Dissociation and Reconstitution of Human Ferroxidase II†

Lee F. Lykins, Christopher W. Akey, Ellen G. Christian, George E. Duval, and Richard W. Topham*

ABSTRACT: The ferroxidase II protein from human serum is large and structurally complex. It possesses protein-bound lipid and copper components which are essential for the maintenance of its catalytic activity. Treatment of ferroxidase II with 8 M urea, 6 M guanidine hydrochloride, or 6 M guanidine hydrochloride and alkylation does not result in the dissociation of the enzyme into subunits. However, treatment with sodium dodecyl sulfate results in the dissociation of ferroxidase II into two nonidentical subunits, designated S-I and S-II. S-I contains little phospholipid, cholesterol, or copper and has a molecular weight of $3.8–3.9 \times 10^5$. In contrast, S-II contains bound phospholipid, cholesterol, and copper and has a molecular weight of $2.2–2.4 \times 10^5$. The lipid composition of S-II is identical with the native enzyme. Sodium dodecyl sulfate-free S-I exhibits no ferroxidase activity. Immediately following removal of sodium dodecyl sulfate, S-II exhibits ferroxidase activity but S-II rapidly loses its activity in the absence of S-I. The separated subunits spontaneously reassociate upon removal of the sodium dodecyl sulfate to yield a fully active enzyme which chemically appears identical with native ferroxidase II. Furthermore, the reconstituted enzyme is stable. Both native and reconstituted ferroxidase II may be stored at 4 °C for 6 weeks without any loss in activity. This suggests that S-II, the copper and lipid-containing subunit, is the catalytic subunit and that S-I is essential for the stabilization of the enzymic activity of S-II. These results provide insight into the molecular structure and chemical composition of ferroxidase II and suggest that the complete native structure of ferroxidase II is required for the maintenance of its functional integrity.

The purification and partial characterization of a nonceruloplasmin ferroxidase (ferroxidase II) from whole human sera and the Cohn IV-1 fraction of human plasma have been previously reported (Topham and Frieden, 1970; Sung and Topham, 1973; Topham and Johnson, 1974; Topham et al., 1975). Kinetic studies (Topham and Johnson, 1974) have shown that ferroxidase II would be capable of functioning as an alternative for ferroxidase I (ceruloplasmin) in human serum and as the major ferroxidase in sera in species that contain low ferroxidase I levels. Copper, phospholipids, and cholesterol remain tightly bound to the ferroxidase II protein purified from human serum. Recent studies (Sung and Topham, 1973; Topham et al., 1975) have shown that the copper and phospholipids tightly bound to the ferroxidase II protein are essential for the maintenance of enzymic activity. The present paper describes the selective dissociation of ferroxidase II into two nonidentical subunits, the chemical characterization of these subunits, and the reconstitution of enzymically active ferroxidase II from these component subunits. These studies provide considerable insight into the chemical composition and molecular structure of this interesting and complex serum enzyme.

Experimental Procedure

Materials

Ferroxidase II. Highly purified ferroxidase II was obtained by a slight modification of the method previously described (Topham and Frieden, 1970). It was necessary to pass partially purified ferroxidase II over an Agarose A-50m column two successive times rather than once to obtain a preparation which yielded a single protein band on polyacrylamide gel electrophoresis. Following purification, the ferroxidase II preparations were stored at 4 °C in the presence of 0.02% sodium azide to prevent bacterial growth. No loss of enzymic activity was observed for periods of 6 weeks of storage.

Ferrous Iron. Crystalline ferrous ammonium sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) was dissolved in deionized, glass-distilled water to a concentration of 4×10^{-4} M and used as substrate.

Apotransferrin. A 2.0% (w/v) solution of iron-free transferrin (apotransferrin, Behring Diagnostics, Sommerville, N.J.) was prepared in deionized, glass-distilled water and extensively dialyzed as previously recommended (Johnson et al., 1970).

Buffers. Sodium acetate ($Na_2C_2H_3O_2 \cdot 3H_2O$) was dissolved in deionized, glass-distilled water to the desired concentration and the pH adjusted with acetic acid.

Lipids. High purity neutral and phospholipid standards for thin-layer chromatography were purchased from Applied Science Laboratories, Inc., State College, Pa.

Dissociation and Alkylation Reagents. Sodium dodecyl sulfate, urea, guanidine hydrochloride, 2-mercaptoethanol, and iodoacetate were purchased from Sigma Chemical Co., St. Louis, Mo.

