The Purification and Characterization of a Phospholipase A in Hamster Heart Cytosol for the Hydrolysis of Phosphatidylethanolamine

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Phospholipases A₁ and A₂ catalyze the hydrolysis of acyl groups of phospholipids at C-1 and C-2, respectively. These phospholipases are important in phospholipid catabolism and the remodeling of the acyl groups of phospholipids. Phospholipase A₁ from hamster heart cytosol was purified by a combination of ion-exchange and gel filtration chromatography. The purity of the enzyme was assessed by nondenaturing polyacrylamide gel electrophoresis, two-dimension polyacrylamide gel electrophoresis, and immunological studies. The purified enzyme exhibited both phospholipase A₁ and A₂ activities toward phosphatidylethanolamine and had the ability to hydrolyze the acyl groups of phosphatidylethanolamine. However, the enzyme was not active toward lysophosphatidylcholine, diacylglycerol, or triacylglycerol. By Sepharose 6B chromatography, the molecular weight of the purified enzyme was estimated to be 140,000. Analysis of the purified enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the enzyme was composed of identical M, 14,000 subunits. At least six subunits in the native enzyme could be cross-linked by dimethyl suberimidate. Both phospholipase A₁ and A₂ activities showed similar pH profiles, exhibited no absolute requirements for divalent metallic cations, but displayed a high degree of specificity for the acyl groups of phosphatidylethanolamine at both C-1 and C-2. The Kₘ of phospholipases A₁ and A₂ for 1-palmitoyl-2-arachidonoylglycerophosphocholine was found to be identical (0.5 mM).

Phosphatidylethanolamine is the major phospholipid in the mammalian heart (6). We have shown in an earlier report (7) that the majority of the newly formed phosphatidylethanolamine (via the CDP-choline pathway) in the hamster heart is subsequently remodeled by the deacylation-reacylation process. Our findings suggest that cardiac phospholipase A₂ is highly active toward phosphatidylethanolamine. In addition, the increase in the level of lysophosphatidylethanolamine and/or fatty acids during cardiac ischemia (8–11) has been implicated as one of the biochemical factors for the production of cardiac arrhythmias. Since both lysophosphatidylcholine and fatty acids are produced in the heart, the nature of these enzymes toward the major phospholipid in the mammalian heart should be carefully examined.

The presence of phospholipases A₁ and A₂ in the hamster heart has been clearly demonstrated (4). These enzymes have the ability to hydrolyze the acyl groups of phosphatidylethanolamine at C-1 and C-2, respectively. In the same report (4), we have also shown that the majority of the cardiac phospholipase A₁ (and some phospholipase A₂) is located in the cytosolic fraction. The presence of a cytosolic phospholipase A has also been reported in rat (12) and canine (13) hearts. In this study, we report the purification of a cytosolic protein in the hamster heart which exhibits both phospholipase A₁ and A₂ activities.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Purification of Phospholipase A in Hamster Heart Cytosol—Hamster hearts were homogenized in a buffer containing 20 mM Hepes (pH 7.4) to obtain a 20% homogenate (w/v). Cytosol was obtained from the homogenate by differential centrifugation at 150,000 x g for 90 min as described previously (4). The percent cytosolic contamination of other subcellular fractions was estimated from the activities of the marker enzymes in the cytosol and in the tissue homogenate. The assay of these marker enzymes is described under "Experimental Procedures." The cytosol was found to contain 4% 5'-nucleotidase activity (sarcolemma marker), 2% glucose-6-phosphatase activity (sarcoplasmic reticulum marker) and 10% acid phosphatase and 6% deoxyribonuclease activity.

1 Portions of this paper (including "Experimental Procedures," Fig. 1M, Table 1M, and additional Refs. 1–23) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 87M-1068, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethyleneminitro)]tetracetic acid; Mε, methylergosterol.

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bonuclease activities (lysosomal markers). In a typical purification process, four to six hamster hearts were used for the preparation of cytosol.

The cytosol was applied to a CM-cellulose column (1 × 21 cm) equilibrated with 50 mM Hepes and 5 mM EDTA (pH 7.4). Subsequent to the application of the cytosol, 120 ml of the same buffer was applied to the column, which was followed by a linear gradient of 0–1 M NaCl in 200 ml of the same buffer. The content in each fraction was assayed for phospholipase A activity with phosphatidylcholine as substrate, and enzyme activity was determined by the amount of radioactivity in the lysophosphatidylcholine fraction after the reaction. The use of this natural substrate (isolated from the heart) would not enable us to differentiate between the phospholipase A1 and A2 activities. However, the labeled phosphatidylcholine with mixed acyl groups would allow us to identify all phospholipase A activity that was eluted from the column, some of which might otherwise remain undetected due to possible acyl specificities of the enzymes.

