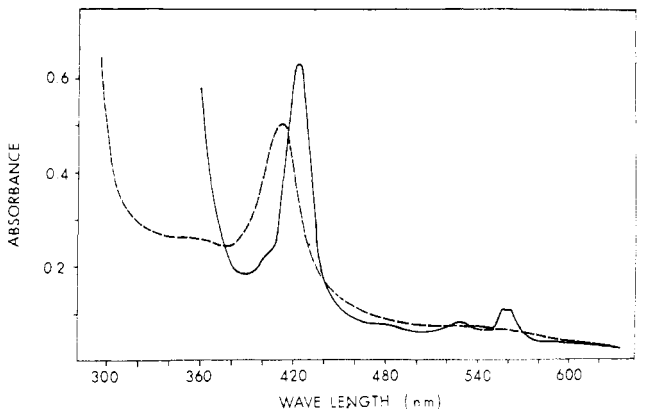

FIGURE 1: Absorption spectra of partially purified bovine cytochrome b_5. The preparation from step 1 (26 nmol/ml) was diluted tenfold with 0.1 M Tris-acetate (pH 8.1): (-----) oxidized; (———) reduced with sodium dithionite.

out with the same buffer, and fractions of 165 ml were collected, at a flow rate of 175 ml/hr. The cytochrome b_5 containing fractions were combined (~360 ml) and concentrated to 15 ml in an Amicon type protein concentrator equipped with a PM-30 membrane. After concentration, the solution was dialyzed against 10 mM Tris-HCl buffer (pH 8.15) containing 1 mM EDTA.

Step 3. Chromatography on DEAE. The concentrated cytochrome preparation from step 2 was applied to a DEAE-cellulose column (2.6 × 70 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.1). After the sample was applied, the column was flushed with 400 ml of the equilibrating buffer. It was then eluted with a linear gradient composed of 400 ml of 10 mM Tris-HCl buffer containing 0.25% deoxycholate and 400 ml of buffer B. The fractions containing the predominant cytochrome b_5 peak were combined, concentrated to 10 ml by ultrafiltration on PM-10 membrane, and dialyzed against 0.01 M potassium phosphate buffer (pH 7.4) containing 0.25% Triton N 01 and 25% glycerol (buffer C). The dialyzed solution was applied to a column (1.5 × 30 cm) of DEAE-cellulose previously equilibrated with buffer C. The column was washed with 300 ml of buffer C, and cytochrome b_5 was eluted with the same buffer containing 0.25 M sodium thiocyanate. The hemoprotein solution was again concentrated to 5 ml and dialyzed against 10 mM Tris-HCl buffer (pH 8.15).

Step 4. Gel Filtration on Sephadex G-100. A 2-ml aliquot of the cytochrome b_5 preparation (2.3 μM) was passed through a Sephadex G-100 column (2.5 × 100 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.15). Fractions of 4 ml were collected at a flow rate of 8 ml/hr. The effluent was assayed for protein and cytochrome b_5 content. Separate amino acid analysis was performed on the cytochrome b_5 containing fractions, and those with identical amino acid composition were combined.

Chymotryptic Hydrolysis and Fractionation of Chymotryptic Peptides. Heme-free bovine apocytochrome was prepared by treatment of the cytochrome with HCl–acetone as follows: to a 0.5-ml aliquot (0.3–0.5 μmol) of cytochrome b_5 in 0.01 M Tris-HCl, 10 ml of cold acetone containing 0.2% HCl (v/v) was added. After 10 min at 5°, the white precipitate of apocytochrome was collected by centrifugation, rapidly dried with a stream of nitrogen, and suspended in 0.5 ml of 0.1 M NH_4HCO_3 (pH 8.5). A 60-μl (5 nM) aliquot of freshly prepared chymotrypsin in 1 mM HCl was added. After 12 hr at 25° another 60-μl aliquot of chymotrypsin was added, and the hydrolysis was continued for 12 hr at 25°. The digest was then lyophilized and dissolved in 1 ml of 30% acetic acid. An initial fractionation of the digest was performed on a Dowex 50 (AG-50W-X2, 200–400 mesh, Bio-Rad) column, 0.9 × 60 cm at 40° at a flow rate of 20 ml/hr, using pyridine–acetate buffer as previously described (Ozols, 1970). Further fractionations were carried out, when necessary, by gel filtration on Sephadex G-100 (3 × 105 cm) in 50% acetic acid.