Chromatographic Materials. DEAE[1]-Sephadex A-50 and Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) were swollen and equilibrated with 0.05 M acetate buffer, pH 5.5. Agarose A-5m and A-50m (Bio-Rad Laboratories, Richmond, Calif.) were obtained in a fully hydrated form and were equilibrated with 0.05 M acetate buffer, pH 5.5. Dowex 1-X2 (Bio-Rad Laboratories, Richmond, Calif.) was equilibrated with 0.05 M acetate buffer, pH 5.5, prior to use. Thin-layer chromatographic plates precoated with Silica Gel H were purchased from Analtech, Newark, Del.

† From the Department of Chemistry, University of Richmond, Richmond, Virginia 23173. *Received August 24, 1976.* This work was supported by a grant-in-aid from Research Corporation and a faculty research grant from the University of Richmond.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

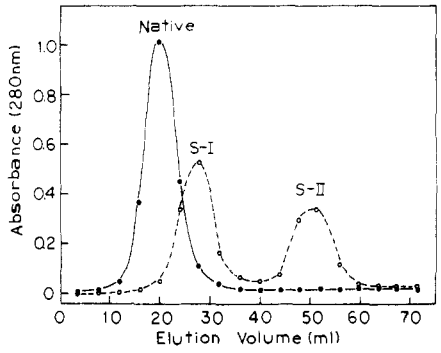

FIGURE 1: Treatment of ferroxidase II with sodium dodecyl sulfate. Treatment with sodium dodecyl sulfate was performed by incubating 4 mL of ferroxidase II (20 mg protein) in 1% sodium dodecyl sulfate for 2 h at 30 °C. Following incubation, a 2-mL sample was applied to a 1.6 × 35 cm column of Sephadex G-200 equilibrated with 1% sodium dodecyl sulfate. Protein was eluted from the column with 0.05 M acetate buffer, pH 5.5, containing 1% sodium dodecyl sulfate. Fractions of 4 mL were collected and monitored for protein at 280 nm. The fractions containing S-I were combined and concentrated to 2 mL using an Amicon Model 12 ultrafiltration cell equipped with a Diaflo PM-10 membrane. The fractions containing S-II were treated in an identical manner.

Methods

Enzymic Assays. Ferroxidase activities were measured spectrophotometrically monitoring Fe(III) transferrin formation as the absorbance change at 460 nm or polarographically monitoring the consumption of oxygen during the ferroxidase-catalyzed reaction. The spectrophotometric and polarographic assays of ferroxidase activity have been described in detail and validated in numerous previous reports (Osaki et al., 1966; Osaki, 1966; Johnson et al., 1967; Topham and Frieden, 1970; Topham and Johnson, 1974).

Spectrophotometric Measurements. All ferroxidase assays and other spectrophotometric measurements were performed on a Beckman Acta Model C-III spectrophotometer equipped with a 0.1 absorbance scale. Protein elution from columns was monitored at 280 nm with an Altex Model 150 absorbance monitor or an ISCO Model UA-5 absorbance monitor equipped with a Type 6 optical unit. Polyacrylamide gels were scanned at 280 nm with the ISCO Model 1310 gel scanning attachment for the Model UA-5 absorbance monitor.

pH Measurements. All pH determinations were made with a Fisher Accumet Model 210 pH meter equipped with a Corning Series 500 combination pH electrode.

Protein Concentrations. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Quantitative Lipid Analyses. Cholesterol analyses were performed according to the method of Zak (1957). Phospholipid analyses were carried out using the method of Bartlett (1959) and an average molecular weight of phospholipid (775 g/mol) was used for the calculation of phospholipid contents.

Qualitative Lipid Analyses. Samples of native ferroxidase II and each subunit were extracted with 20 volumes of chloroform–methanol (2:1, v/v). The resulting lipid extracts were washed using the method of Folch et al. (1957), dried under a stream of nitrogen, and redissolved in a small volume of chloroform–methanol (2:1, v/v). The individual neutral and phospholipids contained in these extracts were identified by separation on thin-layer chromatographic plates coated with Silica Gel H. The plates were activated at least 1 h at 110 °C before chromatography. Chloroform–methanol–acetic acid–water (25:15:4:2, v/v/v/v) was the developing solvent used for the separation of phospholipids. Hexane–diethyl ether–acetic acid (80:20:1, v/v/v) was the developing solvent used for the separation of neutral lipids.

Copper Determinations. Copper analyses were performed by the method of Wharton and Rader (1970).

