originate from histidine residues in the Fc portion of the IgG molecule. It appears that the intact IgG1 and the fragments acquire an increased degree of flexibility at low pH.

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References

Arata, Y., & Ozawa, H. (1976) *J. Magn. Reson. 21*, 67.
Arata, Y., & Shimizu, A. (1979) *Biochemistry 18*, 2513.
Arata, Y., Shimizu, A., & Matsuo, H. (1978a) *J. Am. Chem. Soc. 100*, 3230.
Arata, Y., Ozawa, H., Ogino, T., & Fujiwara, S. (1978b) *Pure Appl. Chem. 50*, 1273.
Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., & Tasumi, M. (1977) *J. Mol. Biol. 112*, 535.
Bull. W.H.O. (1964) *30*, 447.
Cathou, R. E. (1978) *Compr. Immunol. 5*, 37–83.
Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauser, U., & Waxdal, M. J. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 78.
Edmundson, A. B., Ely, K. R., Abola, E. E., Schiffer, M., & Panagiotopoulos, N. (1975) *Biochemistry 14*, 3953.
Kabat, E. A. (1976) in *Structural Concepts in Immunology and Immunochemistry*, 2nd ed., Chapter 9, Holt, Rinehart, and Winston, New York.
Noelken, M. E., Nelson, C. A., Buckley, C. E., & Tanford, C. (1965) *J. Biol. Chem. 240*, 218.
Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B. L., Phizacherley, R. P., & Saul, F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3305.
Porter, R. R. (1958) *Nature (London) 182*, 670.
Putnam, F. W. (1969) *Science (Washington, D.C.) 163*, 633.
Shimizu, A., Honzawa, M., Yamamura, Y., & Arata, Y. (1980) *Biochemistry 19*, 2784.
Steiner, L. A., & Porter, R. R. (1967) *Biochemistry 6*, 3957.
Tanokura, M., Tasumi, M., & Miyazawa, T. (1978) *Chem. Lett.*, 739.
Turner, M. W., Bennich, H. H., & Natvig, J. B. (1970) *Clin. Exp. Immunol. 7*, 627.
Valentine, R. C., & Green, N. M. (1967) *J. Mol. Biol. 27*, 615.
Yguerabide, J., Epstein, H. F., & Stryer, L. (1970) *J. Mol. Biol. 51*, 573.

Roles of Phospholipid and Detergent in Soluble Protein Activation of Squalene Epoxidase†

Leu-Fen Hou Lin*

ABSTRACT: "Soluble protein factor" (SPF) from hog liver stimulates hepatic microsome-associated squalene epoxidase in the presence of phosphatidylglycerol or phosphatidylserine. When SPF and phosphatidylglycerol are preincubated for 30 min at 37 °C before addition to the epoxidase system, this stimulation is abolished. On Sephadex chromatography of the protein–phospholipid mixture, both components appear in the void volume, whereas SPF alone is retarded on the column. These results suggest formation of a SPF–phosphatidylglycerol complex. Treatment of the complex with Tween 80 restores the stimulatory effects of SPF on squalene epoxidase. The stimulation of microsomal squalene epoxidase by SPF was abolished by pretreatment of the membrane with low concentrations of deoxycholate or by solubilizing the enzyme with Triton X-100, implying that an intact membrane system is required for SPF sensitivity. SPF has been purified 1200-fold from hog liver.

Several soluble proteins that stimulate microsomal enzymes involved in the late stages of hepatic cholesterol biosynthesis have been described (Tchen & Bloch, 1957; Ritter & Dempsey, 1970; Srikantaiah et al., 1976; Spence & Gaylor, 1977). Rat hepatic tissue has been the source of soluble proteins in all previous investigations. In contrast, information on such soluble proteins in hepatic tissue other than that of the rat has been missing. In particular, a rat liver cytoplasmic protein, termed "supernatant protein factor" (SPF),[1] is shown to stimulate microsomal squalene epoxidase (Tai & Bloch, 1972; Saat & Bloch, 1976). Although homogeneous SPF can be obtained from this tissue (Ferguson & Bloch, 1977), the yield is low, not exceeding ~2 mg/kg of rat liver. Since the scarcity of the rat material could be a major hindrance to the study of the mechanism of SPF stimulation, it seemed of importance to search for a more convenient source. Hog liver was found to contain moderate SPF activity, whereas no SPF was demonstrable in extracts of fresh or frozen beef liver. This paper accounts for the partial purification and characterization of SPF from hog liver and demonstrates the requirement of anionic phospholipids for the SPF response. Toward the un-

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[1] Abbreviations used: SPF, supernatant protein factor; SPF_{Ac}, crude SPF preparation, 45–75% acetone precipitate (as described under Methods) from hog liver; AMO-1618, (trimethylammonio)-5-carvacryl 1-piperidinecarboxylate chloride; S_{78}, 78000*g* supernatant from hog liver; DTT, dithiothreitol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid.