Tryptic Hydrolysis and Fractionation of Tryptic Peptides. To a 0.5-ml (0.3 μmol) aliquot of horse or pig cytochrome in 10 mM Tris-HCl, a 50-μl (5 nM) aliquot of freshly prepared trypsin in 1 mM HCl was added. After 12 hr at 25°, 10 ml of cold acetone containing 0.2% HCl (v/v) was added. After 10 min at 5°, the precipitate was collected by centrifugation and suspended in 0.5 ml of 0.1 M NH_4HCO_3 (pH 8.1). The HCl–acetone fraction contains free heme, N-terminal tryptic fragment, and several other peptides from the membranous segment of the cytochrome. The precipitate consists predominantly of the heme binding peptide of 80–90 residues. It was further hydrolyzed for 12 hr at 25° by the addition of another 5 mM aliquot of trypsin. The digest was chromatographed on a Dowex 1 (Ag-1W-X2, 200–400 mesh) column (0.9 × 60 cm) at 40° using morpholine–picoline and pyridine–acetate buffers as previously described (Ozols, 1972). The acid–acetone fraction was evaporated to dryness, dissolved in 1.0 ml of 50% acetic acid, and resolved on a column (3 × 105 cm) of Sephadex G-100 in 50% acetic acid. Peptides in effluent fractions were identified by ninhydrin reaction, and degraded by Edman procedure as outlined previously (Nobrega and Ozols, 1971).

Results

Isolation of Cytochrome b_5. The method described here was developed specifically to isolate homogeneous cytochrome b_5 from a variety of mammalian livers in quantities sufficient for studying structure and function of the protein.

In the present study, frozen liver tissue was used as the starting material. The tissue was homogenized with only 2 volumes of buffer. This facilitated centrifugation steps by decreasing the volume of material. Because of the possibility that the preparation might be contaminated with lysosomal cathepsins, the pH of the suspensions was maintained at 8.3 in order to minimize their proteolytic activity. For the same reason, sulfhydryl compounds were not added during the membrane solubilization steps. Although it has been reported that washing microsomes with solutions of high ionic strength results in the removal of large amounts of protein sedimenting initially in the microsomal fraction (Spatz and Strittmatter, 1971; Weihing *et al.*, 1972), this step was deleted in order to circumvent additional centrifugation steps. The treatment of the microsomal preparation with acetone was essential for successful extraction of cytochrome with detergents. Analysis for phospholipid in the preparations before and after acetone treatment demonstrated that 70% of phospholipids were removed in this step. Since polyols have been reported to stabilize microsomal cytochromes (Sato *et al.*, 1969; Fujita *et al.*, 1973), glycerol was added to the membrane preparation prior to removal of lipids with acetone. After elimination of acetone by centrifugation, the preparation was suspended immediately in buffer. In this step the preparation was freed of the remaining acetone as well as of some 20% of total protein without loss of cytochrome b_5.

Treatment of the partially defatted membrane preparation with a solution containing Triton X-100 and sodium deoxycholate released cytochrome b_5, cytochrome b_5 reductase, and

TABLE I: Yield of Cytochrome b_5 Derived from Various Livers.[a]

Liver Source	Amount of Cytochrome b_5 (μmol/10 kg of liver)		Yield (%)
	Step 1	Step 4	
Porcine	37	11	30
Bovine	26	8	30
Equine	28	8	28

[a] Ten kilograms of liver was used as starting material. Experimental details are given in the Materials and Methods.

cytochrome P-450 like proteins. In the development of the various steps, the concomitant isolation of cytochrome P-450 was also considered. Consequently, a special effort was made, when possible, to maintain conditions compatible with the stability of cytochrome P-450. A similar yield of bovine cytochrome b_5 was obtained when Triton X-100 and deoxycholate were replaced by 1.2% Triton N 01 in 0.1 M potassium phosphate (pH 7.4) and 25% glycerol. Fujita *et al.* (1973) report that extraction with Triton N 01 in phosphate buffer containing glycerol provides more suitable conditions for the solubilization of rat P-450 hemoproteins than Triton X-100. When the detergent extract of microsomes was passed through a DEAE-cellulose column, as described in the Experimental Section, cytochrome b_5 was retained on the column. A similar purification was obtained when Triton N 01–glycerol extract was passed through a DEAE-cellulose column previously equilibrated with 0.01 M potassium phosphate (pH 7.4) containing 25% glycerol and 0.25% Triton N 01. Cytochrome b_5 was always eluted when the column was washed with buffer containing 0.15–0.25 M sodium thiocyanate. Figure 1 shows the typical spectra of cytochrome b_5 preparation following DEAE-cellulose chromatography. The overall spectral features of the porcine and equine preparations were essentially identical with that of the bovine protein. The total amount of cytochrome b_5 from 10 kg of liver, recovered at this step, ranged from 26 to 37 μmol. Accurate estimation of cytochrome b_5 concentration prior to step 1 is difficult because these preparations contain several hemoproteins which interfere with cytochrome b_5 assay. Preparations obtained in step 1 contained inert protein, lipid material, and some CO-combining heme proteins. Cytochrome b_5 was separated from the major portion of the membrane proteins and phospholipids by gel filtration on Sephadex G-100, using an eluent consisting of sodium deoxycholate and thiocyanate. Since 20-l. bed-volume columns are not available commonly, gel filtration results using a 2.5 × 105 cm column are shown in Figure 2. More than 80% of the total protein, under these conditions, eluted in the void volume. Relatively small amounts of protein were present in the cytochrome and phospholipid fractions (Figure 2). Such elution profiles suggested that the majority of the acidic microsomal membrane proteins had molecular weights greater than that of cytochrome b_5. Final purification of cytochrome b_5 was achieved using DEAE chromatography and a gradient with increasing thiocyanate concentrations as described in the Experimental Section. Table I illustrates the yields of cytochrome b_5 obtained from livers of the species studied.

