Proc. Nat. Acad. Sci. USA
Vol. 69, No. 11, pp. 3490–3492, November 1972

Resolution and Reconstitution of the Phosphatidate-Synthesizing System of Rat-Liver Microsomes

(Triton X-100/sucrose density gradient centrifugation/glycerolphosphate acyltransferase/ 1-acylglycerolphosphate acyltransferase/asymmetric fatty acid distribution)

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Communicated by F. Lynen, September 18, 1972.

ABSTRACT **The phosphatidate-synthesizing system of rat-liver microsomes was resolved into two component enzymes, glycerolphosphate acyltransferase and 1-acylglycerolphosphate acyltransferase. The resolution is effected by sucrose density gradient centrifugation in the presence of a nonionic detergent, Triton X-100. Combination of both enzymes results in reconstitution of the phosphatidate-synthesizing system. These results establish that two distinct enzymes, glycerolphosphate acyltransferase and 1-acylglycerolphosphate acyltransferase, are required for synthesis of phosphatidic acid from *sn*-glycerol 3-phosphate.**

Furthermore, the 1-acylglycerolphosphate acyltransferase preparation efficiently uses unsaturated (or saturated) fatty acyl-CoA as acyl donor. Our previous studies showed that the glycerolphosphate acyltransferase preparation catalyzes formation of 1-acylglycerol 3-phosphate, using preferentially saturated fatty acyl-CoA as acyl donor. These findings indicate that the reconstituted system is capable of yielding phosphatidic acid with an asymmetric fatty acid distribution.

Phosphatidic acid is an important intermediate in the metabolic pathway leading to synthesis of glycerolipids (1–3). This lipid intermediate is formed by esterification of *sn*-glycerol 3-phosphate by long-chain acyl-CoA thioesters (1). On the basis of different sensitivities to sulfhydryl-binding reagents of glycerolphosphate-acylating and 1-acylglycerolphosphate-acylating activities, Lands and Hart (4) postulated that phosphatidic acid synthesis is mediated by two different enzymes, as shown by reactions **1** and **2**.

sn-Glycerol 3-phosphate + acyl-CoA

→ monoacylglycerol 3-phosphate + CoA (**1**)

Monoacylglycerol 3-phosphate + acyl-CoA

→ phosphatidic acid + CoA (**2**)

Recently, temperature-sensitive mutants of *Escherichia coli* unable to undergo the complete acylation sequence were isolated (5–7). All the mutants with a defect in the activity for reaction **1** catalyzed reaction **2** at normal rates (5, 6). A thermolabile mutant possessing the converse phenotype was also reported (7). The availability of these mutants gives support to the hypothesis postulated by Lands and Hart (4) for the mechanism of phosphatidic acid synthesis. Furthermore, evidence was presented that indicates that in *E. coli* a single glycerolphosphate acyltransferase (acyl-CoA:L-glycerol 3-phosphate *O*-acyltransferase, EC 2.3.1.15) catalyzes acylation of *sn*-glycerol 3-phosphate to form monoacylglycerol 3-phosphate, palmitic acid being esterified to position 1 or unsaturated fatty acid to position 2. However, it was impossible to prove that phosphatidic acid cannot be synthesized by glycerolphosphate acyltransferase alone.

A full understanding of the mechanism of phosphatidic acid synthesis would be achieved by isolation of the enzyme(s) involved in this process. However, since the enzyme system responsible for phosphatidic acid synthesis is bound to microsomal membranes (8), its purification and characterization are extremely difficult. Thus far, no successful attempts to isolate the enzymes catalyzing the individual reactions (reactions **1** and **2**) have been reported. In the present investigation, we have been able to resolve the phosphatidate-synthesizing system into its component enzymes and to reconstitute it by combining the isolated enzymes. The results of these studies are described in this paper.