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derstanding of the so far poorly defined role of SPF as a promotor of the microsome-associated squalene epoxidase system, evidence was presented for the binding of SPF to specific phospholipids which support SPF activity. The interaction of SPF and microsomes was also explored by studying the consequences of exposing the membranes to detergent that disturbs membrane integrity. Results support the suggestion that an intact membrane is essential for SPF sensitivity.

Experimental Procedures

Materials

Hog liver was obtained fresh from Copaco Co. Female rats (strain CD) weighing 201–225 g were obtained from Charles River Breeding Laboratories. DL-[2-^{14}C]Mevalonic acid dibenzylethylenediamine salt was from New England Nuclear. AMO-1618[1] was supplied by Calbiochem. Sigma Chemical Co. supplied Tween 80, FAD, Tris, NADPH, and dithiothreitol (DTT).[1] Phospholipids were purchased from Avanti Biochemicals. DEAE-cellulose (0.7 mequiv/g) and Bio-Beads SM-2 (20–50 mesh) were obtained from Bio-Rad Laboratories. Sephadexes G-25 (medium), G-50 (fine), G-75, and G-100 were products of Pharmacia. All other chemicals were of reagent grade.

Methods

[^{14}C]Squalene was prepared biosynthetically from DL-[2-^{14}C]mevalonic acid as previously described (Ferguson & Bloch, 1977). Phospholipid suspensions in water (10 mg/mL) were prepared by intermittent sonication in N_2 for 4 min with a Branson sonifier. Rat liver microsomes were prepared as previously reported (Tai & Bloch, 1972).

Preparation of Supernatant Fractions from Hog Liver. The following procedures were carried out at 4 °C unless specified. Fresh hog liver was perfused with 100 mL of 0.1 M potassium phosphate buffer, pH 7.5, and either processed immediately or stored frozen at −70 °C. Livers were cut into small pieces and minced with a meat grinder, and the minced tissue was homogenized in a Waring blender for 30 s at top speed in the presence of 2 volumes of 0.1 M potassium phosphate buffer, pH 7.5. The homogenate was centrifuged at 10000*g* for 15 min, and the supernatant was filtered through four layers of cheese cloth to remove the floating material and centrifuged at 78000*g* for 90 min. A remaining floating layer was removed by aspiration and the supernatant decanted (S_{78}).[1] Acetone fractionation was based on that reported earlier (Tai & Bloch, 1972) and performed with 350-mL batches of S_{78}. The S_{78} was chilled to 0 °C in an ethanol–water–dry ice bath at −5 °C. Reagent grade acetone (287 mL) chilled to −65 °C was added to give a final acetone concentration of 45% (v/v), and the bath temperature was lowered to −10 °C. The mixture was stirred for 20 min and centrifuged at 14000*g* for 15 min at −10 °C. The clear supernatant was brought to an acetone concentration of 75% (v/v) with 764 mL of acetone chilled to −65 °C and stirred for 10 min after which the mixture was centrifuged for 10 min as before. The pellet was resuspended in 17.5 mL of 5 mM potassium phosphate buffer containing 0.5 mM DTT, pH 7.5, with the aid of a Teflon pestle, and the suspension was centrifuged for 10 min (12000*g*) to remove particulate matter. Acetone was removed by immediately applying the supernatant to a column of Sephadex G-25 (medium) (5.8 × 38 cm) equilibrated with the 5 mM phosphate buffer containing 0.5 mM DTT, pH 7.5. Void volume fractions were pooled (SPF_{Ac}).[1] This acetone fractionation produced a 12-fold purification over the S_{78} with a quantitative recovery of SPF activity. Both S_{78} and SPF_{Ac} could be stored for several months at −70 °C without substantial loss of activity.

Squalene Epoxidase Activity Assay. [^{14}C]Squalene (40 nmol, 0.25 Ci/mol in benzene) was introduced into a test tube and the solvent evaporated under N_2. Where mentioned, 50 μL of a 0.1% Tween 80 in acetone was also added at this point before solvent evaporation. To the residue were added sequentially 0.05 M Tris-HCl, pH 7.6, 0.5 mM AMO-1618, 5 μM FAD, 0.2 mg of phosphatidylglycerol, and 1 mM NADPH, and the reaction was started by adding 1.2 mg of rat liver microsomal protein. Mixtures were incubated at 37 °C for 30 min, and products were isolated and assayed as described (Tai & Bloch, 1972). Epoxidase activity is expressed as nanomoles of squalene 2,3-oxide formed in 30 min under standard conditions. When tested, SPF was added to the above assay system following the addition of microsomal protein. One unit of SPF activity is defined as 1 incremental nmol of squalene 2,3-oxide produced in 30 min under standard assay conditions in the presence of SPF over controls without SPF. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Purification of SPF. All steps were performed at 4 °C. Concentration of fractions was done by pressure dialysis using an Amicon UM-10 membrane.