Polyacrylamide Gel Electrophoresis. Uniform bore, straight glass tubes were filled with a 4% polyacrylamide separating gel to a height of 80 or 120 mm. To ascertain the homogeneity of protein samples, a basic buffer system (0.188 M Tris–glycine, pH 8.8) was employed for the electrophoresis. To estimate the molecular weight of the subunits of ferroxidase II, a slightly acidic buffer system (0.205 M Tris–acetate, pH 6.6) containing 0.1% sodium dodecyl sulfate was utilized for the electrophoresis. Sufficient solid sucrose was added to the samples to obtain a 5% sucrose solution. Twenty-five microliters of these preparations was applied to the top of the separating gels. With the basic buffer system, the sample was run into the gel at 1 mA/tube and the electrophoresis completed at 2–3 mA/tube. With the sodium dodecyl sulfate–acidic buffer system, the sample was run into the gel at 4 mA/tube and the electrophoresis completed at 6–8 mA/tube. After the electrophoresis, the gels were fixed with 12% Cl_3CCOOH, washed, and scanned for protein at 280 nm with an ISCO Model 1310 gel scanner. The gels were subsequently stained with Coomassie blue. High purity thyroglobulin, urease, pyruvate kinase, and aldolase (Sigma Chemical Co., St. Louis, Mo.) were utilized in the sodium dodecyl sulfate–buffer system for constructing the standard curve for the estimation of the molecular weight of the subunits of ferroxidase II.

Results

Preparation of Subunits of Ferroxidase II

Dissociation of Ferroxidase II. Preliminary estimates (Topham and Frieden, 1970) of the molecular weight of ferroxidase II indicated a value in the vicinity of 800 000. A protein possessing a molecular weight of this magnitude would most probably be multichained. Thus, attempts to promote dissociation of ferroxidase II into smaller subunits were initiated with the hope of providing insight into the molecular structure of this complex serum enzyme.

Purified ferroxidase II samples were treated with 8 M urea, 6 M guanidine hydrochloride, and a combination of 6 M guanidine hydrochloride and mercaptoethanol, followed by alkylation with iodoacetic acid. Two milliliters of the treated ferroxidase II samples were passed through a column of Sephadex G-200 equilibrated with either 8 M urea or 6 M guanidine hydrochloride. No dissociation of ferroxidase II into subunits was observed with any of these classical protein dissociating reagents. Ferroxidase II has been shown to be a lipoprotein (Sung and Topham, 1973; Topham et al., 1975). Thus, it was felt that treatment with detergents might promote the dissociation of ferroxidase II into subunits. Accordingly, dissociation was attempted by treatment of purified ferroxidase II with 1% sodium dodecyl sulfate, an ionic detergent. Passage of 2 mL of the sodium dodecyl sulfate-treated sample through a Sephadex G-200 column equilibrated with sodium dodecyl sulfate resulted in the dissociation of ferroxidase II into two nonidentical subunits (Figure 1). The larger molecular weight subunit was designated S-I, and the smaller, S-II. Treatment with a higher percentage (up to 5%) of sodium dodecyl sulfate did not result in the further dissociation of ferroxidase but yielded the same elution pattern as 1% treated samples upon gel filtration.

Homogeneity of the Subunits. To establish that each of the

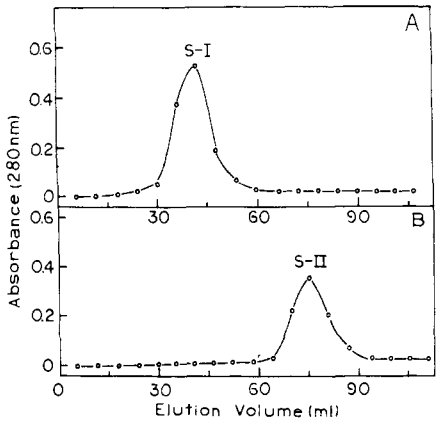

FIGURE 2: Gel filtration of the separated subunits. The concentrated samples of S-I and S-II obtained by the procedure described in Figure 1 were individually applied to a 1.6 × 60 cm column of Sephadex G-200. Protein was eluted from these columns with 0.05 M acetate buffer, pH 5.5. Fractions of 4 mL were collected and monitored for protein at 280 nm.

separated subunits represented a single homogeneous protein band, samples of S-I and S-II, concentrated by ultrafiltration, were individually rechromatographed on a longer column of Sephadex and were subjected to polyacrylamide gel electrophoresis. A single symmetrical band of protein was eluted from the longer Sephadex column with each subunit sample (Figure 2). A single band of protein was also observed for each subunit and the native enzyme when polyacrylamide gels were scanned following electrophoresis (Figure 3). Both subunits migrated significantly faster than native ferroxidase II. It was necessary to use a large pore (4%) gel to get native ferroxidase II to migrate during polyacrylamide gel electrophoresis.

