Purification and Characterization of Glycerophosphate Acyltransferase from Rat Liver Mitochondria*

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Glycerophosphate acyltransferase (GAT) catalyzes the conversion of sn-glycerol 3-phosphate to lysophosphatidic acid (LPA), the first and committed step of triacylglycerol and phospholipid synthesis. In spite of the important regulatory roles GAT may play in this biosynthetic pathway, little information is available on the structure, biochemical properties, and regulation of GAT from eukaryotic cells. We solubilized GAT from rat liver mitochondrial membranes and purified it to an apparent homogeneity by hydroxylapatite chromatography, preparative isoelectric focusing, and gel filtration. The enzyme is composed of a single polypeptide of 85 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration chromatography of the native protein. The GAT activity was completely lost during the purification procedure and required addition of exogenous phospholipids for its reconstitution. Since a high phospholipid to detergent ratio was needed for full reactivation, it is concluded that GAT requires "lipid boundary" for reconstitution. The ability of different phospholipids to reconstitute GAT decreased in the following order: phosphatidylglycerol (PG), phosphatidylyethanolamine (PE), phosphatidylcholine (PC), asolectin, phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin. 1,2-Dioleoyl derivatives of PG and PE were more effective in reconstituting the GAT activity than corresponding dipalmitoyl derivatives. The GAT activation was further increased by using a combination of PG and PE or PG and PC. Regardless of the phospholipid used for reconstitution, palmitoyl-CoA was the best acyl donor and LPA was the only reaction product.

The first committed step of glycerolipid synthesis is the formation of lysophosphatidic acid (LPA). In eukaryotes, LPA is generated either by direct acylation of sn-glycerol 3-phosphate by glycerophosphate acyltransferase (GAT) or by acylation of dihydroxyacetone phosphate to form acyl dihydroxyacetone phosphate followed by its reduction to LPA. The number and roles of the different acyltransferases catalyzing these reactions and the relative contributions of these two pathways to overall glycerolipid biosynthesis remain unclear (1, 2).

At least three triose phosphate acyltransferase activities appear to function in LPA synthesis in mammalian systems: a peroxisomal dihydroxyacetone phosphate acyltransferase, a mitochondrial GAT, and a microsomal activity capable of acylating both glycerol 3-phosphate and dihydroxyacetone phosphate (3). It is not clear whether the microsomal acyltransferase activity is due to a single enzyme with dual specificity or due to two enzymes with different specificities for triose phosphates (3–7). These mammalian acyltransferases are expected to play a regulatory role in glycerolipid synthesis and, therefore, attract significant attention. However, they have proven difficult to solubilize and purify (8–12). Bell and co-workers used molecular biology tools to clone, overexpress, purify and characterize Escherichia coli GAT (13–19).

The research focus in our laboratory has centered on mitochondrial GAT because there is a significant amount of evidence showing that this enzyme is responsible for predominance of saturated fatty acids found in position 1 of naturally occurring glycerophospholipids (20). The asymmetric distribution of fatty acids is considered to be of major importance in maintaining the functional and structural role of phosphoglycerides in biological membranes (21, 22). Consistent with the proposed role, the mitochondrial GAT prefers palmitoyl-CoA four to six times over oleoyl-CoA as an acyl donor while the microsomal enzyme does not show acyl-CoA specificity (23–25). Support for the role of mitochondrial GAT in regulation of the asymmetric distribution of fatty acids in cellular glycerolipids has come from several experiments. First, in Ehrlich ascites tumor cells, where the choline phosphoglycerides do not show the asymmetric distribution of fatty acids (26), the activity of the mitochondrial GAT cannot be detected (25). Second, in cells grown in primary culture, the mitochondrial GAT activity decreases with a concomitant increase in the amount of 18:1 fatty acid in position 1 in choline phosphoglycerides (27). Third, since the principal site for synthesis of complex phospholipids are microsomes, LPA synthesized by mitochondrial GAT must be transported to the endoplasmic reticulum for lipid biosynthesis. Consistent with this hypothesis, we have identified fatty acid-binding protein as the factor responsible for stimulation of mitochondrial GAT and export of LPA from mitochondria (20, 28). Indeed, fatty acid-binding protein inhibited conversion of LPA to phosphatidic acid in mitochondria but stimulated the same reaction in microsomes (20). These observations strengthen the idea that mitochondrially made LPA can at least partially exit the organelle and be transported to endoplasmic reticulum where it serves as a precursor for the synthesis of complex phospholipids.