(*1*) SPF_{Ac} (960 mL) was chromatographed on a DEAE-cellulose column (5 × 14.5 cm) equilibrated with 5 mM potassium phosphate buffer containing 0.5 mM DTT, pH 7.5. The column was eluted with the same buffer, and the unadsorbed material containing most of the activity was pooled. Ammonium sulfate was added to 75% saturation. After centrifugation the pellet was resuspended in a minimal volume (50 mL) of 50 mM Tris-HCl buffer containing 0.5 mM DTT, pH 7.5, and was stable as such if stored at −70 °C. This fraction was desalted on a Sephadex G-25 column by using the same buffer and concentrated to 11.5 mL (48.8 mg/mL) (DEAE-I fraction).

(*2*) The DEAE-I fraction was chromatographed on a 150-mL bed of DEAE-cellulose equilibrated with 5 mM Tris-HCl buffer containing 0.5 mM DTT, pH 8.4. The column was eluted with 2 bed volumes of the same buffer followed by 10 mM KCl in the same buffer, and 21-mL fractions were collected. Active fractions were pooled and concentrated to 27 mL (2.75 mg/mL). Tris-HCl buffer (1 M, pH 7.4, 1.4 mL) was added to the concentrate and stored at −70 °C if necessary (DEAE-II fraction).

(*3*) The above fraction was further concentrated to 6.6 mL and then applied to a Sephadex G-75 column (1.6 × 100 cm) equilibrated with 0.1 M Tris-HCl buffer containing 0.5 mM DTT, pH 7.8, and 6-mL fractions were collected. Active fractions were combined, concentrated to 5.9 mL (8.1 mg/mL), and frozen at −70 °C (G-75 fraction).

(*4*) One-third of the G-75 fractions (1.9 mL) was dialyzed against 300 mL of 5 mM Tris-HCl buffer containing 0.5 mM DTT and 1% glycerol, pH 8.0, with two changes of buffer in 4 h. The protein sample, with 0.52 g of sucrose added, was subjected to isoelectric focusing on an LKB 8101 apparatus (Haglund, 1971) maintained at 4 °C with a Lauda K4R circulating bath. A sucrose gradient (0.5 mM in DTT) containing pH 3.5–10 ampholytes was introduced. The protein sample was layered midway between the dense anode solution and the light cathode solution. The final ampholyte concentration was 13.3 g/L. For the first 15 h, voltage was maintained at 300 V and then it was increased to 500 V. Power was shut down after 42 h of focusing. The column was drained at 2 mL/min and 1-mL fractions were collected. pH, ab-

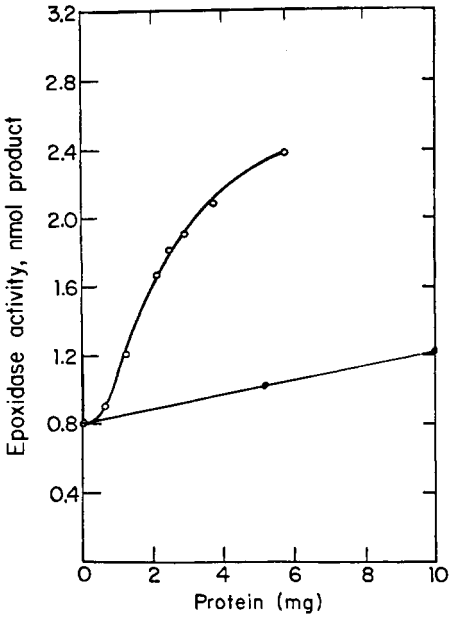

FIGURE 1: Effect of supernatant protein factor on squalene epoxidase activity. The assay conditions were those described under Methods. (○) SPF_{Ac}; (●) S_{78}.

Table I: Effect of Phospholipid and Tween 80 on Supernatant Protein Factor[a]

assay additions	squalene epoxidase act. (nmol/30 min) −SPF	+SPF
none	0.20	0.22
phosphatidylglycerol	0.68	1.80
phosphatidylserine	0.60	1.53
phosphatidylcholine	0.17	0.19
Tween 80	0.40	0.38
Tween 80 plus phosphatidylglycerol	0.44	5.09

[a] Conditions for the epoxidase assay were the same as described under Methods except that phosphatidylglycerol was omitted. Where indicated, 100 μg of each phospholipid and/or 50 μg of Tween 80 was added. SPF_{Ac} (2.5 mg) was the source of SPF.

sorbance at 280 nm, and SPF activity were scanned. Fractions of the highest relative specific activity (in two peaks) were separated, pooled, passed through a bed of Sephadex G-50 (fine) (2 × 35 cm), and eluted with 0.1 M Tris-HCl buffer containing 0.5 mM DTT, pH 7.9, to remove the ampholytes. Void volume fractions with significant absorbance at 280 nm were pooled, concentrated to 0.6 mL, and stored at −70 °C (focused fraction).