Purity and Properties of Cytochromes. Figure 3 illustrates that preparations from step 4 migrated as a single protein band when subjected to acrylamide gel electrophoresis in

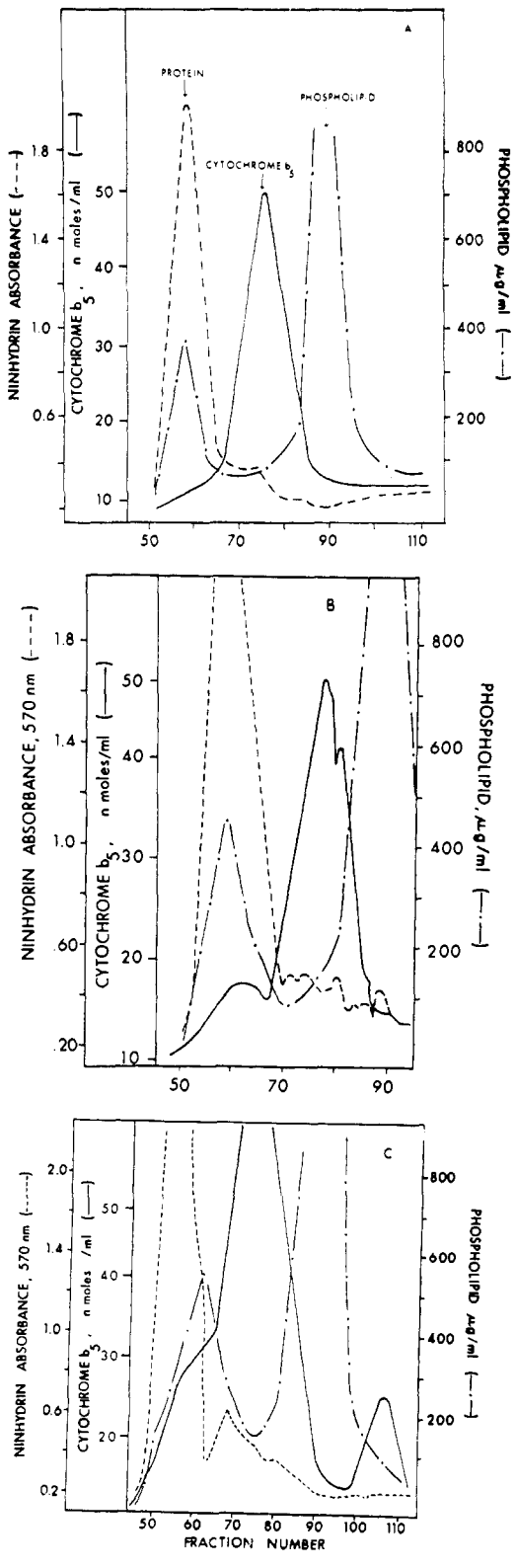

FIGURE 2: Separation of cytochrome b_5 from microsomal proteins and phospholipids on a Sephadex G-100 column (5 × 60 cm) in 10 mM Tris-HCl buffer (pH 8.15) containing 0.25 M sodium thiocyanate, 0.23% sodium deoxycholate, and 1 mM EDTA. Fractions of 5.6 ml were collected at a flow rate of 10 ml/hr and were analyzed for cytochrome b_5, for protein (by the ninhydrin method), and for total phosphorus: (A) 1.7 μmol of bovine cytochrome in 20 ml of the above buffer; 5-μl aliquots were analyzed by the ninhydrin method, and 50-μl aliquots for total phosphorus; (B) 1.8 μmol of equine preparation in 35 ml; 10-μl aliquots were analyzed for protein, and 50-μl aliquots for phosphorus; (C) 5 μmol of porcine preparation in 30 ml; 2.5-μl aliquots were analyzed for protein and 50-μl aliquots for phosphorus.