Despite the fact that GAT plays a regulatory role in glycerolipid synthesis, the systematic study of this enzyme was hampered by its extremely difficult purification. The goals of this work were to establish a purification procedure for isolation of

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The abbreviations used are: LPA, 1-acyllysophosphatidic acid; GAT, acyl-CoA sn-glycerol 3-phosphate O-acyltransferase; PAGE, polyacrylamide gel electrophoresis; PG, phosphatidylglycerol; PE, phosphatidyethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; MES, 4-morpholinoethanesulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]aminomethaneethanesulfonic acid; FMSF, phenylmethylsulfonyl fluoride.
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the mitochondrial GAT and characterize the requirement of the purified protein for its activation by phospholipids.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Sprague-Dawley rats were purchased from Taconic Farms, Germantown, NY. sn-12-HGlycerol-3-phosphate was purchased from American Radiolabeled Chemicals Inc. or synthesized enzymatically from sn-12-HGlycerol (DuPont-NEN) and purified as described previously (25). Bio-Gel HT, silver stain kit, amphotoles, and molecular weight markers for SDS-PAGE were obtained from Bio-Rad, Schönyl, and pHstat. Polyvinylidene difluoride and other protein purification media were from Pharmacia Biotech Inc. Lubrol PX, Triton X-100, CHAPS, and octyl p-glu-dium deoxycholate, all phospholipids, and standards used in gel filtration were obtained from Sigma, and Silica Gel G plates were from Whatman.

**Preparation of Mitochondria**—Rat liver mitochondria were prepared from 150–200-g male rats as described previously (12, 20). Mitochondrial membranes were prepared by resuspending the mitochondria (10 mg/ml) in 20 mM Tris-HCl buffer, pH 8.4, containing 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml each of pepstatin, aprotinin, and leupeptin. The mixture was exposed to sonication for 30 min (30 s exposures with 15-s intervals) using Megasoan Ultrasonic Disintegrator working at nine-tenths of its maximum output. The lysate was then centrifuged at 170,000 g for 90 min, and the sediment containing mitochondrial membranes was resuspended and solubilized as described below.

**Analytical Methods**—GAT activity was measured by following the incorporation of 3H-glycerol 3-phosphate into butanol-extractable phospholipids (25). LPA was identified as the reaction product of purified GAT by thin layer chromatography on silica gel plates as described previously (29). Protein was determined by the method of Lowry et al. (29) and as modified for the presence of detergents (30) or according to Whitaker and Granum (31). SDS-PAGE analysis was performed as described previously (20).

**Purification of Mitochondrial GAT**—The purification procedure started with the solubilization of mitochondrial membranes (mito- chondrial phospholipids) by resuspending them in a buffer containing 20 mM Tris-HCl buffer, pH 8.4, 20% glycerol, 1 mM KCl, 1 mM PMSF, 20 μg/ml each of pepstatin, aprotinin, and leupeptin, and 0.5% Lubrol PX. The mixture was gently homogenized by five strokes in glass-Teflon homogenizer (Wheaton Scientific) and incubated on ice for 30 min with occasional gentle homogenization. The mixture was then centrifuged at 170,000 g for 90 min, and the supernatant was taken as "solubilized membrane." This extract was transferred to a buffer containing 50 mM potassium phosphate, pH 7.5, 10% glycerol, 0.2% CHAPS, and 1 mM PMSF on PD-10 columns (Pharmacia; 2.5-ml aliquots of the extract were loaded on multiple PD-10 columns equilibrated in the above buffer, and the sample was subsequently eluted from each column with 3.5 ml of the same buffer) and loaded on the column of Bio-Gel HT (1 × 10 cm), equilibrated in the same buffer. The column was first washed with 50 ml of this buffer and then eluted with 100 ml of gradient of 50–500 mM potassium phosphate buffer, pH 7.4, containing 0.2% CHAPS and 10% glycerol. Fractions exhibiting GAT activity were combined and transferred to 10 mM Tris, pH 7.5, 10% glycerol, 5 mM β-mercaptoethanol, and 0.2% CHAPS using PD-10 columns as described above. This pooled fraction was supplemented with amphotoles (5–10) at 1.0% (final concentration) and electrofocused at 120 w for 3 h in the Roto- form TF cell (parameters at the beginning: 13 kV, 600 V, parameters at the end: 86 kV, 1150 V) at 4 °C. The fraction with the highest activity of GAT was loaded directly on a column of Sephacryl S-300 (1 × 10 cm) equilibrated in 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% CHAPS. The column was eluted with the same buffer and peak fractions combined. All purification steps were performed at 4 °C and did not take longer than 3 days.