MATERIALS AND METHODS

The enzyme preparation used for the resolution experiment was obtained by Sepharose 2B column chromatography of Triton X-100-treated rat-liver microsomes as described (9, 10). *sn*-[*1*(*3*)-^{14}C]Glycerol 3-phosphate, acyl-CoA thioesters, and a suspension of soya bean phosphatidylcholine (a gift of Nattermann International GmbH, Köln, Germany) were prepared as described (10). 1-Palmityl-[*1*(*3*)-^{14}C]-glycerol 3-phosphate was prepared from *sn*-[*1*(*3*)-^{14}C]glycerol 3-phosphate and palmitoyl-CoA by the action of glycerolphosphate acyltransferase. The procedure was as reported (10), except that the incubation period was prolonged to 1 hr. The radioactive lipid was extracted (10) and purified on a 10 mM Na_2CO_3-impregnated silica gel H plate with chloroform–methanol–acetone–acetic acid–water 200:40:80:40:20 as the developing solvent (11). The lipid, which was extracted from the gel with methanol–chloroform 4:1 and then with methanol, was dispersed in 20 mM Tris·HCl buffer (pH 7.6) by sonic oscillation with a Branson W185D sonifier.

Protein was determined in the presence of deoxycholate by the method of Lowry *et al.* (12) with bovine serum albumin as the standard. Glycerolphosphate acyltransferase activity was also determined (10). 1-Acylglycerolphosphate acyltransferase activity was assayed by measurement of the conversion of 1-palmityl-[*1*(*3*)-^{14}C]glycerol 3-phosphate to [^{14}C]phosphatidic acid. The reaction mixture contained 20 μmol of Tris·HCl (pH 7.6), 2.4 nmol of 1-palmityl-[*1*(*3*)-^{14}C]glycerol 3-phosphate (2,990–4,200 cpm/nmol), 10 nmol of linoleoyl-CoA, 1.75 μmol of $CaCl_2$, 215 nmol of suspended soya bean phosphatidylcholine, and enzyme in a total volume of 0.35 ml. After incubation at 20° for 5 min, the reaction was terminated by addition of 6 ml of chloroform–methanol 2:1. The lipids extracted were sepa-

rated on a thin-layer plate by the same chromatographic procedure as used for purification of 1-palmityl-[1(3)-^{14}C]glycerol 3-phosphate. The area containing phosphatidic acid was scraped into Bray's solution (13) containing 3.5% thixotropic gel (Cab-O-Sil, Packard Instrument Co.) and assayed for radioactivity with a Packard 3320 liquid scintillation spectrometer. No correction was made for the effect of silica gel on the counting efficiency. Under the assay conditions used, the amount of phosphatidic acid formed from 1-palmitylglycerol 3-phosphate was nearly proportional to the enzyme concentration.

RESULTS

Resolution of the phosphatidate-synthesizing system

As reported (10), attempts to purify glycerolphosphate acyltransferase from Triton-treated rat-liver microsomes by centrifugation in a sucrose density gradient yielded an enzyme preparation that catalyzed the conversion of *sn*-glycerol 3-phosphate to 1-acylglycerol 3-phosphate. These results implied that the phosphatidate-synthesizing system was resolved into component enzymes by the centrifugation. In the experiment represented in Fig. 1, therefore, we tested fractions obtained from the sucrose density gradient for 1-acylglycerolphosphate acyltransferase activity, as well as glycerolphosphate acyltransferase activity. The centrifugation procedure used in this experiment was previously shown to permit a good resolution between membrane fragments and proteins dissociated from membranes (9, 10). As is evident from Fig. 1, the two acyltransferase activities were clearly separated from each other. The bulk of glycerolphosphate acyltransferase activity sedimented almost to the bottom of the gradient. 1-Acylglycerolphosphate acyltransferase activity, on the other hand, was largely enriched in the membrane-containing fractions that remained near the top of the gradient after centrifugation. This indicates that 1-acylglycerolphosphate acyltransferase, in contrast to glycerolphosphate acyltransferase, is still bound to membrane fragments.