Results

Supernatant Protein Factor of Hog Liver. Microsomal squalene epoxidase activity was stimulated by adding to the assay mixture a crude preparation of postmicrosomal supernatant (S_{78}) from hog liver (Figure 1). SPF was partially purified from hog S_{78} through precipitation with acetone (45–75%, SPF_{Ac}). As shown in Figure 1, addition of increasing amounts of SPF_{Ac} produced substantial increments of epoxidase activity and the response to SPF was sigmoidal rather than linear. Since hog liver microsomes were nearly inactive enzymatically, squalene epoxidase activity was assayed routinely with microsomes from rat liver. SPF_{Ac} was extremely heat labile, and the stimulatory activity was totally lost after exposure to 50 °C for 5 min. Partially purified SPF was found to be absolutely dependent on phospholipid for activity (Table I). Phosphatidylglycerol or phosphatidylserine could satisfy

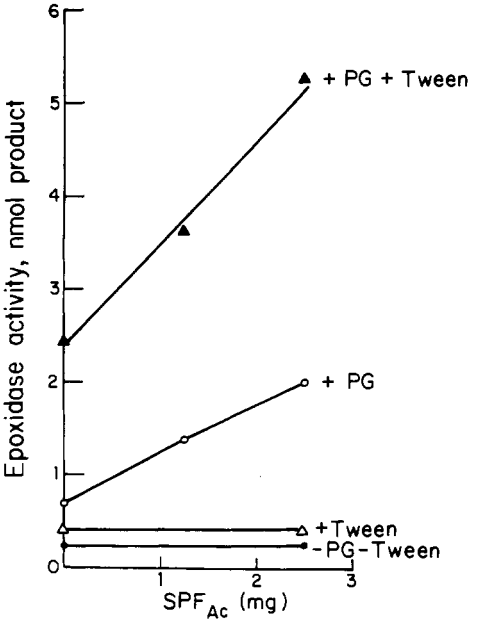

FIGURE 2: Effect of phosphatidylglycerol (PG) and Tween 80 on squalene epoxidase activity in the presence of SPF. Conditions for epoxidase assay were the same as described under Methods except that PG was omitted or 50 μg of Tween 80 added where indicated.

the phospholipid requirement of SPF activity, while phosphatidylcholine was totally inactive. The essential phospholipid added routinely to assay mixtures was phosphatidylglycerol. Detergent Tween 80 added up to 50 μg/mL could not substitute for phospholipid in the SPF assay. It should also be noted that Tween 80, which has no effect by itself or in combination with SPF, in conjunction with phosphatidylglycerol, raises epoxidase activity substantially. However, even in the detergent-activated system, SPF causes a further, severalfold increase in enzyme activity (Table I and Figure 2).

Phosphatidylglycerol–SPF Interactions. In the normal assay for SPF activity, phosphatidylglycerol is premixed with other reaction components and the enzyme reaction started by adding microsomes and SPF. Under these conditions, epoxidase activity is stimulated about threefold (Table II, experiments 1 and 2). However, when SPF and phospholipid were preincubated for 5 or 30 min at 37 °C and then mixed with remaining assay components, SPF no longer stimulated epoxidase activity (Table II, experiments 3 and 4). On preincubation of SPF and phosphatidylglycerol at 4 °C, about half of the stimulatory effect remained (Table II, experiment 5). Subsequent addition of phosphatidylglycerol to the preincubation mixture could not restore the SPF activity (Table II, experiment 6). Therefore, the possibility was ruled out that the phenomenon was caused by suppression of phosphatidylglycerol during the preincubation with SPF. Since SPF preincubated alone for 30 min at 37 °C showed full activity provided phosphatidylglycerol was present in the subsequent assay mixture (Table II, experiment 7), instability of SPF does not account for the observed phenomenon; phosphatidylglycerol was also stable during preincubation (Table II, experiment 8). Significantly, SPF, when preincubated with phosphatidylcholine, retained its normal activating effect provided phosphatidylglycerol was subsequently added to the preincubated mixture along with the other assay components (Table II, experiment 9). The interaction between SPF and phosphatidylglycerol which abolishes epoxidase stimulation is reversible by detergent. As shown in experiment 10, Table II, SPF preincubated with phosphatidylglycerol, when added to an assay mixture containing Tween 80, causes