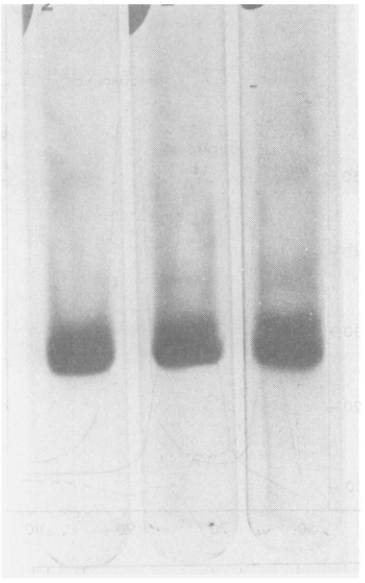

FIGURE 3: Sodium dodecyl sulfate electrophoresis of various cytochromes b_5. Standard 11% acrylamide gels were run as described by Neville (1971), and as indicated under Methods. Each gel contained 50 μg of equine (E), bovine (B), and porcine (P) cytochrome b_5. The direction of electrophoresis was from top to bottom.

sodium dodecyl sulfate. Even at relatively high concentrations, only traces of other proteins were seen either in the gels or on their upper surfaces. Occasionally preparations from step 4, upon overloading the gel, did exhibit several minor bands. In such cases the presence of inhomogeneity was also evident in the amino acid composition of the sample. Rechromatography of the preparation on a short DEAE column, using conditions described in step 3, removed these components.

The amino acid composition of cytochromes b_5 is given in Table II. The number of residues of each amino acid in the protein was calculated on the basis of 1 mol of heme bound per mole of protein (Ozols, 1970). The residues given are based on rounded-off averages for the duplicate determination after hydrolysis for 22, 48, and 92 hr. The composition reported for the "high molecular weight" preparations from human (Ozols, 1972) and rabbit (Spatz and Strittmatter, 1971) livers has also been included in Table II.

The absorption spectrum of the bovine cytochrome b_5 is shown in Figure 4. The spectral features of the equine and porcine cytochromes were essentially identical with the bovine preparation. No changes in absorbance occurred when either oxidized or dithionite reduced cytochrome preparations were treated with carbon monoxide, compatible with the complete absence of other heme proteins or partially denatured cytochrome b_5.

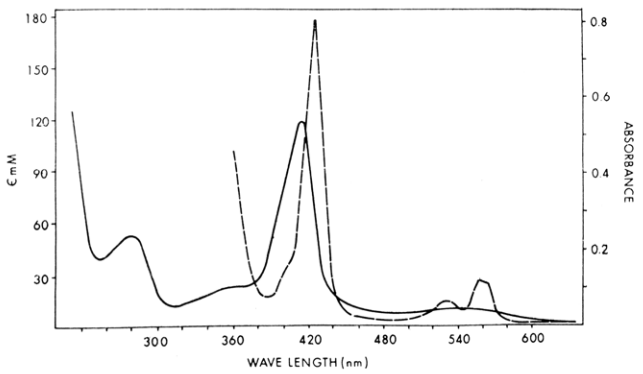

FIGURE 4: Absorption spectra of bovine cytochrome b_5, in 10 mM Tris buffer (pH 8.1) at 25°, containing 4.62 nM of cytochrome/ml: (———) oxidized form; (------) after reduction with solid sodium dithionite.

TABLE II: Amino Acid Composition of Various Cytochromes b_5.[a]

Amino Acid	Human[b]	Porcine[c]	Bovine[c]	Equine[c]	Rabbit[d]
	Amino Acid Residues/ Molecule of Cytochrome b_5				
Lysine	9	10	10	11	11
Histidine	8	7	6	7	7
Arginine	4	4	4	5	4
Aspartic acid	13	15	14	15	16
Threonine	10	11	11	11	10
Serine	10	13	13	10	10
Glutamic acid	22	22	21	21	15
Proline	5	4	7	5	5
Glycine	6	8	7	8	7
Alanine	11	9	9	12	10
Cystine (half)			0.3		
Valine	8	8	6	10	7
Methionine	4	2	1	2	3
Isoleucine	8	8	10	10	8
Leucine	12	11	13	12	15
Tyrosine	5	6	6	6	5
Phenylalanine	3	5	5	4	4
Tryptophan	3–4	3	4	3	4

[a] Amino acid compositions were determined on 20-, 48-, and 92-hr acid hydrolysates of the predominant preparation with the Spinco Model 120C amino acid analyzer (Ozols, 1970). Cytochrome b_5 concentration was determined from its absorption spectra as described in Methods. Entries represent the closest integral average values of at least two analyses. Values for amino acids released slowly, such as valine and isoleucine, have been taken from hydrolysates of maximum time. No corrections for destruction of serine or threonine are incorporated. Tryptophan was determined from 20-hr acid hydrolysate containing 3% thioglycolic acid, and half-cystine was determined as cysteic acid after performic acid oxidation. [b] Data from Ozols (1972). [c] Data from this study. [d] Data from Spatz and Strittmatter (1971).

