LIPID COMPONENTS OF HUMAN FERROXIDASE-II*

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Cholesterol and phospholipids remain tightly associated with the ferroxidase-II protein from human serum following extensive purification. Purified ferroxidase-II preparations show a consistent ratio of protein, phospholipid, and cholesterol. Thin-layer chromatographic analyses indicate that phosphatidyl choline accounts for 70% of the bound phospholipid. Treatment of purified ferroxidase-II with phospholipase C or A results in a loss of ferroxidase activity which parallels the hydrolysis of phospholipid. A lipid-depleted form of ferroxidase-II can be prepared by gel-filtration following treatment with phospholipase C. However, hydrolysis, not removal, of the lipid is sufficient for the loss of ferroxidase activity. These studies indicate that the bound lipid components are essential to the maintainence of the catalytic activity of ferroxidase-II.

INTRODUCTION

The purification and partial characterization of a non-ceruloplasmin ferroxidase (ferroxidase-II) has recently been reported (1,2). Highly purified ferroxidase-II migrated in a manner similar to serum lipoproteins during immunoelectrophoresis and stained with oil red, a lipoprotein stain (1). This report describes the identification and quantitative analyses of the lipid components bound to ferroxidase-II and preliminary evidence which suggests that intact phospholipids are required for the maintainence of ferroxidase activity.

EXPERIMENTAL PROCEDURES

High purity ferroxidase-II preparations, which exhibited a single protein component on disc-gel and immunoelectrophoresis, were obtained by the method previously reported (1). Ferroxidase activities were measured spectrophotometrically following Fe(III)-transferrin formation at 460 nm as has been extensively described in numerous previous reports (1-5). Protein concentrations were determined by the method of Lowry et al. (6).

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TABLE I

Lipid Content of Ferroxidase-II

Enzyme Prep.	Specific Activity ($\frac{\mu MFe^{3+}\text{-}Tf^{*}}{\text{min./mg protein}}$)	Protein (mg/ml)	Phospholipid (mg/ml)	Cholesterol (mg/ml)	Protein / Phospholipid / Cholesterol
I	196	7.4	0.92	0.72	10 / 1.3 / 1
II	172	3.7	0.43	0.36	10 / 1.2 / 1
III	160	5.0	0.72	0.49	10 / 1.4 / 1
IV	187	4.7	0.60	0.47	10 / 1.3 / 1

*Tf = transferrin

TABLE II

Gel-filtration of Phospholipase C Treated Ferroxidase-II

Enzyme Prep.	Treatment	Phospholipid / Protein	Cholesterol / Protein	Specific Activity ($\frac{\mu MFe^{3+}\text{-}Tf^{*}/min.}{mg\ protein}$)
V	Incubated 2 hrs., No Phospholipase C	0.153	0.120	192
	Incubated 2 hrs., + Phospholipase C, followed by Gel-filtration	0.052	0.022	21

*Tf = transferrin

Cholesterol analyses were performed according to the method of Zak (7). Lipid phosphorus was determined by the method of Bartlett (8) and an average molecular weight of phospholipid (775 g. per mole) was used for calculation of phospholipid concentrations. Individual phospholipids were separated by thin-layer chromatography on plates coated with Silica Gel H (Analtech, Newark, Del.). The plates were activated at least 1 hour at 110° before chromatography. Chloroform-methanol-acetic acid-water (25:15:4:2,v/v) was used as the developing solvent.

Phospholipase C treatments were performed by incubating flasks containing 2 ml (10 mg protein) of purified ferroxidase-II, 0.1 mg phospholipase C (Type I, Sigma Chemical Co., St. Louis, Mo.) and 0.02 ml of 0.4 M $CaCl_2$ for various time periods. Control flasks containing ferroxidase-II and calcium but no phospholipase C were incubated for identical time periods. The ferroxidase activity of each flask was determined and the lipids extracted with 20 volumes of chloroform:methanol (2:1 v/v). The extracts were washed by the procedure of Folch et al. (9) and the phospholipid contents of the washed extracts determined.

In the gel-filtration experiments, 20 ml (100 mg protein) of purified ferroxidase-II, 1 mg phospholipase C, and 0.2 ml of 0.4 $CaCl_2$ were incubated for 2 hours at 30°. Immediately following the incubation, a 15 ml sample from this reaction mixture was applied to a column (3 x 45 cm) of Agarose A-50m (Bio-Rad Laboratories, Richmond, Calif.). The ferroxidase-II protein was eluted with 0.05 M acetate buffer, pH = 5.5, concentrated by ultrafiltration, and the ferroxidase activity, phospholipid content, and cholesterol content determined.

Treatment with phospholipase A was performed by incubating flasks containing 2 ml (10 mg protein) of purified ferroxidase-II, 70 μl of a solution (1 mg/ml) of phospholipase A (From Vipera russelli, Sigma Chemical Co., St. Louis, Mo.), and 0.02 ml of 0.4 M $CaCl_2$ for various time intervals at 30°. Control flasks containing ferroxidase-II and calcium but no phospholipase A were incubated for identical time intervals. The ferroxidase activity of each flask was determined and the lipids extracted just as described for phospholipase C. Phospholipase A hydrolysis of phosphatidyl choline results in the formation of lysophosphatidyl choline and fatty acyl groups. Thus, phospholipid hydrolysis could not be monitered by following the disappearance of lipid phosphorus in the extracts but was monitered by thin-layer chromatography.