Large Scale Preparations of the Subunits. For physical and chemical characterization studies, large quantities of S-I and S-II were obtained by treating 30 mL of purified ferroxidase II (10–20 mg of protein/mL) with 1% sodium dodecyl sulfate and passing the entire 30 mL of sodium dodecyl sulfate-treated ferroxidase II through a preparative size column (4.5 × 50 cm) of Sephadex G-200 equilibrated with 1% sodium dodecyl sulfate. The large samples of ferroxidase II were completely dissociated into the component subunits (S-I and S-II) and the subunits were well resolved by the preparative Sephadex column.

Physical and Chemical Characterization of the Subunits

Molecular Weights of the Subunits. The molecular weights of native ferroxidase II, S-I, and S-II were estimated by gel filtration on a column of Sepharose 6B calibrated with standard proteins of known molecular weight. The ratios of elution volume to void volume (V_e/V_0) for native ferroxidase II, S-I, and S-II were compared with those obtained with the standard proteins. These results indicated that native ferroxidase II, S-I, and S-II had molecular weights respectively of 6.2×10^5, 3.9×10^5, and 2.4×10^5.

The molecular weights of the subunits were independently estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis utilizing the same standard proteins. The relative mobilities obtained for S-I and S-II were compared with those obtained for the subunits of the standard proteins. These results indicated that S-I and S-II had molecular weights respectively of 3.8×10^5 and 2.2×10^5.

The molecular weights for S-I and S-II estimated by gel filtration and sodium dodecyl sulfate–polyacrylamide gel electrophoresis were in good agreement.

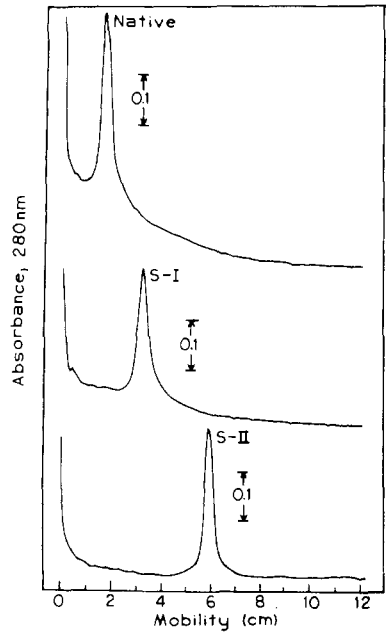

FIGURE 3: Polyacrylamide gel electrophoresis of native ferroxidase II, S-I, and S-II. Polyacrylamide gel electrophoresis was performed as described in Methods. Twenty-five microliters (ca. 40 μg) of samples of native ferroxidase II, S-I, and S-II were applied to the top of the separating gels.

Analysis of the Lipid Contents, Copper Contents, and Enzymic Activity of the Subunits. Lipids, primarily in the form of phosphatidylcholine and cholesterol, have been shown to remain tightly bound to the ferroxidase II protein following purification (Sung and Topham, 1973). Digestion of the bound phospholipids with phospholipase C or A resulted in loss of ferroxidase activity which paralleled the extent of hydrolysis of the bound phospholipids (Sung and Topham, 1973). Recent studies (Topham et al., 1975) indicated that the copper atoms bound to ferroxidase II were essential for enzymic activity and that intact lipid components were necessary for the binding of the copper to the protein. Thus, each of the subunits obtained from the sodium dodecyl sulfate-promoted dissociation of ferroxidase II was examined for its lipid content and composition, its copper content, and the possibility that it might possess enzymic activity in the absence of the other subunit. One of the subunits, S-II, contained virtually all the phospholipid and copper and most of the cholesterol (Table I). The phospholipid and copper contents of S-II per unit weight of protein are approximately double those of the native enzyme. This is reasonable since S-I, the subunit containing little phospholipid or copper, accounts for approximately half the protein of the native enzyme.