Phospholipase A activity was resolved into two peaks by CM-cellulose chromatography (Fig. 1). The first peak, which accounted for about 20% of total enzyme activity, did not appear to bind to the column. The second peak was eluted after the application of the NaCl gradient and accounted for 80% of the total activity eluted from the column. The total enzyme activity obtained after CM-cellulose chromatography was 55% higher than the total enzyme activity found in the cytosolic fraction (Table I). It appears that either the cytosolic enzyme was activated after chromatography or an inhibitor of the enzyme in the cytosol was removed during the purification.

The active fractions from the major activity peak of the CM-cellulose column chromatography were pooled, and the volume was reduced by ultrafiltration to 1 ml. The concentrated sample was applied to a Sepharose 6B column (1.5 × 75 cm) which was equilibrated with 50 mM Hepes and 5 mM EDTA (pH 7.4). The enzyme was subsequently eluted from the column with the same buffer. Phospholipase A activity was eluted as a sharp peak, which was located between fractions 41 and 46 (Fig. 2). Calibration of the Sepharose 6B column by proteins of known molecular weights revealed that the enzyme had a mean molecular weight of 140,000. The peak fractions (fractions 43–44) collected were pooled, concentrated by ultrafiltration, and stored at −20 °C. The result of the purification is summarized in Table I.

Assessment of Purity—An aliquot of the sample obtained after Sepharose 6B chromatography was subjected to polyacrylamide gel electrophoresis. Protein samples containing 0.03 mg of protein each were applied to the tube gels (0.7 × 9.5 cm), and electrophoresis was carried out at 4 °C at 4 mA/gel. After electrophoresis, one of the gels was stained with Coomassie Blue, and another gel was sliced in 0.25-cm segments. The proteins in the segments were eluted from the polyacrylamide gel by incubation at 4 °C with 0.2 ml of 50 mM Hepes and 5 mM EDTA (pH 7.4) overnight, and enzyme activity in the eluents was determined. As depicted in Fig. 3, when the gel was stained with Coomassie Blue, only one protein band was observed, which corresponded to the segments where enzyme activity was detected. The purified enzyme sample (0.03 mg) was also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins with known molecular weights were used as standards. For comparative purposes, the cytosolic proteins were also analyzed under identical conditions. After electrophoresis, the proteins were stained with Coomassie Blue. Only one protein band was detected from the purified enzyme which was estimated to have a molecular weight of 14,000 (Fig. 4). When the enzyme was analyzed by a two-dimensional polyacrylamide gel electrophoresis, only one spot was observed, which also displayed a molecular weight of 14,000 (Fig. 5).

Immunological Studies—Antibodies for the purified phospholipase A were obtained from rabbit serum as described under "Experimental Procedures." Control serum was obtained from the same rabbit prior to immunization. The antiserum reacted with the purified enzyme and yielded a single sharp precipitin line after double diffusion analysis in an Ouchterlony dish (Fig. 6). A sharp single precipitin line was also obtained in the double diffusion study when the antiserum reacted with the phospholipase A in cytosol. Surprisingly, the antiserum cross-reacted with the supernatant obtained from Triton X-100-treated microsomal and mitochondrial fractions. The ability of the rabbit antibodies to inhibit the activity of phospholipase A was also investigated. Purified phospholipase A (25 µg) was incubated with 0.06 ml of antiserum for 2 h at 25 °C. The mixture was centrifuged (5000 × g for 10 min), and an aliquot of the supernatant was used for the determination of enzyme activity. Normal sera obtained from the rabbit prior to immunization were used as controls.