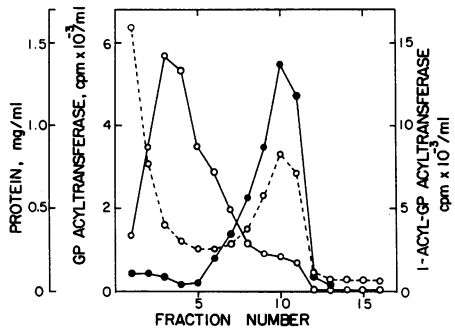

FIG. 1. Resolution of the phosphatidate-synthesizing system by sucrose density gradient centrifugation. A two-layered sucrose gradient was constructed; the lower layer was 0.3 ml of 2 M sucrose, and the upper layer was 3.4 ml of a linear sucrose gradient (0.5–1.1 M) containing 20 mM glycine·NaOH buffer (pH 8.6) and 0.5 mM dithiothreitol. Upon this gradient was applied 1.5 ml of the enzyme preparation (eluate from Sepharose 2B column chromatography, see *Methods*). The tube was centrifuged at 65,000 rpm for 210 min in a Beckman SW 65L rotor. Sixteen fractions were collected and analyzed for glycerolphosphate acyltransferase (O——O), 1-acylglycerolphosphate acyltransferase (●——●), and protein (O---O). Centrifugation from right to left.

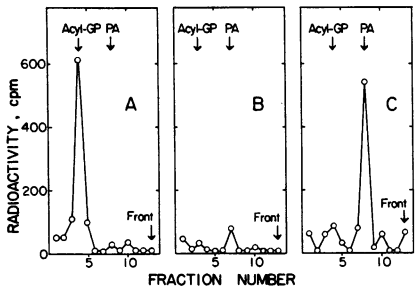

FIG. 2. Reconstitution of the phosphatidate-synthesizing system. The component enzymes were prepared as described for Fig. 1 with the following modifications: 10 ml of the Sepharose 2B eluate was layered upon a two-layered gradient consisting of 2 ml of 2 M sucrose and 20 ml of the linear sucrose gradient, and the tube was centrifuged at 25,000 rpm for 15 hr in a Beckman SW 25.1 rotor. The fractions containing maximal activity of each enzyme were collected. 138 μg of glycerolphosphate acyltransferase (*A*), 80 μg of 1-acylglycerolphosphate acyltransferase (*B*), or a mixture of both enzymes (*C*) was incubated at 20° for 5 min in a reaction mixture containing 40 μmol of Tris·HCl, pH 7.6, 0.5 μmol of *sn*-[1(3)-^{14}C]glycerol 3-phosphate (4,200 cpm/nmol), 20 nmol of palmitoyl-CoA, 10 nmol of linoleoyl-CoA, 3.5 μmol of $CaCl_2$, and 0.43 μmol of soya bean phosphatidylcholine in a total volume of 0.7 ml. The lipid was extracted (10) and separated on a 10 mM Na_2CO_3-impregnated silica gel H plate with ch'oroform–methanol–acetone–acetic acid–water 200:40:80:40:20 as the developing solvent (11). Equal segments of the entire chromatogram were scraped and counted for radioactivity. *Acyl-GP*, 1-acylglycerol 3-phosphate; *PA*, phosphatidic acid.

Reconstitution of the phosphatidate-synthesizing system

Fig. 2 illustrates the results of a reconstitution experiment in which we used preparations of glycerolphosphate acyltransferase and 1-acylglycerolphosphate acyltransferase obtained by the sucrose density gradient centrifugation. When both enzymes were mixed at fairly high concentrations and then incubated with labeled *sn*-glycerol 3-phosphate and acyl donors in the presence of $CaCl_2$ and soya bean phosphatidylcholine, radioactive phosphatidic acid was formed (Fig. 2*C*). Deletion of 1-acylglycerolphosphate acyltransferase resulted in an accumulation of monoacylglycerol 3-phosphate (Fig. 2*A*). It is to be noted here that both palmitoyl- and linoleoyl-CoA were used as acyl donors. When glycerolphosphate acyltransferase was omitted, no appreciable amount of lipid products resulted (Fig. 2*B*). These results clearly establish that two distinct enzymes, glycerolphosphate acyltransferase

TABLE 1. *Substrate specificity of 1-acylglycerolphosphate acyltransferase*

Acyl donor	Phosphatidate formed cpm
Palmitoyl-CoA	990
Stearoyl-CoA	758
Oleoyl-CoA	986
Linoleoyl-CoA	891
Arachidonoyl-CoA	733

The enzyme was prepared as described in Fig. 2. Activity was assayed with 19.6 μg of the enzyme as described in *Methods* except that linoleoyl-CoA was replaced by 10 nmol of various acyl-CoA thioesters.

and 1-acylglycerolphosphate acyltransferase, are required for phosphatidic acid synthesis from *sn*-glycerol 3-phosphate.