Table II: Effect of Preincubating SPF and Phosphatidylglycerol Prior to Squalene Epoxidase Assay[a]

expt	preincubation	additions to assay mixture	epoxidase act. (nmol/ 30 min)
1	none	PG[b]	0.48
2	none	SPF and PG	1.66
3	SPF and PG, 30 min, 37 °C	none	0.29
4	SPF and PG, 5 min, 37 °C	none	0.27
5	SPF and PG, 30 min, 4 °C	none	0.76
6	SPF and PG, 30 min, 37 °C	PG	0.29
7	SPF, 30 min, 37 °C	PG	1.42
8	PG, 5 min, 37 °C	SPF	1.58
9	SPF and PC[c], 30 min, 37 °C	PG	1.47
10	SPF and PG, 30 min, 37 °C	Tween	5.96
11	none	SPF, PG, and Tween	4.93
12	none	Tween and PG	2.33

[a] SPF_{Ac} (2.5 mg) and 200 μg of phosphatidylglycerol were preincubated in 5 mM potassium phosphate buffer containing 0.5 mM DTT, pH 7.5, in a final volume of 0.42 mL, at the indicated temperatures and for the periods given. The preincubated mixtures were then assayed as described under Methods except that phosphatidylglycerol was omitted from the assay mixture unless mentioned. [b] PG, phosphatidylglycerol. [c] PC, phosphatidylcholine.

the same two- to threefold increase in epoxidase activity that is observed in control assays when all components are added simultaneously (Table II, experiments 11 and 12). Preincubation of SPF_{Ac} with phosphatidylglycerol appears to produce a complex that no longer stimulates epoxidase but can be dissociated by detergent.

Evidence for interaction between phosphatidylglycerol and SPF was also obtained by gel filtration. On Sephadex G-100 columns, SPF activity was located as expected in the retarded fractions (Figure 3B), but, when the preincubated mixture (at 4 °C) of phosphatidylglycerol and SPF was chromatographed, none of the eluent fractions raised the basal epoxidase activity. In this instance a protein peak (280 nm) appeared in the void volume (Figure 3A) not seen when SPF alone is chromatographed. Sonicated phosphatidylglycerol also emerged in the void volume of the Sephadex G-100 column. When fractions from the column loaded with the preincubated SPF–phosphatidylglycerol mixture were assayed in the presence of Tween, some epoxidase-stimulating activity was found in the void volume fraction and also in the pooled retarded fractions (Figure 3C). Since both preincubation and chromatography were conducted at 4 °C, the formation of the inactive complex is expected to be incomplete, judging from the results of Table II.[2] At any rate, the data of Figure 3 complement those presented in Table II showing that SPF and phosphatidylglycerol interact to form a complex that is inactive but regains SPF activity after exposure to Tween 80.

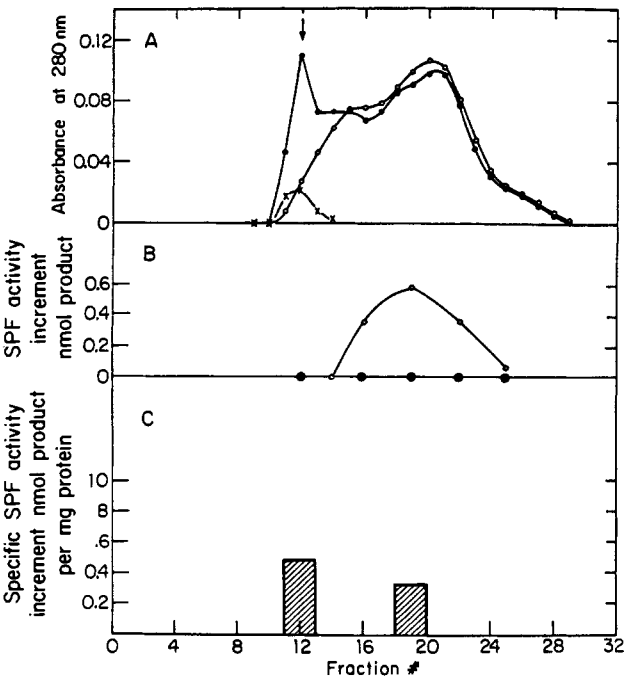

FIGURE 3: Binding of SPF to phosphatidylglycerol. SPF_{Ac} (2.7 mg of protein in 0.44 mL of 5 mM potassium phosphate buffer containing 0.5 mM DTT, pH 7.5) was preincubated with (●) or without (○) 200 μg of phosphatidylglycerol for 30 min at 4 °C and the mixture chromatographed on a Sephadex G-100 column (1.6 × 41 cm) equilibrated with 5 mM Tris-HCl, pH 7.6. Phosphatidylglycerol vesicles (200 μg) were separately chromatographed on the same column (×). (Panel A) Absorbance of 2.5-mL fraction at 280 nm. The void volume is indicated by the arrow. (Panel B) SPF activity of lyophilized eluate fraction (2.5 mL) under standard conditions. (Panel C) Pooled fractions from the lower curve of panel B (●) were lyophilized and assayed for SPF activity after addition of 50 μg of Tween 80 under standard conditions.