![Figure 1: Elution profile of phospholipase A from hamster heart cytosol by CM-cellulose chromatography.](image)

**Table I**

<table>
<thead>
<tr>
<th>Purification of phospholipase A from hamster heart cytosol</th>
<th>Total activity (nmol/h)</th>
<th>Protein (mg)</th>
<th>Specific activity (nmol/h/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>610</td>
<td>88.72</td>
<td>6.9</td>
<td>76</td>
</tr>
<tr>
<td>CM-cellulose chromatography</td>
<td>945</td>
<td>1.80</td>
<td>526.7</td>
<td>187</td>
</tr>
<tr>
<td>Sepharose 6B chromatography</td>
<td>381</td>
<td>0.33</td>
<td>1154.5</td>
<td></td>
</tr>
</tbody>
</table>
Hamster Heart Cytosolic Phospholipase A

**FIG. 2.** Purification of hamster heart cytosolic phospholipase A by Sepharose 6B chromatography. The major active fractions obtained from CM-cellulose chromatography were pooled, concentrated by ultrafiltration to 1 ml, and applied to a Sepharose 6B column (1.5 x 75 cm) equilibrated with Hepes (pH 7.4) and 5 mM EDTA. Fractions (2.5 ml) were collected, and enzyme activity (●) in each fraction was assayed with phosphatidyl[methyl-^3H]choline as substrate. Protein concentration (——) in each fraction was monitored at A_280 nm.

**FIG. 4.** Analysis of hamster heart cytosolic phospholipase A by polyacrylamide gel electrophoresis. Enzyme samples after Sepharose 6B chromatography (0.03 mg) and cytosolic proteins in 0.02 ml of cytosol were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cytosolic protein (lane a), cytosolic phospholipase A after Sepharose 6B chromatography (lane b), and protein standards (lane c) are depicted. The dye front of the gel is indicated by the arrow.

**FIG. 5.** Two-dimensional polyacrylamide gel electrophoresis of cytosolic phospholipase A. Enzyme sample after Sepharose 6B chromatography (0.03 mg) was first analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions and subsequently in the presence of sodium dodecyl sulfate as described under “Experimental Procedures.” Protein standards were included after the first electrophoresis. The arrow indicates the dye front of the second electrophoresis.

controls. A maximum of 85% inhibition of enzyme activity was obtained when the enzyme was incubated with 0.15 ml of antiserum.

**Properties of Cytosolic Phospholipase A**—When the enzyme sample obtained from Sepharose 6B chromatography was rechromatographed by the same column, the active fraction was eluted as a single and symmetrical peak, with an estimated molecular weight of 138,000–140,000. The enzyme activity profile corresponded to the protein profile after chromatography. Phospholipase A activity in hamster heart cytosol was slightly enhanced by the addition of phenylmethylsulfonyl fluoride (1 mM) or pepstatin A (1 μM) during the preparation of tissue homogenates. However, the specific activity of the enzyme sample at different stages of purification was not affected by the presence of these protease inhibitors.
The inclusion of the protease inhibitors did not affect the molecular weight of the enzyme estimated by Sepharose 6B chromatography or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme preparation was found to be stable when stored frozen (−20 °C) in 50 mM Hepes and 5 mM EDTA (pH 7.4) for 48 h. About 75% of the enzyme activity was recovered when the enzyme was stored at −20 °C for 1 week. Storage at −70 °C did not improve the stability of the enzyme.

The results obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis provide strong evidence that the enzyme is composed of identical subunits of $M_r 14,000$. The number of subunits in the native enzyme was investigated by cross-linking studies. The enzyme (0.3 mg) obtained after Sepharose 6B chromatography was cross-linked by dimethyl suberimidate (0.5 mg) in 1 ml of 0.2 M triethanolamine (14). Analysis of the cross-linked product by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that at least six products could be identified (Fig. 7). The molecular weights of these products ranged from $M_r 15,000-90,000$ and appeared to be in multiples of $M_r 14,000-15,000$. The reason for the shift of the molecular weight of the lowest subunit from $M_r 14,000$ to 15,000 after dimethyl suberimidate treatment was not clear, but the slight increase in molecular weight might result from the reaction between the enzyme subunits and the cross-linking reagent. Our results suggest at least six subunits of the native enzyme can be cross-linked.