The individual phospholipids and neutral lipids associated with each subunit were isolated by chloroform–methanol extraction and identified by thin-layer chromatography. The phospholipid components bound to the native enzyme and S-II were identical (Figure 4A). The major phospholipid bound to both was phosphatidylcholine with lesser amounts of lysophosphatidylcholine, sphingomyelin, and phosphatidylethanolamine. Thin-layer chromatographic analyses indicated that S-I contained little if any phospholipid compared with S-II. This finding is consistent with the quantitative results presented in Table I.

When similar thin-layer plates were developed in a solvent system suitable for the separation of neutral lipids, both subunits were found to contain neutral lipids (Figure 4B). The

TABLE I: Chemical Characterization of the Subunits of Ferroxidase II.[a]

Sample	Phospholipid/ Protein (mg/mg of protein)	Cholesterol/ Protein (mg/mg of protein)	Copper/ Protein (nmol/mg of protein)	Mol. Wt	Sp Act.[b]
Native	0.133	0.109	12.0	$6.0–7.0 \times 10^5$	160
Subunit I	0.023	0.056	1.0	$3.8–3.9 \times 10^5$	0
Subunit II	0.237	0.159	20.4	$2.2–2.4 \times 10^5$	292

[a] Phospholipid, cholesterol, copper, and protein contents and enzymic activities were determined as described in Methods. All values represent an average of at least three separate experiments. [b] In μmol of Fe(III) transferrin min^{-1} (mg of protein)$^{-1}$.

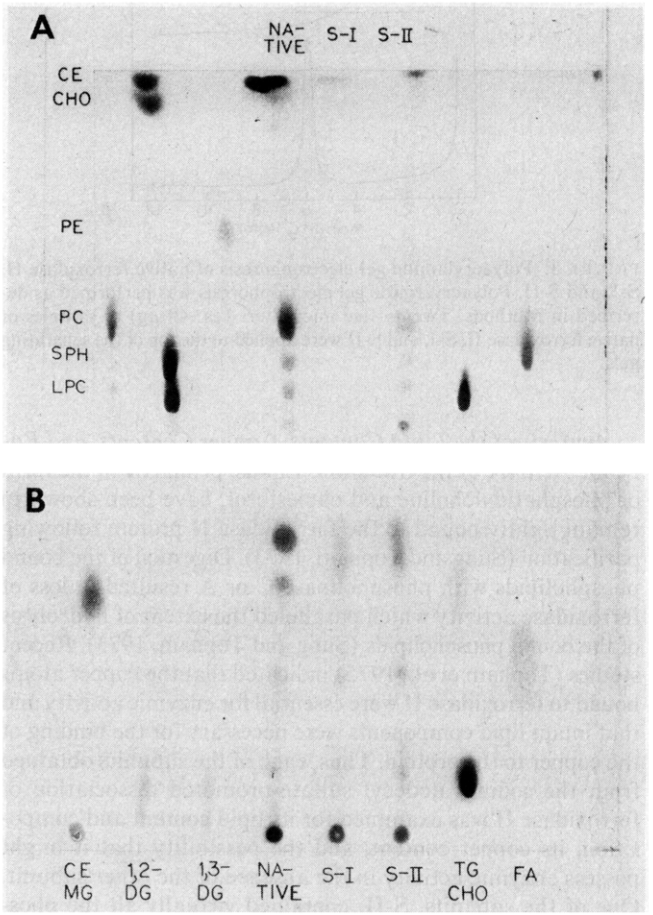

FIGURE 4: Identification of the lipid components associated with the subunits of ferroxidase II. The extraction of lipids from samples of ferroxidase II, S-I, and S-II and the thin-layer chromatographic analyses of the resulting extracts were performed as described in Methods. In A: LPC, lysophosphatidylcholine; SPH, sphingomelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CHO, cholesterol; CE, cholesterol ester. In B: CE, cholesterol ester; MG, monoglyceride; DG, diglyceride; TG, triglyceride; CHO, cholesterol; FA, fatty acid.

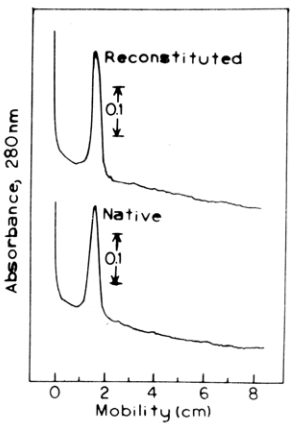

FIGURE 5: Electrophoretic comparison of native and reconstituted ferroxidase II. Polyacrylamide gel electrophoresis was performed as described in Methods. Twenty-five microliters (ca. 40 μg protein) of samples of native and reconstituted ferroxidase II were applied to the top of the separating gels.

neutral lipid compositions of the subunits were the same as the native enzyme and consisted of cholesterol, some cholesterol esters, free fatty acids, and di- and triglycerides. However, for comparable extracts, the spots for S-II were considerably more intense. This finding was also consistent with the quantitative results obtained for cholesterol (Table I).

