Separation of 1-Acylglycerolphosphate A cyltransferase and 1-Acylglycerolphosphorylcholine A cyltransferase of Rat Liver Microsomes (Triton X-100/sucrose density gradient centrifugation/stability/acyl-donor specificity/asymmetric fatty acid distribution)

SATOSHI YAMASHITA, NORIKO NAKAYA, YOSHINOBU MIKI, AND SHOSAKU NUMA

Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto 606, Japan

Communicated by F. Lynen, November 11, 1974

ABSTRACT 1-Acylglycerolphosphate acyltransferase (EC 2.3.1.15) and 1-acetylglycerolphosphorylcholine acyltransferase (EC 2.3.1.23) of rat liver microsomes were separated from each other. The separation was achieved by sucrose density gradient centrifugation of the enzyme preparation that was obtained by solubilizing microsomes with a nonionic detergent, Triton X-100, and subjecting the solubilized microsomes to molecular-sieve chromatography. The two acyltransferases are distinguishable from each other also with respect to their stabilities to heat and to Triton X-100. Hence, it is concluded that these acyltransferases are distinct enzymes. These results, together with our previous finding that glycerolphosphate acyltransferase is also a separate enzyme, demonstrate the presence of distinct acyltransferases responsible for the acylation of the different acyl acceptors. Furthermore, the acyl-donor specificities of these acyltransferases provide the enzymatic basis for the nonrandom distribution of fatty acids in naturally occurring glycerolipids.

The acyl constituents of naturally occurring glycerolipids are distributed usually in a nonrandom manner (1–3). Saturated fatty acids are esterified predominantly at the C-1 position of the glycerol moiety, and unsaturated fatty acids at the C-2 position. Until recently, there had been very little information concerning the enzymatic mechanism responsible for the specific introduction of fatty acids into glycerolipids. In an attempt to elucidate this mechanism, we have recently been able to resolve the phosphatidate-synthesizing system of rat liver microsomes into glycerolphosphate acyltransferase (acyl-CoA:sn-glycerol-3-phosphate O-acyltransferase; EC 2.3.1.15) and 1-acetylglycerolphosphate acyltransferase (acyl-CoA:1-acyl-sn-glycerol-3-phosphate O-acyltransferase; EC 2.3.1.15) (4). The resolution was effected by sucrose density gradient centrifugation of an enzyme preparation derived from microsomes that were solubilized with the use of a nonionic detergent, Triton X-100. Moreover, this synthetic system was reconstituted by combining the two component enzymes (4). Thus, phosphatidic acid is formed by the sequential acylation of sn-glycerol 3-phosphate mediated by the two distinct acyltransferases. In addition, it was demonstrated that glycerolphosphate acyltransferase catalyzes the acylation at the C-1 position preferentially with saturated fatty acyl-CoA thioesters, such as palmitoyl-CoA and stearoyl-CoA, to form 1-acyl-sn-glycerol 3-phosphate (5). In contrast, 1-acetylglycerolphosphate acyltransferase utilizes monoenoic and dienoic fatty acyl-CoA thioesters, including oleyl-CoA, linoleoyl-CoA, and palmitoleoyl-CoA, more efficiently than saturated fatty acyl-CoA derivatives, but is hardly active toward arachidonoyl-CoA (6). Furthermore, the 1-acetylglycerolphosphate acyltransferase preparation was found to exhibit also the activities of 2-acylglycerolphosphate acyltransferase (acyl-CoA:2-acyl-sn-glycerol-3-phosphate O-acyltransferase; EC 2.3.1.15) and 1-acylglycerolphosphorylcholine acyltransferase (acyl-CoA:1-acyl-sn-glycerol-3-phosphorylcholine O-acyltransferase; EC 2.3.1.23) (6). Studies with this preparation showed that 2-acylglycerolphosphate acyltransferase is specific for stearoyl-CoA and palmitoyl-CoA, whereas 1-acylglycerolphosphorylcholine acyltransferase utilizes arachidonoyl-CoA most efficiently (6). These results on the acyl-donor specificities of the four acyltransferases provided the enzymatic basis for the concept that the monoenic and dienoic species of glycerolipids are produced by the synthesis de novo by way of phosphatidic acid of the corresponding species as intermediate, whereas arachidonic acid is introduced into glycerolipids by the decacylation-recacylation cycle (7) operating after the synthesis de novo to yield tetraenoic phosphatidylcholine. The separation of all these acyltransferases would impart further information on the enzymatic mechanism underlying the asymmetric fatty acid distribution in glycerolipids, but has thus far been hampered by the difficulty of isolating membrane-bound enzymes except in the case of glycerolphosphate acyltransferase (4, 5).

The present investigation deals with the successful separation of 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase of rat liver microsomes. Studies on the stabilities of these acyltransferases to heat and to Triton X-100, as well as on the acyl-donor specificities of the separated enzymes, are also included. The results of these studies, together with those of our previous work (4–6), demonstrate the presence of distinct acyltransferases mediating the acylation of the different acyl acceptors and contribute to the elucidation of the enzymatic mechanism responsible for the specific introduction of fatty acids into glycerolipids.