From the results obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4), phospholipase A appears to be one of the major proteins in the hamster heart cytosol. Since fatty acid-binding protein in mammalian heart has a low molecular weight and is present in significant amount in the cytosol (15, 16), it could be argued that the cytosolic phospholipase A may also be the fatty acid-binding protein. In order to investigate this possibility, the partially purified enzyme obtained after CM-cellulose chromatography was analyzed for fatty acid-binding protein activity by the procedure of Said and Schulz (16). The enzyme sample was incubated with 1 nmol of [9,10-3H]oleic acid at 37 °C for 25 min. Subsequent to incubation, the mixture was applied to a Sepharose 6B column. The fractions eluted from the column were analyzed for protein content, as well as fatty acid-binding protein activity (by the determination of radioactivity in each fraction) and phospholipase A activity. As depicted in Fig. 1M (see the Miniprint), the radioactive peak ($M_r 50,000$) was distinct from the phospholipase activity peak. In addition, the radioactive peak did not contain any detectable protein. Analysis of the radioactive peak revealed that it was composed of only oleic acid. The results from this study clearly show that the cytosolic phospholipase A after CM-cellulose chromatography did not contain any fatty acid-binding protein activity.

Additional evidence to differentiate between the cytosolic phospholipase A and fatty acid-binding protein was obtained from immunological studies. Fatty acid-binding protein was

FIG. 6. Double diffusion analysis of anti-phospholipase A antisera obtained from rabbit. Double diffusion analysis of anti-phospholipase A antisera was performed in an Ouchterlony dish. The precipitin lines were stained with Amido black. The center well contained 20 μl of antisera. The side wells contained purified phospholipase (well A), hamster heart cytosol (well B), Triton X-100-treated mitochondrial fraction (well C), and Triton X-100-treated microsomal fraction (well D).

FIG. 7. Cross-linking of the phospholipase A subunits. Phospholipase A obtained from Sepharose 6B chromatography was cross-linked with dimethyl suberimidate as described under "Experimental Procedures." The cross-linked products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein standards (lane A), phospholipase A-treated with dimethyl suberimidate (lane B), untreated enzyme (lane C), and cross-linking reagents without enzyme (lane D) are depicted. The arrow indicates the dye front after electrophoresis.
partially purified from heart by gel filtration chromatography (16). The sample obtained from the pooled active fractions did not form any immunoprecipitant (Ouchterlony) with the anti-phospholipase A antibodies. The result of this study shows that the fatty acid-binding protein and cytosolic phospholipase A are immunologically distinct. In addition, the amino acid composition of hamster heart cytosolic phospholipase A was analyzed. One striking aspect from this analysis was the complete absence of sulfur-containing amino acids. In contrast, fatty acid-binding protein from rat liver contained 1 cysteine and 6 methionine residues. Furthermore, the amino acid composition of the two proteins were quite distinct from each other (see Table 1M in the Miniprint). These studies show quite conclusively that the hamster heart cytosolic phospholipase A is different from the fatty acid-binding protein.

**Substrate Specificity of Purified Hamster Heart Phospholipase A**—The specificity of the purified cytosolic phospholipase A toward various lipid substrates was investigated. The activities of the enzyme toward phosphatidyl[methyl-3H]choline and phosphatidyl[1,2,14C]ethanolamine were monitored by the formation of labeled lysophosphatidylcholine and lysophosphatidylethanolamine, respectively. The activities of the enzyme toward 1-[14C]palmitoylglycerophosphocholine (lysophosphatidylcholine), 1-acetyl-2-[14C]arachidonylglycerol, 1,2-di-[14C]oleoyl glycerol (diacylglycerol), and 1,2,3-tri-[14C]oleoylglycerol (triaxoylglycerol) were monitored by the formation of labeled fatty acids. The reaction mixture contained 1.0 mM labeled substrate, 0.02 mg of enzyme protein in 50 mM Hapes (pH 7.4). As shown in Table II, the enzyme was able to hydrolyze the acyl groups of phosphatidycholine and phosphatidylethanolamine. The ability of the enzyme to act as a phospholipase C or D or phosphodiesterase on phosphatidyl[methyl-3H]choline was also investigated. No labeled phosphocholine or choline was detected after the incubation, which suggests that the enzyme was not able to hydrolyze the phosphodiester group of the phospholipid. It was not active on lysophosphatidylcholine, diacylglycerol, or triacylglycerol.