**Reconstitution of GAT Activity**—To determine the GAT activity in defined phospholipid environment, homogenous GAT was reconstituted by modification of method of Scheider and Bell (17). CHAPS was exchanged for Lubrol PX in the preparation of purified GAT on a Bio- gel HT column (0.5 × 1 cm). After the sample was loaded, the column was washed with 5 ml of buffer containing 50 mM potassium phosphate, pH 7.5; 10% glycerol; 0.1% Lubrol PX and GAT eluted with buffer containing 500 mM potassium phosphate, pH 7.5, 10% glycerol, and 0.1% Lubrol PX. An aliquot of purified enzyme in this buffer was added to a dish containing 1 ml of a phospholipid vesicle suspension prepared from pure commercially available phospholipids by removal of solvent under nitrogen and sonication in 100 mM MES-TESE-glyceryl- cin buffer, pH 7.5, containing 10 mM β-mercaptoethanol; the phospholipid dispersion was then centrifuged in order to remove large lipid aggregates and titaniu m shed. Following the addition of MES-TESE-glyceryl-glycerine buffer to achieve concentration 40 mM in the final volume of reaction mixture (0.5 ml), 100 μl of 5 times concentrated mixture of reaction components (10 mM MgCl2; 250 μM palmitoyl-CoA; 5 mM palmitoyl-CoA) was added. The reaction mixture was incubated on ice for 15 min, and at 37 °C for another 3 min. The reaction was initiated by the addition of 50 μl of 7.5 mM sn-12-HGlycerol 3-phosphate, allowed to proceed at 37 °C for 5 min and stopped by adding 0.5 ml of 1-M butanol.

**Preparation of Crude Dilipidated Mitochondrial Membrane Proteins**—Mitochondrial membranes were prepared by resuspending the mitochondria (10 mg/ml) in 20 mM Tris-HCl buffer, pH 8.4, containing 50 mM potassium phosphate buffer, pH 7.4, 0.5% Lubrol PX, and 10% glycerol. The fraction containing the highest concentration of protein was transferred to a buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.5% Lubrol PX, 1 mM PMSF, and 20 μg/ml each of pepstatin, aprotinin, and leupeptin. Different aliquots were assayed for GAT activity either alone or in combination with purified GAT.

**Tryptin Treatment of Mitochondrial Membrane Proteins**—Crude dilipidated mitochondrial membrane proteins (6 mg/ml) were incubated 1 h on ice with trypsin (120 μg/ml) in buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.5% Lubrol PX. Soybean trypsin inhibitor was added together with the inhibitor to one aliquot, while the other aliquot received the inhibitor after 1 h of incubation. Both aliquots (60 μg each) were combined with purified GAT and assayed for GAT activity.

**Preparation of Total Mitochondrial Lipids**—Isolated mitochondria were extracted three times with chloroform/methanol (2:1, v/v). Extracts were combined and the solvent removed under nitrogen. The residue was resuspended by sonication in 100 mM MES-TESE-glycerol-glycerine buffer, pH 7.5, containing 10 μM β-mercaptoethanol. The phospholipid suspension was then centrifuged in order to remove large lipid aggregates and titaniu m shed. Following the addition of MES-TESE-glycerol-glycerine buffer, pH 7.5, containing 10 mM β-mercaptoethanol; the phospholipid dispersion was then centrifuged in order to remove large lipid aggregates and titanium shed. Following the addition of MES-TESE-glycerol-glycerine buffer, pH 7.5, containing 10 mM β-mercaptoethanol; the phospholipid dispersion was then centrifuged in order to remove large lipid aggregates and titanium shed. Following the addition of MES-TESE-glycerol-glycerine buffer, pH 7.5, containing 10 mM β-mercaptoethanol; the phospholipid dispersion was then centrifuged in order to remove large lipid aggregates and titanium shed. Following the addition of MES-TESE-glycerol-glycerine buffer, pH 7.5, containing 10 mM β-mercaptoethanol; the phospholipid dispersion was then centrifuged in order to remove large lipid aggregates and titanium shed. Following the addition of MES-TESE-glycerol-glycerine buffer, pH 7.5, containing 10 mM β-mercaptoethanol; the phospholipid dispersion was then centrifuged in order to remove large lipid aggregates and titanium shed.
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Solubilization of GAT from mitochondrial membranes