NH_2-terminal analysis of preparations denatured by sodium dodecyl sulfate using the dansyl technique (Gray, 1972) were negative. A combination of this technique with a stepwise degradation by the Edman procedure also failed to reveal α-dansyl derivatives in these cytochrome preparations.

Hydrazinolysis of the cytochromes yielded 0.3–0.5 residue of aspartic acid per mole of protein.

Isolation and Characterization of NH_2-Terminal Peptides. In order to confirm the homogeneity of preparations and the results of NH_2- and COOH-terminal analysis, preparations were fragmented by trypsin or chymotrypsin. Resolution of the chymotryptic digest of bovine apocytochrome yielded peptides essentially accounting for the amino acid composition of the protein. Only the terminal segments are reported in this communication.

Peptide C-1 (Residues 1–12): (Asx,Glx,Glx,Glx,Ala,Ser)-Ser-Lys-Ala-Val-Lys-Tyr. This acidic peptide was isolated in good yield by Dowex 50 chromatography of chymotryptic digest of bovine apocytochrome (Ozols, 1970) and rechromatography on Sephadex G-100. The composition of this peptide and the failure of repeated Edman degradations to remove any amino acid residues suggested that it was the NH_2-terminal segment of the bovine cytochrome. Moreover, it overlapped peptide Ser-Lys-Ala-Val-Lys-Tyr-, the amino terminal segment, of the low molecular weight bovine cytochrome preparation (Ozols and Strittmatter, 1968). Hydrolysis of peptide C-1 (0.1 μmol) with 0.05 mg of trypsin followed by chromatography on Dowex 1 (Ozols, 1970) yields an acidic and a neutral peptide. The acidic peptide has an amino acid composition corresponding to the eight NH_2-terminal residues of peptide C-1; it again failed to react with the Edman reagent. The neutral peptide was subjected to three cycles of Edman degradation which established this peptide as the four COOH-terminal residues of peptide C-1.

Resolution of the HCl–acetone extract of tryptic digest of porcine cytochrome b_5 on Sephadex G-100 yielded an acidic hexapeptide with the following amino acid composition: Lys, 0.92; Asp, 1.05; Ser, 0.77; Glu, 2.30; Ala, 0.97. No NH_2-terminal amino acid was revealed by Edman degradation. Dilute acid hydrolysis of this peptide (0.03 N HCl, 14 hr, 107°) released 1.0 residue of aspartic acid. Two Edman degradation cycles of the reaction mixture removed one residue of alanine, and one residue of serine, in the aforementioned order. Since no other residue was lost, the sequence of the peptide is proposed as: Glx-Glx-Asp-Ala-Ser-Lys.

A peptide with an identical amino acid composition was also obtained by gel filtration of the HCl–acetone extract of tryptic digest of equine cytochrome b_5. Edman degradation of the peptide again showed an absence of a free NH_2 group. Since no other tryptic peptides have a blocked NH_2-terminus, and since it is homologous to the terminus of porcine cytochrome, it must be therefore the terminal segment of the equine cytochrome molecule.

Isolation and Characterization of COOH-Terminal Segments. Resolution of the water-soluble tryptic peptides of the equine apocytochrome by Dowex 1 chromatography (Ozols, 1972) yielded an acidic hexapeptide in addition to the expected peptides. It was eluted in pure form in the fractions following peptide T-1 peak (Figure 1, Ozols, 1972). Five cycles of Edman degradation established its amino acid sequence as: Ile-Tyr-Thr-Ala-Glu-Asp. Since basic residues are absent in this tryptic peptide, and since the terminal residue coincides with the COOH-terminus of the equine apocytochrome, it must correspond, therefore, to the COOH-terminal segment of the equine cytochrome b_5. The homologous peptide from the tryptic digest of the porcine protein was inadvertently lost during malfunctioning of the fraction collector. The homologous COOH-terminal fragment of this peptide was isolated from the chymotryptic digest of bovine apocytochrome. Dowex 50 chromatography (Ozols, 1970) of this hydrolysate revealed an acidic tetrapeptide, in the fractions preceding peptide C-9 peak. Three cycles of Edman degradation established its sequence as: Thr-Ser-Glu-Asp. Since its terminal residues were homologous to the COOH-terminal segment of equine apocytochrome, this tetrapeptide was positioned at the COOH-terminus of the bovine protein. The complete amino acid sequence of all tryptic and chymotryptic peptides from these cytochrome preparations will be described separately.

Discussion

In the past, elucidation of the entire covalent structure of cytochrome b_5 solubilized by detergents has been hindered by the limited quantity of homogeneous preparations available. This problem has been overcome in the present communication through the use of a large-scale procedure enabling us to process 10-kg quantities of liver in 1 day. Moreover, it is applicable to the isolation of cytochrome b_5 from a number of mammalian species.