Deoxycholate Treatment. If microsomes (3 mg of protein/mL) were exposed to 0.025% deoxycholate at 0 °C and then sedimented to remove the detergent, the basal epoxidase activity in the resuspended particles was barely affected. However, SPF stimulation of epoxidase activity in deoxycholate treated microsomes was reduced by one-third compared to the untreated control. The low concentration of deoxycholate used is known to cause little perturbation to the microsomal membrane (Kreibich & Sabatini, 1974), and, yet, microsomes were rendered less sensitive to SPF stimulation. These results suggest that membrane integrity is essential for the SPF response. If the concentration of deoxycholate was raised to 0.05% during the treatment, both basal epoxidase activity and SPF activity were decreased.

Triton Treatment. Triton slightly stimulated the epoxidase activity only in a narrow range of detergent concentration around 0.3%, and it was inhibitory otherwise. In the presence of 0.3% Triton, however, addition of SPF showed no further stimulatory effect on epoxidase activity (Table III, experiment 1). Microsomal epoxidase could be released from the membrane with the treatment of 2% Triton (Ono & Bloch, 1975), but the solubilized enzyme showed elevated basal epoxidase activity which no longer responded to SPF (Table III, experiment 2). If the detergent was removed from the solubilized enzyme by adsorption to Bio-Beads (Holloway, 1973), no epoxidase activity was detectable in either the presence or absence of SPF. However, enzyme activity was restored by the addition of Triton X-100 (Table III, experiment 3). Be-

[2] SPF_{Ac} gradually lost activity in dilute solution as well as during concentration via lyophilization. It is difficult therefore to recover quantitatively the SPF activity after column chromatography. This may explain the lack of detectable SPF activity in the retarded fractions even though the inactivation is not complete in the lower curve of Figure 3, B (●). Since the assay is more sensitive in the presence of Tween 80 (Table I), the residual SPF activity in the retarded fractions could then be detected in Figure 3C. The extremely low recovery of SPF activity after these procedures at 4 °C makes it unfeasible to try preincubating at 37 °C to give complete inactivation of SPF activity followed by chromatography at the same temperature.

Table III: Effect of Triton X-100 on Microsomal Squalene Epoxidase[a]

expt	enzyme source	assay addition	epoxidase act. (nmol/30 min)
1	E-1 (microsomes)	none	0.60
		SPF	1.37
		Triton	0.92
		Triton plus SPF	0.98
2	E-2 (Triton-solubilized E-1)	none	1.45
		SPF	1.52
3	E-3 (Triton-depleted E-2)	none	0.06
		SPF	0.08
		Triton	0.97
4	E-4 (E-3 incorporated into PC)	none	0.09
		SPF	0.09
		Triton	0.82
		Triton plus SPF	0.83

[a] 1.2 mg of each of the following enzyme sources was used in the assay. To microsomes (E-1) of 36 mg of protein in 1.5 mL of 0.1 M Tris-HCl buffer, pH 7.6, was added 0.375 mL of 10% Triton X-100 (final concentration of Triton, 2%). The mixture was gently stirred for 20 min at 4 °C, diluted with 5.625 mL of 1 mM EDTA and 1 mM DTT to lower the concentration of detergent to 0.5%, and centrifuged at 105000*g* for 60 min. The supernatant (E-2) was stirred with a batch of Bio-Beads for 2 h at 4 °C to remove Triton as described (Holloway, 1973). The Triton-depleted supernatant (E-3) was added to phosphatidylcholine (PC) at a ratio of 1.9 mL of E-3/2.5 mg of PC and the mixture sonified for 3 min in ice bath (E-4). Where indicated, SPF_{Ac} (2.5 mg) or Triton X-100 (to a final concentration of 0.3%) was added to the standard assay.

sides indicating that Triton had direct effect on the epoxidase activity per se, the results are also consistent with the view that membrane structure is crucial for SPF activity since Triton-solubilized epoxidase is no longer sensitive to SPF. Attempts to incorporate the detergent-depleted solubilized enzyme into an artificial membrane to recover the SPF sensitivity of the enzyme have been unsuccessful in the sense that the resulting preparation showed epoxidase activity only in the presence of Triton and no SPF stimulation was detected (Table III, experiment 4).