Mitochondrial membranes were prepared as described under "Experimental Procedures," resuspended in a mixture containing 20% glycerol, 1 mM KCl, 1 mM PMSF, and 20 μg/ml each of pepstatin, aprotinin, and leupeptin. This suspension was aliquoted and individual aliquots incubated with occasional homogenization on ice for 30 min with different amounts of detergents. The lysates were centrifuged at 170,000 × g for 90 min and supernatants assayed for GAT activity. Results are expressed as percent of GAT activity recovered in supernatant.

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Concentration</th>
<th>Detergent/protein ratio</th>
<th>Yield of GAT activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lubrol PX</td>
<td>0.1</td>
<td>0.22</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.44</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.10</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.20</td>
<td>82</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1</td>
<td>0.15</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.30</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.75</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.50</td>
<td>26</td>
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<tr>
<td>Nonidet P-40</td>
<td>0.2</td>
<td>0.24</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.62</td>
<td>64</td>
</tr>
<tr>
<td>Cholate</td>
<td>0.2</td>
<td>0.48</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.96</td>
<td>83</td>
</tr>
<tr>
<td>Deoxycholate</td>
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<td>0.48</td>
<td>52</td>
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<td></td>
<td>0.5</td>
<td>0.96</td>
<td>83</td>
</tr>
<tr>
<td>CHAPS</td>
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<td>0.24</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.48</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.96</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.92</td>
<td>21</td>
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</table>

**TABLE II**

Purification of GAT

<table>
<thead>
<tr>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>nmol/min</td>
<td>nmol/min/mg</td>
<td>%</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>320.00</td>
<td>169.6</td>
<td>0.53</td>
</tr>
<tr>
<td>Mitochondrial membranes</td>
<td>121.00</td>
<td>150.0</td>
<td>1.24</td>
</tr>
<tr>
<td>Solubilized membranes</td>
<td>103.00</td>
<td>76.2</td>
<td>0.74</td>
</tr>
<tr>
<td>Bio-Gel HT</td>
<td>8.60</td>
<td>76.2</td>
<td>5.68</td>
</tr>
<tr>
<td>Rotofor*</td>
<td>0.53</td>
<td>17.0</td>
<td>32.14</td>
</tr>
<tr>
<td>Sephacryl S-300:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reconstitution in asolectin</td>
<td>0.17</td>
<td>7.3</td>
<td>43.23</td>
</tr>
<tr>
<td>Reconstitution in PG/PE*</td>
<td>0.17</td>
<td>32.3</td>
<td>190.00</td>
</tr>
</tbody>
</table>

* Asolectin was used for reconstitution of GAT activity as described under "Experimental Procedures."

* GAT activity was reconstituted in binary PG/PE mixture as described under "Experimental Procedures" and "Results" (Fig. 6).

Homogeneity of the final preparation was determined by SDS-PAGE (7%) and Coomassie Blue staining. Only one band, corresponding to 85 kDa, was visible on the gel (Fig. 4). This result suggests that GAT was isolated as a monomer. The larger molecular mass obtained by gel filtration compared to the result of SDS-PAGE can be explained by a higher binding of detergent CHAPS by GAT than by proteins used for calibration of Sephacryl S-300 column. The reaction product of homogeneous, reconstituted GAT was identified by thin layer chromatography to be only LPA.

Phospholipid Activation of Purified GAT—Since relatively low recoveries of GAT activity were obtained in each purification step, it was suspected that GAT activation is not fully restored by simple addition of asolectin suspension to GAT assay mixture. Therefore, a systematic study of phospholipid requirement of GAT was performed. Kinetic analyses of activation of membrane enzymes using phospholipid effectors are best performed employing detergent/phospholipid mixed micelle systems (32-35). Variety of detergents (Triton X-100, Lubrol PX, CHAPS, cholate, deoxycholate, digitonin, β-ocetyl glucoside, Zwittergent 3-14) were tested for their ability to support GAT.
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**Fig. 4. SDS-PAGE of purified GAT.** Purified GAT (4 μg; pooled fractions 24–26 of Sephacryl S-300 column) was analyzed on 7% SDS-PAGE gel and stained with Coomassie Blue. Molecular mass standards included β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (22 kDa).