**Acyl Specificity of Purified Hamster Heart Phospholipase A**—The acyl specificity of the enzyme toward phosphatidylcholine was also investigated. In this study, molecular species of phosphatidylcholine with a labeled acyl group at C-2 were synthesized and were used as substrates in the assay. The reaction mixture contained 1.0 mM labeled substrate, 0.02 mg of enzyme protein in 50 mM Hapes (pH 7.4). Subsequent to the reaction, the products were analyzed by thin-layer chromatography. Phospholipase A1 and A2 activities were calculated from the amount of radioactivity found in the lysophosphatidylcholine and fatty acid fractions, respectively. In order to ensure that the release of labeled fatty acid was not caused by the sequential reactions catalyzed by phospholipase A and lysophospholipase, control experiments were conducted under identical assay conditions with phosphatidyl[methyl-3H]choline as substrate. After the reaction, the amount of labeled glycerophospho[methyl-3H]choline formed was determined. No detectable amount of glycerophospho[methyl-3H]choline was found in the reaction, which indicates that the release of labeled fatty acid was not caused by the action of lysophospholipase on the lysolipid formed. As depicted in Table III, the enzyme displayed both phospholipase A1 and A2 activities toward molecular species of phosphatidylcholine with an arachidonyl group at C-2. 1-Stearoyl-2-arachidonylglycerophosphocholine was the preferred substrate for phospholipase A1 activity, whereas 1-palmitoyl-2-arachidonylglycerophosphocholine was the substrate which elicited the highest phospholipase A2 activity. The unambiguous presence of both phospholipase A1 and A2 activities and the acyl specificities of

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### Table II

**Substrate Specificity of Hamster Heart Cytosolic Phospholipase A**

A purified enzyme preparation obtained after Sepharose 6B chromatography (see Table I) was used in this study. The reaction mixture consisted of 1.0 mM labeled substrate, 0.02 mg of enzyme protein in 50 mM Hapes (pH 7.4). The reaction mixture was incubated at 37°C for 1 h, and the product was separated from the substrate by thin-layer chromatography. The results represent the mean ± S.D. of at least three separate experiments, each assayed in duplicate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (nmol/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine (methyl-3H)</td>
<td>974 ± 12.2</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine ([1,2,14C]ethanolamine)</td>
<td>775 ± 10.5</td>
</tr>
<tr>
<td>Lysophosphatidylcholine (1-14C)palmitoyl</td>
<td>ND</td>
</tr>
<tr>
<td>Diacylglycerol (1-acetyl-2-[14C]arachidonyl)</td>
<td>ND</td>
</tr>
<tr>
<td>Diacylglycerol (1,3-[14C]oleoyl)</td>
<td>ND</td>
</tr>
<tr>
<td>Triaxoylglycerol (1,2,3-[14C]oleoyl)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not detectable.

### Table III

**Acyl Specificity of phospholipase A from hamster heart cytosol**

A purified enzyme preparation obtained after Sepharose 6B chromatography (see Table I) was used in this study. The reaction mixture consisted of 1.0 mM labeled phosphatidylcholine, 0.02 mg of enzyme protein in 50 mM Hapes (pH 7.4). The reaction mixture was incubated at 37°C for 1 h, and the labeled fatty acid as well as lysophosphatidylcholine were separated from phosphatidylcholine by thin-layer chromatography. The results represent the mean ± S.D. of three separate assays.

<table>
<thead>
<tr>
<th>Molecular species of phosphatidylcholine</th>
<th>Phospholipase A1</th>
<th>Phospholipase A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Palmitoyl-2-[14C]palmitoyl ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1-Palmitoyl-2-[14C]oleoyl</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1-Palmitoyl-2-[14C]linoleoyl</td>
<td>14 ± 4</td>
<td>18 ± 8</td>
</tr>
<tr>
<td>1-Palmitoyl-2-[14C]arachidonyl</td>
<td>142 ± 50</td>
<td>232 ± 64</td>
</tr>
<tr>
<td>1-[14C]Palmitoyl-2-arachidonyl</td>
<td>178 ± 69</td>
<td>251 ± 82</td>
</tr>
<tr>
<td>1-Stearoyl-2-[14C]palmitoyl</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1-Stearoyl-2-[14C]oleoyl</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1-Stearoyl-2-[14C]linoleoyl</td>
<td>21 ± 9</td>
<td>24 ± 18</td>
</tr>
<tr>
<td>1-Stearoyl-2-[14C]arachidonyl</td>
<td>420 ± 112</td>
<td>12 ± 10</td>
</tr>
<tr>
<td>1-[14C]Stearoyl-2-arachidonyl</td>
<td>481 ± 70</td>
<td>12 ± 10</td>
</tr>
</tbody>
</table>

*ND, not detectable.