Purification of SPF. Since a nonlinear response had been observed, different amounts of the protein had to be incubated in each assay to measure the increment produced by addition of the cytosolic protein. Specific activity was calculated from the minimal amount of protein needed to produce a maximal response. A summary of the purification procedure for SPF is shown in Table IV. Preparative isoelectric focusing of the G-75 fraction in pH 3–10 gradients resulted in separation of several peaks of absorbance at 280 nm while two of the peaks have SPF activity (p*I* = 7.0 and 8.5, respectively). When the two active focused fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the two gel patterns were strikingly similar with five bands in each. The overall purification of SPF was 1200-fold with a 33% recovery of activity. Attempts to obtain a homogeneous preparation of hog liver SPF have so far been unsuccessful.

Discussion

Hog liver SPF shares the following properties with the corresponding preparations from rat liver (Tai & Bloch, 1972): heat sensitivity (total loss of activity after 5 min at 50 °C) and an absolute dependence of the stimulatory effects on anionic phospholipid. It is likely that a similar hepatic SPF exists in the two species.

Earlier studies with squalene epoxidase (Saat & Bloch, 1976) involving preincubation of microsomes with squalene

Table IV: Purification of SPF from Hog Liver (442 g)

fraction	total protein (mg)	sp act. (units/mg)	purifcn fraction (*x*-fold)	total act. (units)	recovery (%)
S_{78}	27246	0.04	1.0	1090	100
SPF_{Ac}	2636	0.47	11.8	1239	114
DEAE-I	534	1.20	30.0	641	59
DEAE-II	73	7.10	177.5	518	48
G-75	24	21.44	536.0	515	47
focused					
peak I	5.7	50.0	1250.0	285	26
peak II	1.7	46.5	1162.5	79	7

in the presence and absence of SPF were interpreted as showing that SPF may facilitate the transport of substrate to a specific site or compartment within the endoplasmic reticulum. However, the fact that no binding or aggregation of SPF with substrate was observed (Tai & Bloch, 1972) is inconsistent with the hypothesis that SPF acts as a carrier for the substrates within the microsomal membrane. Other modes of action for SPF have to be considered. It is now shown that SPF binds to phosphatidylglycerol, an anionic phospholipid that supports SPF activity. At the moment the SPF–phospholipid interaction appears to play an important role in the stimulation of epoxidase by SPF. At the completion of this work, Caras et al. (1980) demonstrated, with the availability of radiolabeled SPF from rat liver, the binding of SPF to vesicles of anionic phospholipids, confirming independently the finding in this report. However, radioactivity rather than SPF activity was traced in their binding studies. Since an artifact could not be ruled out if only inactive SPF would bind to phospholipid, the present work in which SPF activity was monitored for binding studies really complements that reported by Caras et al. (1980), to offer a stronger evidence that SPF–phospholipid interaction might play an important if not essential role in the stimulation of epoxidase by SPF. However, it remains to be ascertained why an anionic phospholipid such as phosphatidylglycerol is required for expression of the SPF effect. The protein and phosphatidylglycerol form a complex which can be isolated by column chromatography, but paradoxically this complex or aggregate does not stimulate the epoxidase system unless it is disassociated by Tween. It should be noted that previously Tween has always been added to the epoxidase assay system to disperse substrate squalene (Tchen, 1963; Tai & Bloch, 1972; Saat & Bloch, 1976; Nakamura & Sato, 1979). It is clear from the present study that the role of Tween is more complex than merely solubilizing the water-insoluble squalene. Besides, the amount of microsomes used in the system is enough to disperse squalene.[3] It is advisable therefore that Tween be omitted from the assay system especially when studying the mechanism of SPF effect.

Squalene epoxidase solubilized by disrupting the membrane with Triton X-100 loses the requirement for SPF, showing that intact membranes are required for expression of the SPF effect. Similar results have also been obtained previously with rat liver SPF (Ono & Bloch, 1975). Furthermore, when microsomes were exposed to a low concentration of deoxycholate or limited digestion with phospholipase A_2,[4] epoxidase activity was rendered insensitive to SPF. Such treatments perturb the membrane only minimally in the sense that they do not change the sedimentation properties of the particles or the distribution of labeled phospholipids (Kreibich & Sabatini, 1974). These results demonstrate directly the requirement of intact mem-

[3] J. B. Ferguson, unpublished observation.
[4] L.-F. H. Lin, unpublished observation.

branes for the response of squalene epoxidase to SPF.