**Fig. 5. Specific phospholipid requirement for GAT activity.** Homogenous GAT was reconstituted in different phospholipids as described under “Experimental Procedures,” and data are presented as the final ratio (mole/mole) of phospholipid to detergent in the assay. Phospholipids used for reconstitution were: dioleoyl (●, line 1; ○, line 3) and dipalmitoyl (×, line 2; ■, line 4) derivatives of PG (●, line 1; ×, line 2) or PE (○, line 3; ■, line 4) (Panel A) and PC (●, line 1), asolectin (○, line 2), PI (×, line 3), PS (■, line 4), and CL (△, line 5) (Panel B). The data represent the average of three experiments which agreed within 5%.

Lubrol PX, has a long saturated hydrocarbon tail and also supported GAT activity, although to a lower level. Triton X-100 was even less capable of supporting GAT activity. Bile salts, cholate, deoxycholate, and CHAPS completely inhibited GAT activity, probably because of high concentrations required to achieve the formation of micelles. These results suggest that GAT activity can be reconstituted only in the presence of detergents which have a long saturated hydrocarbon tail and are nonionic or zwitterionic. Similar results were reported for GAT purified from *E. coli* membranes (17). In a mixed phospholipid/detergent micelle these detergents would be expected to permit the efficient side-to-side packing of adjacent phospholipid acyl chains. However, without addition of phospholipids to the reconstitution assay, the GAT activity was not detectable even in the presence of these two detergents. This result shows that 1) during the purification GAT was efficiently delipidated and 2) GAT requires specific interaction with phospholipids for reconstitution of its activity. All tested phospholipids (PG, PE, PC, asolectin, PI, PS, and CL) activated GAT in the presence of Lubrol PX. However, the efficiency of reconstitution of GAT activity by different phospholipids varied greatly (Fig. 5). The highest GAT activity was achieved in the presence of 1,2-dioleoyl derivative of PG (145 nmol/mg.min), followed by 1,2-dioleoyl derivative of PE (120 nmol/mg.min) and PC (85 nmol/mg.min). All other phospholipids (PI, PS, CL, 1,2-dipalmitoyl derivatives of PG and PE and mixture of soybean phospholipids asolectin) stimulated GAT activity with lower efficiency (Fig. 5). Also, half-maximal activation of the enzyme activity required different concentrations of individual phospholipids: 0.54, 1.2, and 1.4 mol of phospholipid/mol of Lubrol PX were required for 50% activation of GAT when PG (1,2-dioleoyl), PE (1,2-dioleoyl), or PC were used, respectively. The effect of binary phospholipid mixtures on GAT activity was determined using GAT reconstituted in PG (1,2-dioleoyl)/Lubrol PX mixed micelles (1.5 mol PG/mol Lubrol PX; Fig. 6). Addition of PE (1,2-dioleoyl) or PC resulted in further 30 or 28% activation of GAT activity, respectively. On the contrary, addition of LPA or PA almost completely inhibited the enzyme activity (Fig. 6). These data suggest that mitochondrial GAT belongs to a group of membrane enzymes which require lipid activation involving a “lipid boundary” (35).

Since mitochondrial GAT is thought to be responsible for preferential incorporation of saturated fatty acids in position 1
of naturally occurring glycerophospholipids, it was of interest to determine the specificity of purified GAT reconstituted in defined phospholipid environment (Table III). Under these conditions GAT exhibited Michaelis-Menten kinetics and was able to use myristoyl-CoA, palmitoyl-CoA, stearoyl-CoA, or oleoyl-CoA as acyl donors. Double reciprocal plots of initial velocity measurements showed that the best acyl donor is palmitoyl-CoA. The phospholipids used for reconstitution of GAT exhibited significant effect on both the $K_m$ and $V_{max}$ values of all substrates. The lowest $K_m$ and highest $V_{max}$ values for all acyl donors and glycerol 3-phosphate were observed when GAT was reconstituted in PG (1,2-dioleoyl).