Prior to testing each subunit for ferroxidase activity, bound sodium dodecyl sulfate was removed by a slight modification of the Dowex 1-X2 column chromatographic method of Weber and Kuter (1971). To remove sodium dodecyl sulfate from the individual subunits of ferroxidase II, it was not necessary to include urea in the samples or in the column eluting buffer. The buffer used was 0.05 M sodium acetate, pH 5.5, rather than the Tris–acetate, pH 7.8, employed by Weber and Kuter. Sodium dodecyl sulfate-free S-I exhibited no ferroxidase activity. However, immediately following removal of sodium dodecyl sulfate, S-II, the copper and lipid containing subunit, possessed ferroxidase activity. The specific activity of S-II was approximately double the specific activity of the native enzyme (Table I). This is reasonable since S-II accounts for approximately half of the protein of native ferroxidase II. However, S-II rapidly lost its activity. In 72 h, the specific activity of S-II had diminished by 80%. In contrast, native ferroxidase II remains fully active upon storage for 6 weeks.

Reconstitution of Ferroxidase II. Ferroxidase II was dissociated into subunits by treatment with 1% sodium dodecyl sulfate. The subunits (S-I and S-II) were separated by gel filtration on a column of Sephadex G-200 equilibrated with 1% sodium dodecyl sulfate. The separated subunits were recombined in equimolar amounts. Removal of sodium dodecyl sulfate from the recombined subunits and reconstitution of the subunits was achieved using the Dowex slurry procedure of Weber and Kuter (1971). Two modifications of this procedure were made. No urea was added to samples or buffers and the final dialysis was done with 0.05 M sodium acetate buffer, pH 5.5, rather than 0.05 M Tris–acetate, pH 7.4.

To verify that reconstitution had been achieved, native and reconstituted ferroxidase II were compared electrophoretically. The native and reconstituted samples both appeared as a single protein band and migrated identical distances in polyacrylamide gel electrophoresis (Figure 5).

The gel filtration elution patterns of native and reconstituted

TABLE II: Chemical Comparison of Native and Reconstituted Ferroxidase II.[a]

Sample	Phospholipid/ Protein (mg/mg of protein)	Cholesterol/ Protein (mg/mg of protein)	Copper/ Protein (nmol/mg of protein)	Sp Act.[b]
Native	0.150	0.110	12	160
Reconstituted	0.140	0.109	13	140

[a] Analyses of phospholipid, cholesterol, and copper contents and enzymic activities were performed as described in Methods. All values represent the average of at least three separate experiments. [b] In μmol of Fe(III) transferrin min^{-1} (mg of protein)$^{-1}$.

ferroxidase II were also compared. Both eluted from a Sephadex G-200 column as a single band of protein and the elution volume of the reconstituted protein was identical with native ferroxidase II (Figure 6). Furthermore, treatment of the reconstituted ferroxidase II with 1% sodium dodecyl sulfate resulted in its redissociation into nonidentical subunits. These subunits eluted from the Sephadex G-200 column with the same elution volumes as the subunits obtained by an identical treatment of native ferroxidase II (Figure 6).

The chemical compositions and enzymic activities of native and reconstituted ferroxidase II were also compared (Table II). The phospholipid, cholesterol, and copper contents of the reconstituted protein were all comparable to the native protein. The specific enzymic activity of the reconstituted ferroxidase II was 85% of that of native ferroxidase II. The reconstituted enzyme demonstrated a stability equal to that of the native ferroxidase II. No significant loss in enzymic activity was observed when the reconstituted ferroxidase II was stored for 6 weeks at 4 °C in 0.05 M sodium acetate buffer, pH 5.5, containing 0.02% sodium azide.