Substrate specificity of 1-acylglycerolphosphate acyltransferase

The asymmetric distribution of fatty acids in glycerolipids is considered to be of major importance in the functional and structural roles of these lipids in living organisms. This fatty acid distribution in *E. coli* is effected during the synthesis of phosphatidic acid from *sn*-glycerol 3-phosphate (6, 14). However, the enzymatic mechanism conferring the nonrandom distribution of fatty acids in glycerolipids of mammalian tissues has been the subject of much dispute. Several workers reported that phosphatidic acid is synthesized with a random distribution of fatty acids (15–17), while some recent studies showed that this synthetic process occurs in an asymmetric manner (18–20). We have recently shown that partially purified glycerolphosphate acyltransferase from rat-liver microsomes catalyzes formation of 1-acylglycerol 3-phosphate, using saturated fatty acyl-CoA as acyl donor, and that unsaturated fatty acyl-CoA thioesters are poor substrates for this enzyme (9, 10). Hence, it is of interest to examine the substrate specificity of 1-acylglycerolphosphate acyltransferase, which catalyzes acylation at position 2 of the glycerol backbone. The results of such studies with various acyl-CoA thioesters are recorded in Table 1. The enzyme activity was assayed by determination of the rate of phosphatidic acid formation from 1-palmityl-[*1*(*3*)-^{14}C]glycerol 3-phosphate. This enzyme, in contrast to glycerolphosphate acyltransferase, efficiently used unsaturated fatty acyl-CoA thioesters such as oleoyl-, linoleoyl-, and arachidonoyl-CoA, although palmitoyl- and stearoyl-CoA were also effective. When the effects of palmitoyl- and linoleoyl-CoA as acyl donors were compared at various concentrations (15–55 μM) of the thioesters, both acyl donors were found to be almost equally active at all concentrations examined.

Recently, Okuyama and Lands (21) have demonstrated that the acyl donor selectivity of 1-acylglycerolphosphate acyltransferase in liver microsomes is variable depending upon the incubation conditions, especially upon the concentration of 1-acylglycerol 3-phosphate. It has been shown that palmitate and arachidonate tend to be excluded from position 2 of phosphatidic acid at low concentrations (below 10 μM) of 1-acylglycerol 3-phosphate, whereas relatively nonselective acylation occurs at high concentrations (around 20 μM) of the acyl acceptor. In the experiment shown in Table 1, a relatively high concentration (about 7 μM) of 1-acylglycerol 3-phosphate was used. It is quite probable that the monoacylglycerol 3-phosphate concentration *in vivo* is quite low, since this intermediate does not accumulate in microsomes during formation of phosphatidic acid from *sn*-glycerol 3-phosphate (4, 9, 10, 15).

DISCUSSION

In the present investigation, we have been able to resolve the phosphatidate-synthesizing system of rat-liver microsomes into two component enzymes and to reconstitute it by combining these enzymes. The reconstituted system catalyzes phosphatidic acid synthesis through the following sequence of reactions:

sn-Glycerol 3-phosphate + saturated acyl-CoA
$\xrightarrow{\text{glycerolphosphate acyltransferase}}$ 1-acylglycerol 3-phosphate + CoA

1-Acylglycerol 3-phosphate + unsaturated (or saturated) acyl-CoA
$\xrightarrow{\text{1-acylglycerolphosphate acyltransferase}}$ phosphatidic acid + CoA

This sequence of reactions obviously contributes to the asymmetric distribution of fatty acids in glycerolipids. Recent studies of Okuyama *et al.* (22) have demonstrated that in liver microsomes acylation of 2-acylglycerol 3-phosphate occurs only at about one-tenth the rate observed for 1-acylglycerol 3-phosphate. In addition, Tamai (23) has recently shown by an isotope-trapping technique that virtually no 2-acylglycerol 3-phosphate is produced from *sn*-glycerol 3-phosphate and either palmitoyl- or oleoyl-CoA in liver microsomes. These findings support the view that the sequence of reactions catalyzed by our reconstituted system represents the major pathway of phosphatidic acid synthesis in mammalian tissues.

This investigation was supported in part by research grants from the Ministry of Education of Japan, the Toray Science Foundation, the Japanese Foundation of Metabolism and Diseases, and the Tanabe Amino Acid Research Foundation.

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