MATERIALS AND METHODS

Triton X-100 was a product of Rohm and Haas (Philadelphia, Pa.). Lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, and arachidonic acids were obtained from Applied Science Laboratory (Pennsylvania State University, University Park, Pa.). Acyl-CoA thioesters were prepared by the mixed anhydride method of Wieland and Rueff (8) and were purified as described by Seubert (9). The acyl constituent of the acyl-CoA preparations was examined as follows: the fatty acids released by mild alkaline hydrolysis of the thioesters
were esterified with diazomethane, and the methyl esters were analyzed by gas-liquid chromatography at 165° with the use of a column (2 mm × 1.8 m) containing 15% ethylene glycol succinate coated on Neopak 1A (60–80 mesh; Nishikogyo, Tokyo, Japan). The results indicated that the purity of the acyl-CoA preparations with respect to their acyl constituent was 99% or more. [1-14C]Oleyl-CoA was prepared enzymatically and purified as reported (6). The concentration of acyl-CoA thioesters was determined as described by Zahler et al. (10). 1-Acyl-sn-glycero-3-phosphate (5) and 1-acyl-sn-glycero-3-phosphorylcholine (6) were prepared enzymatically from egg phosphatidylcholine by described procedures.

1-Acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase were separated from each other as follows. All operations were conducted at 0–4°. Rat liver microsomes were prepared as reported (5) and were solubilized with 6 mM Triton X-100 in the presence of 20 mM glycine·NaOH buffer (pH 8.6) and 20% (v/v) ethylene glycol; the protein concentration was 10 mg/ml. This mixture (6 ml) was applied to a column (2.6 × 16-cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden) that had been equilibrated with 20 mM glycine·NaOH buffer (pH 8.6) containing 0.25 mM Triton X-100 and 20% (v/v) ethylene glycol. Material was eluted from the column with the same solution. The fractions containing the acyltransferase activities were combined and subjected to 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 20% (v/v) ethylene glycol. Upon this gradient was applied 1.5 ml of the enzyme solution eluted from the Sepharose 2B column. The tube was centrifuged in a Beckman SW 65L rotor at 65,000 rpm for 16 hr. After the centrifugation, 16 fractions were collected and assayed for the activities of 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase, as well as for protein. The fractions exhibiting the highest activities (usually, fractions no. 1 and no. 2 for 1-acylglycerolphosphorylcholine acyltransferase and fractions no. 11 and no. 12 for 1-acylglycerolphosphate acyltransferase; see Fig. 1) were combined and used for experiments on the acyl-donor specificities and on the reaction products of the acyltransferases.

The partially purified enzyme preparation used to study the stabilities of the acyltransferases to heat and to Triton X-100 was obtained by a described procedure (6). The concentration of Triton X-100 used for the solubilization of rat liver microsomes was 5 mM, and the sucrose density gradient centrifugation was carried out in a Beckman SW 25.1 rotor at 25,000 rpm for 16 hr.

The activities of 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase were assayed by measuring the acyl-acceptor-dependent release of CoA spectrophotometrically with the use of 5,5′-dithiobis(2-nitrobenzoic acid) as described (6). Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as the standard; particulate protein was solubilized with 48 mM deoxycholate prior to the determination.

**RESULTS**

*Separation of 1-Acylglycerolphosphate Acyltransferase and 1-Acylglycerolphosphorylcholine Acyltransferase.* The procedure used to separate 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase of rat liver microsomes from each other was similar to that used for the resolution of the phosphatidate-synthesizing system (4). Since the two acyltransferases were found to be associated with each other when rat liver microsomes were solubilized with 5 mM Triton X-100 as used in most of the previous experiments (6), the concentration of the detergent was raised to 6 mM; the adequate concentration of Triton X-100 varied slightly with different batches of microsomes. Moreover, it was necessary to stabilize 1-acylglycerolphosphate acyltransferase by the addition of ethylene glycol. In Fig. 1 are illustrated the results of a typical experiment to separate the two enzymes by means of sucrose density gradient centrifugation. 1-Acylglycerolphosphorylcholine acyltransferase sedimented almost to the bottom of the gradient, whereas 1-acylglycerolphosphate acyltransferase remained near the top of the gradient. The direction of centrifugation is from right to left.

1-Acylglycerolphosphate acyltransferase activity assayed with oleyl-CoA (○) or with palmitoyl-CoA (●); 1-acylglycerolphosphorylcholine acyltransferase activity assayed with arachidonoyl-CoA (△) or with linoleyl-CoA (▲); protein (×).
FIG. 2. Heat inactivation of 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase. The partially purified enzyme preparation (protein concentration, 0.46 mg/ml) obtained as described in Materials and Methods was incubated at 43° for the various lengths of time indicated and was chilled at 0°. The activities of 1-acylglycerolphosphate acyltransferase (○) and 1-acylglycerolphosphorylcholine acyltransferase (△) were then assayed with oleyl-CoA and with arachidonyl-CoA, respectively, as described in Materials and Methods.