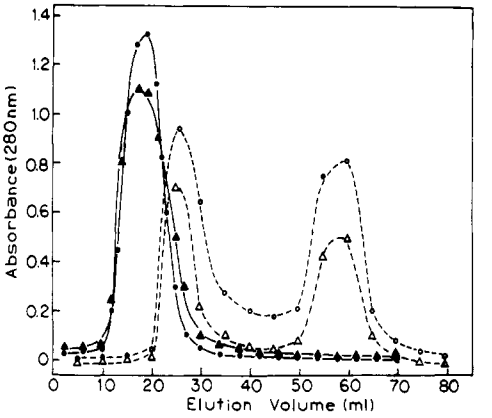

FIGURE 6: Gel filtration comparison of native and reconstituted ferroxidase II. (●—●) Native ferroxidase II; (▲—▲) reconstituted ferroxidase II; (○ - - - ○) sodium dodecyl sulfate-treated native ferroxidase II; (△ - - - △) sodium dodecyl sulfate-treated reconstituted ferroxidase II. Two-milliliter samples (10 mg of protein/mL) of native and reconstituted ferroxidase II were each applied to a column (1.6 × 40 cm) of Sephadex G-200. Protein was eluted from the column with 0.05 M acetate buffer, pH 5.5. The protein content of the column was continuously monitored at 280 nm. In the sodium dodecyl sulfate studies, 3-mL samples of ferroxidase II (8.9 mg of protein/mL) were treated with 1% sodium dodecyl sulfate and incubated for 2 h at 30 °C. Following incubation, a 2-mL sample was applied to a column (1.6 × 40 cm) of Sephadex G-200 equilibrated with 1% sodium dodecyl sulfate. Three-milliliter samples of reconstituted ferroxidase II (5.3 mg of protein/mL) were processed in an identical manner. The protein contents of the column eluents were continuously monitored at 280 nm.

Discussion

Ferroxidase II is a serum enzyme with an interesting and novel structure. It contains tightly integrated protein, lipid, and copper components (Sung and Topham, 1973; Topham et al., 1975). Furthermore, the association among these three components has been shown to be indispensable to the catalytic function of this enzyme (Topham et al., 1975). The studies described in this paper suggest that ferroxidase II is composed of two nonidentical subunits. The subunits obtained by sodium dodecyl sulfate treatment are large and may still be multichained. However, if multichained, treatment with larger concentrations of sodium dodecyl sulfate, urea, guanidine hydrochloride, or mercaptoethanol and alkylation does not result in the further dissociation of these subunits. The sum of the molecular weights of the subunits is approximately equal to the molecular weight of native ferroxidase II which suggests that native ferroxidase II is composed of one molecule of each subunit.

Only S-II, the smaller subunit, binds significant quantities of phospholipid and copper. This eliminates the possibility that S-II could be derived from the further dissociation of the larger subunit, S-I, which contains very little phospholipid or copper. Both subunits bind neutral lipids but S-II binds substantially more than S-I. The fact that both contain neutral lipids may suggest that neutral lipids play a role in the association of the two subunits in the native enzyme. Of the various reagents tried, only a detergent promoted the dissociation of ferroxidase II into subunits. This observation may also indicate that the bound lipid components play a role in the maintenance of the native structure of ferroxidase II. Following removal of sodium dodecyl sulfate, only S-II, the copper and lipid binding subunit, exhibited ferroxidase activity. This suggests that it is the catalytic subunit. This is reasonable since the copper and phospholipid components bound to native ferroxidase II must be present for the demonstration of enzymic activity (Topham et al., 1975). However, the ferroxidase activity of S-II rapidly diminishes in the absence of S-I. Both undissociated ferroxidase II and ferroxidase II reconstituted from its subunits are stable when stored for 6 weeks. These observations suggest that S-I, the noncatalytic subunit, stabilizes S-II, the catalytic subunit, in the native enzyme. Thus, S-I is essential for the maintenance of the functional integrity of ferroxidase II even though it exhibits no enzymic activity of its own.

In summary, these results provide considerable insight into the chemical composition and molecular structure of ferroxidase II from human serum. The purification, characterization, and comparison of ferroxidase II from the sera of other species are currently in progress.

References

Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 449.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol.*

Chem. 226, 497.
Johnson, D. A., Osaki, S., and Frieden, E. (1967), *Clin. Chem. 13,* 142.
Johnson, D. A., Osaki, S., and Frieden, E. (1970), *Clin. Chem. 16,* 65.
Lowry, O. H., Rosebrough, J. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Osaki, S. (1966), *J. Biol. Chem. 241,* 5053.
Osaki, S., Johnson, D. A., and Frieden, E. (1966), *J. Biol. Chem. 241,* 2746.
Sung, S. M., and Topham, R. W. (1973), *Biochem. Biophys. Res. Commun. 53,* 824.
Topham, R. W., and Frieden, E. (1970), *J. Biol. Chem. 245,* 6698.
Topham, R. W., and Johnson, D. A. (1974), *Arch. Biochem. Biophys. 160,* 647.
Topham, R. W., Sung, S. M., Morgan, F. G., Prince, W. D., and Jones, S. H. (1975), *Arch. Biochem. Biophys. 167,* 129.
Weber, K., and Kuter, D. J. (1971), *J. Biol. Chem. 246,* 4505.
Wharton, D. C., and Rader, M. (1970), *Anal. Biochem. 33,* 226.
Zak, B. (1957), *Am. J. Clin. Pathol. 27,* 583.