Cytochrome b_5 is bound tenaciously to the endoplasmic reticulum and its solubilization requires proficient means of membrane isolation and fractionation. A cytochrome b_5 preparation containing about 80–90 residues, however, is readily released from endoplasmic membranes upon mild proteolysis of the intact microsomal preparations (Strittmatter, 1967; Kajihara and Hagihara, 1968; Ozols and Strittmatter, 1969; Nobrega *et al.*, 1969). Proteases from liver lysosomes can fragment the native form of cytochrome b_5 to yield the 80–90-residue fragment. Procedures for isolating the intact hemoprotein, therefore, require preparation of pure microsomal fractions by careful differential centrifugation of liver homogenates. Because of the small size of microsomal particles, the isolation of large quantities of microsomes is a formidable task, and the quantity of pure membranes generated by such procedures is not large.

Treatment of the partially defatted membrane preparation with nonionic detergents such as Triton X-100, at pH 8.1, or Triton N 01, at pH 7.4, in 25% glycerol, solubilizes several microsomal hemoproteins. Effective resolution of cytochrome b_5 from other proteins and phospholipids was obtained by chromatography on DEAE-cellulose and gel filtration. Cytochrome b_5 was separated from a major portion of the membrane proteins and phospholipids by gel filtration on Sephadex G-100, using eluent consisting of sodium deoxycholate and thiocyanate. As shown in Figure 2, more than 80% of the total protein, under these conditions, eluted in the void volume. Relatively small amounts of protein were present in the phospholipid fractions. Such elution profiles suggested that the majority of the acidic microsomal membrane proteins have molecular weights greater than that of cytochrome b_5.

The effectiveness of chaotropic ions, such as thiocyanate, to solubilize membrane preparations has been reported by Hatefi and Hanstein (1970). According to these investigators, the action of chaotropic ions is related to their ability to decrease the "ordered" structure of water and increase its lipophilicity. These changes in the structure and lipophilicty of water result in a weakening of the hydrophobic bonds of membrane preparations suspended in aqueous media and in the resolution of their component proteins and lipids (Hatefi and Hanstein, 1970).

Every partially purified cytochrome preparation, when chromatographed on DEAE-cellulose, using gradient elution, revealed minor cytochrome b_5 components. The reason for the presence of these multiple minor forms remains a question. There is no evidence from these and previous studies (Ozols, 1970) whether these multiple forms of cytochrome b_5 exist in the membrane *per se* or are artifactual, the products of partial proteolytic degradation.

By using the procedure described herein, 10 μmol of homogeneous cytochrome b_5 can be obtained from 10 kg of liver (Table I). In a single laboratory-scale procedure, one human liver (~1.2 kg) yields approximately 1 μmol of cytochrome (Ozols, 1972), comparable in molecular weight to the preparations described in the present communication.

TABLE III: Partial Amino Acid Sequences of Terminal Segments of Cytochromes b_5.

Species		Ref
	NH_2-Terminus	
Human	(Asx,Glx)Glu-Glu-Ala-Ser- Asp-Glu-Ala-Val-Lys-Tyr	*a*
Bovine	(Asx,Glx,Glx, Glx,Ala, Ser) Ser- Lys-Ala-Val-Lys-Tyr	*b*
Porcine	Glx-Glx-Asp-Ala-Ser- Lys	*b*
Equine	(Asx,Glx,Glx) Ala-Ser- Lys	*b*
Rabbit	(Glx, Ala,Ala) Ser-Asp-Lys-Asp-Val-Lys-Tyr	*c*
	COOH-Terminus	
Human	Leu-Tyr-Met-Ala-Glu-Asp-COOH	*d*
Bovine	Thr- Ser-Glu-Asp-COOH	*b*
Equine	Ile- Tyr-Thr- Ala-Glu-Asp-COOH	*b*

[a] Ozols (1972). [b] This paper. [c] Ozols (1970). [d] Unpublished observation.

When microsomes are solubilized by proteolytic means, somewhat larger quantities of cytochrome b_5 are usually obtained. All of these cytochrome preparations, however, contain 50–60 residues less than the detergent-solubilized preparations. Indeed, when hepatic microsomes of man, monkey, pig, and chicken are solubilized with pancreatic lipase followed by trypsin, about 0.2–2 μmol of homogeneous cytochrome b_5, having molecular weight of 11,000 daltons, can be obtained from 1 kg of liver tissue (Strittmatter, 1967; Nobrega *et al.*, 1969). High yields of this protein can also be obtained from calf liver microsomes by using pancreatic lipase, containing steapsins, as the solubilizing agent (Strittmatter, 1967).