These two activities were confirmed when similar results were obtained with molecular species of phosphatidylcholine with a labeled acyl group at C-1 (1-14C)palmitoyl-2-arachidonoylglycerophosphocholine and 1-14C)stearoyl-2-arachidonoylglycerophosphocholine). The saturated and mono-unsaturated species of phosphatidylcholine were not hydrolyzed at all by the enzyme. It appeared that phospholipase A1 activity of the enzyme preferred a stearoyl group, whereas the phospholipase A2 activity preferred a palmitoyl group at C-1. Both phospholipase A1 and A2 activities were more active with an arachidonyl group at C-2.

**Characterization of Hamster Heart Phospholipase A1 and A2**—From studies above, 1-palmitoyl-2-arachidonoylglycerophosphocholine appeared to be the substrate of choice for the display of both phospholipase A1 and A2 activities in the purified enzyme. Hence, 1-palmitoyl-2-[14C]arachidonoylglycerophosphocholine was used in all subsequent studies. Phospholipase A1 activity was calculated from the amount of label associated with lysophosphatidylcholine fraction, and phospholipase A2 activity was determined by the amount of label in the fatty acid fraction after the reactions. The reaction rates of both activities were found to be linear for at least 90 min of incubation, and they were also linear with up to 0.05 mg of enzyme protein. Both phospholipase A1 and A2 activities displayed broad pH profiles, and both showed optimal activi-
trophoresis. Additional evidence for the purity of the enzyme was shown to be homogeneous by nondenaturing polyacrylamide gel electrophoresis, and two-dimensional gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the antibodies were highly inhibitory to the enzyme activity. The results of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis clearly indicate that the enzyme is made up of multiple copies of subunits of $M_r$ 14,000. This finding is in agreement with reports of phospholipase A from several mammalian species which were also found to have $M_r$ 14,000 subunits (5). The molecular weight of the native enzyme obtained by gel filtration analysis (140,000) suggests that the enzyme is composed of 10 subunits. The result obtained from the cross-linking of the subunits with dimethyl suberimidate provides direct evidence that the native enzyme consists of at least six identical subunits. At this time, the exact number of the $M_r$ 14,000 subunits in the enzyme remains undefined.

The purified phospholipase A is active toward the acyl groups of phosphatidylycholine and phosphatidylethanolamine, but not lysophosphatidylycholine or neutral lipids. This differentiates it from the less specific activity of phospholipase B from bovine pancreas, which is active toward both lysophospholipids and phospholipids (17). It is also different from the extrahepatic lipoprotein lipase (18, 19), which hydrolyzes triacylglycerol and diacylglycerol. It is rather intriguing that the phospholipase A in the cytosol has a much higher activity toward phosphatidylethanolamine than phosphatidylycholine (4), and yet the purified enzyme depicts slightly higher activity toward phosphatidylcholine. One facile explanation for this apparent discrepancy is that the hydrolysis of phosphatidylcholine was inhibited in the cytosol by an inhibitor which was removed during purification. The increase in enzyme activity after CM-cellulose chromatography in spite of only partial recovery of the active fractions certainly substantiates this supposition.

The existence of a soluble endogenous inhibitor of phospholipase A with a molecular weight of 37,000 from a mammalian source has been reported (20). Another possibility is that the cytosol may contain an enzyme with a very high specific activity toward phosphatidylethanolamine which was removed during enzyme purification.

The use of synthetic molecular species of phosphatidylcholine with defined acyl groups at C-1 or C-2 enabled us to show unambiguously that the enzyme has both phospholipase A$_1$ and A$_2$ activities at physiological pH. It is clear that the release of acyl groups from C-1 did not result from the combined action of phospholipase A$_2$ and lysophospholipase activities since lysophospholipase activity was not detected in the enzyme preparation. Furthermore, our studies demonstrate for the first time that the activity of phospholipase A$_1$ is not only dependent on the C-1 acyl group of phosphatidylycholine, but is also dependent on the acyl group at C-2. Similarly, the activity of phospholipase A$_2$ is not only dependent on the C-2 acyl group of the substrate, but is also dependent on the C-1 acyl group. For example, both phospholipases favor a highly unsaturated acyl group at C-2, but the phospholipase A$_1$ activity prefers a C-1 stearoyl group. On the other hand, phospholipase A$_2$ activity exhibits a higher specificity toward a C-1 palmitoyl group. The specificity of phospholipase A$_2$ for a highly unsaturated acyl group at C-2 is similar to the hamster heart microsomal enzyme (4) and to the enzyme from human platelets (21). It should be noted that the acyl specificities exhibited by the purified enzyme makes it unlikely that this enzyme is the only phosphatidylycholine-hydrolyzing phospholipase A in hamster heart. Our recent studies on the remodeling of newly synthesized phosphatidylycholine in hamster heart clearly demonstrated that