A Comparison of Human Prothrombin, Factor IX (Christmas Factor), Factor X (Stuart Factor), and Protein S†

Richard G. Di Scipio, Mark A. Hermodson, Stanley G. Yates, and Earl W. Davie*

ABSTRACT: Human prothrombin, factor IX, and factor X have been isolated in high yield and characterized as to their amino-terminal sequence, molecular weight, amino acid composition, and migration in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. An additional human plasma protein, called protein S, has also been purified and its properties have been compared with those of prothrombin, factor IX, and factor X. Prothrombin (mol wt 72 000), factor IX (mol wt 57 000), and protein S (mol wt 69 000) are single-chain glycoproteins, while factor X (mol wt 59 000) is a glycoprotein composed of two polypeptide chains held together by a disulfide bond(s). The amino-terminal sequence of the light chain of human factor X is homologous with prothrombin, factor IX, and protein S. The heavy chain of human factor X is slightly larger than the heavy chain of bovine factor X and differs from bovine factor X in its amino-terminal sequence.

The formation of fibrin during the coagulation process involves the participation of a number of plasma proteins which require vitamin K for their biosynthesis (Davie and Fujikawa, 1975). These include prothrombin, factor VII, factor IX, and factor X.[1] Vitamin K is required for carboxylation of a number of specific glutamic acid residues located in the amino-terminal end of these proteins (Stenflo et al., 1974; Magnusson et al., 1974; Nelsestuen et al., 1974). This leads to the formation of γ-carboxyglutamic acid (Gla) which participates in the binding of calcium and the interaction of these coagulation factors with phospholipid. Stenflo (1976) has recently purified and characterized a fifth plasma protein from bovine plasma containing γ-carboxyglutamic acid. The biological role of this protein, called protein C, however, is not known (Esmon et al., 1976; Kisiel et al., 1976).

The purification of human factor IX has been reported by Østerud and Flengsrud (1975), Andersson et al. (1975), and Rosenberg et al. (1975a). It is composed of a single polypeptide chain with an amino-terminal tyrosine (Fryklund et al., 1976). Human prothrombin has been well characterized (Shapiro and Waugh, 1966; Lanchantin et al., 1968; Kisiel and Hanahan, 1973; Downing et al., 1975), and major portions of its structure have been determined (Pirkle et al., 1973; Thompson et al., 1974; Walz and Seegers, 1974; Butkowski et al., 1976). Human factor X has been isolated and partially characterized by Aronson et al. (1969) and Rosenberg et al. (1975b). No physical-chemical characterization of the final preparation was made. Properties of the corresponding bovine coagulation factors, however, are well known (Fujikawa et al., 1974a), and the amino acid sequences for bovine prothrombin and bovine factor X have been completed (Magnusson et al., 1975; Enfield et al., 1975; Titani et al., 1975).

In the present communication, we report the isolation and characterization of human factor IX, factor X, and a new plasma protein of unknown function, called protein S.[2] A comparison of the properties of these three proteins with those of human prothrombin and the corresponding proteins from bovine plasma is also presented.

Experimental Section

Materials

Heparin sodium salt (grade I, 150 USP units/mg), soybean trypsin inhibitor (type I-S), imidazole (grade I), morpholinoethanesulfonic acid (Mes),[3] morpholinopropanesulfonic acid

† From the Departments of Biochemistry and Medical Genetics, University of Washington, Seattle, Washington 98195. *Received October 1, 1976.* This work was supported in part by Grants HL 16919-02 and GM 15253 from the National Institutes of Health.

[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

[2] The new protein was arbitrarily called protein S in reference to its isolation and characterization in Seattle. When its function is discovered, a more appropriate name can be assigned to this plasma protein.

[3] Abbreviations used are: Mes, morpholinoethanesulfonic acid; Mops, morpholinopropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Dip-F, diisopropyl fluorophosphate; $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.