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References

Caras, I. W., Friedlander, E. J., & Bloch, K. (1980) *J. Biol. Chem. 255*, 3575–3580.
Ferguson, J. B., & Bloch, K. (1977) *J. Biol. Chem. 252*, 5381–5385.
Haglund, H. (1971) *Methods Biochem. Anal. 19*, 1–104.
Holloway, P. W. (1973) *Anal. Biochem. 53*, 304–308.
Kreibich, G., & Sabatini, D. D. (1974) *Methods Enzymol. 31*, 215–225.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Nakamura, M., & Sato, R. (1979) *Biochem. Biophys. Res. Commun. 89*, 900–906.
Ono, T., & Bloch, K. (1975) *J. Biol. Chem. 250*, 1571–1579.
Ritter, M. C., & Dempsey, M. E. (1970) *Biochem. Biophys. Res. Commun. 38*, 921–929.
Saat, Y. A., & Bloch, K. E. (1976) *J. Biol. Chem. 251*, 5155–5160.
Spence, J. T., & Gaylor, J. L. (1977) *J. Biol. Chem. 252*, 5852–5858.
Srikantaiah, M. V., Hansbury, E., Loughran, E. D., & Scallen, T. J. (1976) *J. Biol. Chem. 251*, 5496–5504.
Tai, H.-H., & Bloch, K. (1972) *J. Biol. Chem. 247*, 3767–3773.
Tchen, T. T. (1963) *Methods Enzymol. 6*, 505–512.
Tchen, T. T., & Bloch, K. (1957) *J. Biol. Chem. 226*, 921–930.

Magnetic Resonance Studies of Apolipoprotein C-I Nitroxide Labeled or [^{13}C]Methyl Enriched at Methionine-38†

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ABSTRACT: One of the three proposed lipid-binding regions of the human apolipoprotein C-I (apoC-I) is an amphipathic helix which extends from residue 33 to residue 53 and includes a single methionine at sequence position 38. The involvement of the sequence around methionine-38 in phospholipid binding has been evaluated with paramagnetic and nuclear reporter groups attached to the thiomethyl moiety. This moiety has been spin-labeled with *N*-(2,2,6,6-tetramethylpiperidinyl-1-oxy)bromoacetamide or ^{13}C enriched with $^{13}CH_3I$. As determined from its EPR spectrum, the nitroxide at Met-38 of apoC-I had a rotational correlation time (τ_c) of 0.22 ns. When dimyristoylphosphatidylcholine (DMPC) was bound to the spin-labeled apoprotein, τ_c increased to 0.35 ns, indicating decreased motion for the methionyl side chain. The line width ($\nu_{1/2}$) and spin–lattice relaxation time (T_1) for the thiomethyl resonance of ^{13}C-enriched apoC-I in 10 mM phosphate buffer was 6.0 Hz and 320 ms, respectively. When the protein solution was made 1.6 M in Gdn·HCl, these values changed to 2.6 Hz and 970 ms, respectively. Upon addition of DMPC multilamellar liposomes to [^{13}C]apoC-I in 1.6 M Gdn·HCl, the line width increased to 4.7 Hz and the T_1 decreased to 380 ms. These results strongly suggest that methionine-38 of apoC-I resides in a region of the apoprotein which undergoes significant secondary and/or tertiary structural change upon disaggregation/unfolding in Gdn·HCl and upon interaction with phospholipid.

Apolipoprotein C-I (apoC-I)[1] is the smallest of the well-characterized human plasma apolipoproteins. It accounts for about 10 and 2% of the proteins in human plasma very low density lipoproteins and high density lipoproteins, respectively (Schaefer et al., 1978). The amino acid sequence of apoC-I has been determined by Jackson et al. (1974a) and Shulman et al. (1972, 1975) (Figure 1). The protein contains 57 residues which include a single methionine but exclude cystine, cysteine, tyrosine, and histidine. The total synthesis of apoC-I by the solid-phase method has been reported by Harding et al. (1976) and by Sigler et al. (1976). In aqueous solution at neutral pH, the apoprotein undergoes self-association which is attended by alteration in secondary structure. In acidic solution (e.g., pH 2.5), the apoprotein is monomeric and its secondary structure appears to be independent of protein concentration (Osborne et al., 1977a).

ApoC-I is involved in both the metabolism and structure of lipoproteins. It stimulates the activity of lecithin:cholesterol acyltransferase to the same extent with saturated and unsaturated phospholipid acyl donors (Soutar et al., 1975). Synthetic peptides containing residues 32–57, 24–57, and 17–57 stimulate enzymatic activity to the extent of 50, 60, and

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[1] Abbreviations used: Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; apoC-I, apolipoprotein C-I isolated from human very low density lipoproteins; DMPC, dimyristoyl-L-α-phosphatidylcholine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid; VLDL, very low density lipoproteins ultracentrifugally isolated at $d < 1.006$ g/mL; LDL, low density lipoproteins isolated at d 1.019–1.063 g/mL; HDL, high density lipoproteins isolated at d 1.063–1.210 g/mL.

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