The reaction products formed by the actions of the separated 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase were identified by thin-layer chromatography as phosphatidic acid and phosphatidylcholine, respectively. For this purpose, each enzyme was incubated with [1-14C]oleyl-CoA and the respective acyl acceptor in a reaction mixture similar to that used for the enzyme assay, except that 5,5′-dithiobis(2-nitrobenzoic acid) was omitted. The radioactive lipid product formed was extracted with chloroform–methanol 2:1 and chromatographed on a thin-layer plate as described (6) with chloroform–methanol–acetone–acetic acid–water 200:40:80:40:20 (12) (for phosphatidic acid) or chloroform–methanol–acetic acid–water 25:15:4:2 (13) (for phosphatidylcholine) as developing solvent.

Stability to Heat and Triton X-100. In an attempt to provide further evidence indicating that 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase are distinct enzymes, a partially purified preparation exhibiting the activities of both these enzymes was subjected to heat treatment at 43°. As evident from the data of Fig. 2, the activity of 1-acylglycerolphosphate acyltransferase was much more stable at this temperature than that of 1-acylglycerolphosphorylcholine acyltransferase; the half-lives for heat inactivation of these enzymes were 62 min and 12 min, respectively.

Table 1 shows the stabilities of the two enzyme activities to Triton X-100. The activity of 1-acylglycerolphosphorylcholine acyltransferase was more resistant to the detergent than that of 1-acylglycerolphosphate acyltransferase.

<table>
<thead>
<tr>
<th>Triton X-100 (mM)</th>
<th>1-Acylglycerolphosphate acyltransferase (%)</th>
<th>1-Acylglycerolphosphorylcholine acyltransferase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>22</td>
<td>60</td>
</tr>
<tr>
<td>1.5</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2.0</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

The partially purified enzyme preparation obtained as described in Materials and Methods was treated with Triton X-100 in the various concentrations indicated at 0° for 60 min; the protein concentration was 0.30 mg/ml. The activities of the acyltransferases were then assayed as described for Fig. 2.
Protein Nat. Acad. Sci. USA 72 (1975)

Fig. 4. Aeryl-donor specificity of 1-acylglycerolphosphorylcholine acyltransferase. The activity was assayed with 50 µg of the enzyme as described in Materials and Methods, except that the acyl-CoA concentration was varied as indicated. The symbols denote the same acyl donors as in Fig. 3.

In Fig. 4 is shown the acyl-donor specificity of the separated 1-acylglycerolphosphorylcholine acyltransferase. The best acyl donor was arachidonyl-CoA. Linoleyl-CoA and oleyl-CoA were also fairly effective. Palmitoleyl-CoA, lauril-CoA, myristyl-CoA, palmitoyl-CoA, and stearyl-CoA were utilized at very low rates.

DISCUSSION

In the present investigation, 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase of rat liver microsomes were separated clearly from each other. Furthermore, these acyltransferases were shown to differ from each other in their stabilities to heat and to Triton X-100. It is concluded from these results that 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase are distinct enzymes. In addition, we have evidence indicating that 2-acylglycerolphosphate acyltransferase is also an enzyme distinct from the two acyltransferases just mentioned; under the conditions used in the present work, the half-life for heat inactivation at 43° of 2-acylglycerolphosphate acyltransferase was 20 min, which was longer than those of 1-acylglycerolphosphate acyltransferase (62 min) and 1-acylglycerolphosphorylcholine acyltransferase (12 min), and 2-acylglycerolphosphate acyltransferase was more resistant to Triton X-100 than the two other acyltransferases. Moreover, our previous studies demonstrated that glycerolphosphate acyltransferase is an enzyme separate from the above-mentioned three acyltransferases (4, 6). All these results indicate that liver microsomes contain distinct acyltransferases responsible for the acylation of the different acyl acceptors.

The acyl-donor specificities of the separated 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase are in general agreement with those observed in our previous work (6) with the partially purified preparation containing both these enzymes. The only difference to be noted is the fact that linoleyl-CoA and oleyl-CoA are fairly good substrates for 1-acylglycerolphosphorylcholine acyltransferase, as shown in the present investigation. It was reported previously that the partially purified 1-acylglycerolphosphorylcholine acyltransferase was specific for arachidonyl-CoA (6). However, subsequent experiments with similar enzyme preparations revealed that, although arachidonyl-CoA was the most effective acyl donor for this acyltransferase, linoleyl-CoA and oleyl-CoA were also utilized fairly efficiently, just as observed with the separated enzyme. The reason for the discrepant results obtained previously remains to be clarified. In the present experiments, freshly prepared acyl-CoA thioesters were used, and their purity with respect to the acyl constituent was 99% or more as examined by gas-liquid chromatography (see Materials and Methods).

This investigation was supported in part by research grants from the Ministry of Education of Japan, the Mitsubishi Foundation, the Japanese Foundation of Metabolism and Diseases, the Yakuin Foundation, and the Japanese Medical Association.