Of all the livers investigated heretofore, rabbit liver appears to be the source of cytochrome b_5 which gives the highest yield. In earlier studies, in which pancreatic lipase was used to solubilize microsomes, 8 μmol of cytochrome was obtained from 1.3 kg of liver (Ozols, 1970). This preparation consisted of 97 residues. Somewhat higher yields of this protein were obtained from rabbit livers by Kajihara and Hagihara (1968) when trypsin was used to disrupt the microsomes. Their preparation, however, which was obtained in a crystalline form, contained fewer amino acid residues than the lipase solubilized product.

Table II compares the amino acid composition of the various cytochrome b_5's. In spite of the diversity of species from which the preparations were obtained, there was remarkable conservation of acidic, basic, and hydrophobic residue content. The acidic residues appear to be the dominating set of amino acids. All preparations show a ratio of acid to basic amino acids of about 1.7. Presumably, because of this property, cytochrome b_5 preparations are effectively separated from the basic membrane proteins using DEAE-cellulose chromatography.

The nature of the differences in the total residue content of

TABLE IV: Amino Acid Composition of Polar and Membranous Segment of Various Cytochromes b_5.[a]

	Polar				Membranous			
Amino Acid	Human[b]	Porcine[b]	Bovine[c]	Rabbit[d]	Human[e]	Porcine[f]	Bovine[f]	Rabbit[g]
Lysine	7	6	9	10	2	4	1	1
Histidine	7	6	5	7	1	1	1	
Arginine	3	3	3	3	1	1	1	1
Aspartic acid	9	9	9	10	4	6	5	6
Threonine	6	6	7	7	4	5	4	3
Serine	5	4	8	7	5	9	5	3
Glutamic acid	14	13	14	14	8	9	7	1
Proline	3	2	3	3	2	2	4	2
Glycine	6	6	6	6		2	1	1
Alanine	4	4	4	5	7	5	5	5
Valine	4	4	4	4	4	4	2	3
Methionine	1			1	3	2	1	2
Isoleucine	4	4	5	4	4	4	5	4
Leucine	7	8	8	9	5	3	5	6
Tyrosine	3	3	4	3	2	3	2	2
Phenylalanine	3	3	3	3		2	2	1
Tryptophan	1	1	1	1	2–3	2	3–4	2–3

[a] Composition of the polar segment is from the amino acid sequence. Values for the "membranous" segment represent the difference in composition between the cytochrome b_5 obtained by detergents (Table II) and by proteolytic action (polar). [b] Data from Nobrega and Ozols (1971). [c] From Ozols and Strittmatter (1969). [d] From Ozols (1970). [e] From Ozols (1972). [f] From this study. [g] From Spatz and Strittmatter (1971).

TABLE V: Distribution of Amino Acid Groups in Various Cytochrome b_5 Preparations.

		% of Each Group of Amino Acids[a]			
Preparation	Total[a] Residues	Acidic Asx, Glx	Basic Lys, His, Arg	Aromatic Tyr, Phe, Trp	Hydrophobic Val, Met, Leu, Ile, Phe, Trp
Detergent	145	24	15	9	21
Polar[b]	90	21	19	8	22
Membranous	53	20	7	11	34

[a] Values represent the averages of amino acid composition of preparations listed in Tables II and III. [b] Values from Dayhoff (1972).

the various preparations is not understood despite the observation that the NH_2- and COOH-termini are very similar (Table III). Some understanding of the differences may become evident after the covalent structure of the entire molecule is established. Table IV demonstrates the composition of the catalytically active polar moiety and the composition of the segment(s) which is presumably interacting with the membrane. The compositions of membranous segments for bovine and porcine proteins were obtained by subtracting the composition of the polar moiety from the composition of the preparation obtained by detergent action.

A survey of the distribution of the amino acid groups in the polar and membranous moieties of four cytochrome b_5 preparations is presented in Table V. The values for detergent and membranous preparations represented the averages of amino acid compositions of the four preparations listed in Tables II and IV. The values for the polar segment represent averages from a somewhat larger sample of the cytochrome b_5 family (Dayhoff, 1972). When amino acid groups of polar and membranous segments are compared, a decrease in basic and an increase in hydrophobic residues in the membranous segment are apparent. Such a distribution of amino groups is not surprising: the unique character of the membranous segment, which is buried in the hydrocarbon interior of the lipid bilayer, requires fewer polar groups for thermodynamic reasons.

The high content of Glx and Asx residues in the membranous segment of three species is of interest and raises a question about their function and orientation in the membrane. It is tempting to speculate that the acidic segments of the membranous peptide are involved in electrostatic interactions with the polar groups of the phospholipids. If this were so, one would predict that cytochrome b_5 binding to membrane involves both hydrophobic and electrostatic associations between nonpolar and polar segments of the polypeptide chain and the membrane. While such data allow some speculation on the nature of membrane proteins, the precise distribution of amino acid residues in the various segments of an amphipathic protein will be known after the entire covalent structure is established.