To determine if our reconstitution procedure supplies all factors required for full activity of GAT, solubilized mitochondrial membranes were added to the assay mixture containing purified GAT reconstituted in PG (dioleoyl) (Table IV). Unexpectedly, the mixture of purified GAT and solubilized membranes exhibited significantly higher activity than the calculated additive activity of purified GAT and solubilized mitochondrial membranes assayed separately. When a total lipid fraction was prepared from solubilized mitochondrial membranes (or whole mitochondria) and subsequently added to the assay of purified GAT, no increase in activity was observed. Also, treatment of solubilized membranes with trypsin abolished the stimulation of GAT activity by solubilized membranes. To confirm this result and to gather more information about an extent of the stimulation, mitochondrial membranes were solubilized and proteins concentrated and delipidated on a Bio-Gel HT column, and different aliquots assayed for GAT activity either alone or with purified GAT (Fig. 7). The GAT activity in mixtures containing both mitochondrial membrane proteins and purified GAT was dramatically increased over values corresponding to additive activity of purified GAT and mitochondrial membranes assayed separately. The results indicate that some protein factor(s) in mitochondrial membranes stimulate(s) GAT activity or improve(s) the efficiency of reconstitution of GAT activity.

**DISCUSSION**

In this report, we present purification and characterization of GAT from rat liver mitochondria. This membrane protein proved extremely difficult to purify and we had to depart from the purification protocol we had worked out previously (12). The reason for this change was that GAT after a hydrophobic interaction chromatography on octyl-Sepharose CL-4B column was very unstable, and the activity was rapidly lost which prevented further purification. In addition to this change, we also eliminated the ion exchange chromatography on Sepharose Q Fast Flow, because this step did not yield sufficient purification. The affinity chromatography on palmitoyl-CoA or glycerol 3-phosphate-agarose (12) did not prove to be very reproducible and often led to complete loss of activity when used as the last purification step. The purification protocol described here features three steps and yields virtually homogeneous protein. The "critical" purification step was the preparative isoelectric focusing in the Rotofor cell. The successful outcome of this purification step depended heavily on the level of purification achieved by chromatography on Bio-Gel HT column. To avoid protein precipitation in the Rotofor cell, only peak fractions (47-54) of the Bio-Gel HT chromatography were collected and used for further purification.

The reconstitution of purified GAT in defined phospholipid environment (binary mixture of dioleoyl derivatives of PG and PE) increased the specific enzyme activity to about 190 nmol/min/mg and also improved the apparent level of purification of GAT (to 358-fold, Table II). SDS-PAGE analysis (Fig. 4) of our preparation of purified GAT revealed presence of a single polypeptide of about 85 kDa. However, our preparation of purified GAT still exhibits about 35-fold lower specific activity than purified E. coli GAT (15). This can be explained assuming that both enzymes under an "ideal" assay and reconstitution conditions have different specific activity. Another possible explanation is inefficiency of our reconstitution procedure and/or inactivation of the enzyme during purification. Given the sig-
Reactivation of purified GAT by PG, PC, and to a lesser extend also by PE, followed a sigmodial concentration dependence (Fig. 5) suggesting that the enzyme interacts with more than one phospholipid molecule. Such apparent cooperativity has been observed with several lipid-dependent enzymes, among them also enzymes of lipid metabolism (35). Purified GAT showed some head-group specificity, preferring PG over PE (Fig. 5) for both dioleoyl and dipalmitoyl derivatives. However, more striking was the dependence of GAT reactivation on saturation of the acyl chains in activating phospholipids. The activity of GAT reconstituted in 1,2-dioleoyl derivatives of PG or PE was 3-fold higher than in corresponding dipalmitoyl derivatives. Considering that rat liver mitochondrial GAT preferentially incorporates saturated fatty acids in cellular phospholipids (Table III), stimulation of its activity by phospholipids with unsaturated acyl chains may have profound role in regulating the ratio of saturated and unsaturated acyl chains in lipid bilayers. This ratio strongly influences the fluidity of membranes and also the activity of some membrane enzymes (47, 48). This idea also fits nicely with the proposed role of fatty acyl-CoA binding protein in export of LPA from mitochondria (20).

The availability of mammalian or yeast GAT gene (40,49) will permit structure/function studies which would provide more insight into GAT regulation and interaction with phospholipids at the molecular level.

REFERENCES

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