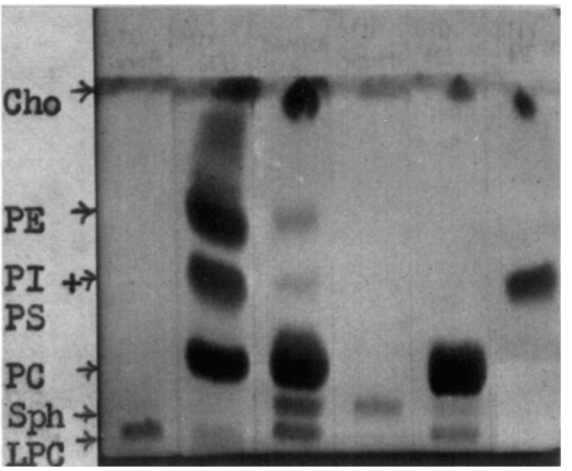

Figure 1. Thin-layer Chromatographic Identification of Phospholipids Bound to Ferroxidase-II. Cho, cholesterol; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; PC, phosphatidyl choline; Sph, sphingomyelin; LPC, lysophosphatidyl choline. Vertical columns, left to right: column 1, standard LPC; column 2, mixed standard (cho, PE, PI, PS, PC, Sph, LPC); column 3, lipid extract of ferroxidase-II; column 4, standard Sph; column 5, standard PC; column 6, standard PI + PS.

RESULTS AND DISCUSSION

The lipid contents of four separately purified ferroxidase-II preparations of high specific activity were determined. The ferroxidase-II preparations contained lipid both in the form of cholesterol and phospholipid. All preparations showed a consistent ratio of protein, phospholipid, and cholesterol (Table I). The purification procedure for ferroxidase-II involves extensive dialysis, ultrafiltration, and gel-filtration which should result in the loss of any unbound or loosely bound lipids from the ferroxidase-II preparations. Thus, these lipid components must be tightly associated with the purified enzyme.

Thin-layer chromatographic analyses were carried out to ascertain which individual phospholipids were bound to ferroxidase-II. Phosphatidyl choline accounts for the majority of the bound phospholipid with small amounts of sphingomyelin and lysophosphatidyl choline accounting for most of the remaining phospholipid (Fig. 1).

To determine whether these phospholipids were essential for the maintainence of enzymic activity, purified ferroxidase-II was treated with

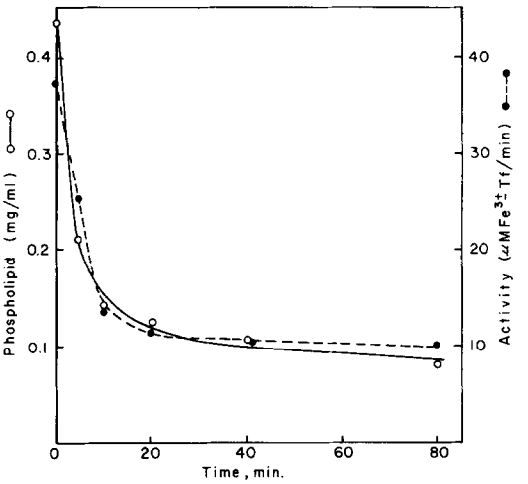

Figure 2. Phospholipase C Treatment of Ferroxidase-II. Phospholipase and ferroxidase activities were assayed as described in "Experimental Procedures". Each value reported represents an average of triplicate flasks.

phospholipase C. A loss of ferroxidase activity paralleling the hydrolysis of phospholipid was observed (Fig. 2). No ferroxidase activity was lost from the control samples incubated for identical time intervals without phospholipase C. To determine if the lipid could be removed from the ferroxidase-II protein following phospholipase C treatment, treated samples were subjected to gel-filtration. Both phospholipid and cholesterol were removed (Table II). The lipid-depleted enzyme had little ferroxidase activity. However, hydrolysis, not removal, of the phospholipid was sufficient for the loss of ferroxidase activity.

Treatment of purified ferroxidase-II with phospholipase A also resulted in the loss of ferroxidase activity. Disappearance of the phosphatidyl choline spot was observed on thin-layer chromatography of the lipid extracts from samples treated with phospholipase A. No disappearance of ferroxidase activity or phosphatidyl choline was observed with the control samples.

The results presented in this report establish the lipoprotein nature of ferroxidase-II previously suggested by the enzyme's immunoelectrophoretic mobility and staining properties. The lipid bound to ferroxidase-II is primarily phosphatidyl choline and cholesterol. Furthermore, even though

these lipids represent only 20% by weight of ferroxidase-II, intact phospholipids appear essential for the maintainence of enzymic activity. Studies concerning the reconstitution of the lipid-depleted form of ferroxidase-II and possible structural changes occurring during lipid-depletion are currently in progress.

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