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Concerning the Quaternary Structure of Ascorbate Oxidase†

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ABSTRACT: When exposed to either sodium dodecyl sulfate or guanidinium chloride, ascorbate oxidase (molecular weight 140,000, 8–10 copper atoms) dissociates into two copper-free subunits, each of about half the molecular weight of the native enzyme. Removal of the denaturant results in aggregation of the subunits and it has not been found possible to restore the copper and activity. All evidence indicates that the two subunits of molecular weight 65,000, comprising the native enzyme, are identical. Treatment of the native enzyme with sodium dodecyl sulfate and either 2-mercaptoethanol or 2-mercaptoethylamine results in two new bands on sodium dodecyl sulfate electrophoresis. The components corresponding to these bands have molecular weights of 38,000 (A chain) and 28,000 (B chain). The same two components are obtained when disulfides of the native enzyme are cleaved with cyanide. It is proposed, therefore, that the native enzyme is a tetramer composed of two A chains and two B chains. Each AB pair is cross-linked by one or two disulfide bonds. The two resulting subunits are held together by noncovalent forces. A comparison of the spectral properties of ascorbate oxidase and *Polyporus* laccase indicates that both enzymes contain the same three types of cupric copper, but that ascorbate oxidase contains twice as much of both the type 1 and the electron spin resonance nondetectable cupric copper per mole of protein. This premise, plus the similiarity of the reactions catalyzed by the two enzymes, suggests that ascorbate oxidase has two "laccase-type" active sites per molecule of molecular weight 140,000. This hypothesis is consistent with the proposed quaternary structure of ascorbate oxidase, since each subunit can contain one active site.

Ascorbate oxidase (EC 1.10.3.3, 1-ascorbate:O_2 oxidoreductase) belongs to the group of enzymes usually referred to as the "blue" copper oxidases. The two other members of this group are laccase and ceruloplasmin. All these enzymes possess very similar spectral properties related to copper, and a recent review of the state of copper in these enzymes has appeared (Malkin and Malmström, 1970). *Polyporus* laccase remains the best understood member of the group with respect to the types of copper found in the "blue" oxidases.

Ascorbate oxidase has a molecular weight of 140,000 and a copper content of 8–10 atoms per molecule (Lee and Dawson, 1973a). The native enzyme has no detectable sulfhydryl groups, but on treatment with a suitable denaturant 10 sulfhydryl groups and four disulfide bonds are exposed (Stark and Dawson, 1962). When titrated to pH 11, ultracentrifuge experiments indicate a decrease in molecular weight to about half that of the native enzyme (Clark *et al.*, 1966), suggesting that ascorbate oxidase possesses a quaternary structure.

There appears to be a proportionality between copper content and molecular weight of the "blue" copper oxidases. The results of this investigation provide an explanation for this observation and also the many other similarities of these enzymes. Failure to appreciate the instability of disulfide bonds at high pH and the use of average molecular weights of apparently heterogeneous protein samples complicate the interpretation of earlier work on the quaternary structure of *Polyporus* laccase (Butzow, 1968) and ceruloplasmin (Poillon and Bearn, 1966).

Experimental Section

Materials. Ascorbate oxidase was prepared from zucchini squash (*Cucurbita pepo medullosa*) as previously described (Lee and Dawson, 1973a), and was homogeneous by the criteria of disc electrophoresis at pH 8.0 and 9.5 at gel concentrations of 7.5 and 5.0%.

Protein was determined by the Lowry method (Lowry *et al.*, 1951; Lee and Dawson, 1973a). Copper determinations were done according to Stark and Dawson (1958). Enzymatic activity was measured at 25° under concentration and buffer conditions previously described (Dawson and Magee, 1957), in a reaction volume of 1.5 ml using a Clark oxygen electrode on a Gilson Model KM Oxygraph.

All aqueous solutions were prepared using deionized water. Guanidinium chloride was obtained from Fisher and was recrystallized twice from absolute ethanol–benzene (2:1 v/v). Urea was Mallinckrodt reagent grade. It was recrystallized from 95% ethanol. A 10 M aqueous urea solution was prepared and deionized with Amberlite MB-3 just before use. Sodium dodecyl sulfate was obtained from Matheson Coleman and Bell. Bio-Gel A-5M 100–200 mesh was supplied by Bio-Rad Labs. All other chemical were of the highest purity available.

Methods. Electrophoresis. Disc electrophoresis at pH 9.5 was carried out according to Davis (1964), except that a sample gel was not used and, in some cases, no stacking gel was used. Electrophoresis at pH 8.0 was performed using a buffer system described by Williams and Reisfeld (1964).

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