**FIG. 8. Double reciprocal plots of phospholipase A$_1$ and A$_2$ activities versus phosphatidylcholine concentration.** The double reciprocal plots of phospholipase A$_1$ (A) and A$_2$ (B) activities versus 1-palmitoyl-2-arachidonylglycerophosphocholine concentrations (mM) are depicted. Enzyme activities were assayed at pH 7.5, and each assay contained 0.05 mg of enzyme protein. Enzyme activity is expressed as nanomoles of product formed per hour/milligram of protein.

**DISCUSSION**

This is the first report on the isolation of a mammalian cytosolic phospholipase A which is specific for the hydrolysis of only phospholipids. Although the presence of phospholipase A$_1$ and A$_2$ activities in heart cytosol has been documented (4, 12, 13), very little information was available on the specificity of these two enzyme activities toward the different molecular species of phosphatidylycholine. In order to detect all the phospholipase A activities in the fractions after chromatography, it was important to prepare a substrate which could be utilized by phospholipase A$_1$ or A$_2$ in spite of any differences in acyl specificities of these enzymes. The labeled phosphatidyl[methyl-$^3$H]choline prepared from isolated hamster heart satisfies this requirement. It is composed of different molecular species (with different acyl groups at C-1 and C-2) and is also the natural substrate for both enzymes.

The enzyme preparation after Sepharose 6B chromatography was shown to be homogeneous by nondenaturing polyacrylamide gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and two-dimensional gel electrophoresis. Additional evidence for the purity of the enzyme was obtained from immunological studies. The antibodies directed against phospholipase A produced only one sharp precipitin line against hamster heart cytosol in the double diffusion analysis, and the antibodies were highly inhibitory to the enzyme activity. The results of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis clearly indicate that the enzyme is made up of multiple copies of subunits of $M_r$ 14,000. This finding is in agreement with reports of phospholipase A from several mammalian species which were also found to have $M_r$ 14,000 subunits (5). The molecular weight of the native enzyme obtained by gel filtration analysis (140,000) suggests that the enzyme is composed of 10 subunits. The result obtained from the cross-linking of the subunits with dimethyl suberimidate provides direct evidence that the native enzyme consists of at least six identical subunits. At this time, the exact number of the $M_r$ 14,000 subunits in the enzyme remains undefined.

The purified phospholipase A is active toward the acyl groups of phosphatidylycholine and phosphatidylethanolamine, but not lysophosphatidylycholine or neutral lipids. This differentiates it from the less specific activity of phospholipase B from bovine pancreas, which is active toward both lysophospholipids and phospholipids (17). It is also different from the extrahepatic lipoprotein lipase (18, 19), which hydrolyzes triacylglycerol and diacylglycerol. It is rather intriguing that the phospholipase A in the cytosol has a much higher activity toward phosphatidylethanolamine than phosphatidylycholine (4), and yet the purified enzyme depicts slightly higher activity toward phosphatidylcholine. One facile explanation for this apparent discrepancy is that the hydrolysis of phosphatidylcholine was inhibited in the cytosol by an inhibitor which was removed during purification. The increase in enzyme activity after CM-cellulose chromatography in spite of only partial recovery of the active fractions certainly substantiates this supposition.

The existence of a soluble endogenous inhibitor of phospholipase A with a molecular weight of 37,000 from a mammalian source has been reported (20). Another possibility is that the cytosol may contain an enzyme with a very high specific activity toward phosphatidylethanolamine which was removed during enzyme purification.

The use of synthetic molecular species of phosphatidylcholine with defined acyl groups at C-1 or C-2 enabled us to show unambiguously that the enzyme has both phospholipase A$_1$ and A$_2$ activities at physiological pH. It is clear that the release of acyl groups from C-1 did not result from the combined action of phospholipase A$_2$ and lysophospholipase activities since lysophospholipase activity was not detected in the enzyme preparation. Furthermore, our studies demonstrate for the first time that the activity of phospholipase A$_1$ is not only dependent on the C-1 acyl group of phosphatidylycholine, but is also dependent on the acyl group at C-2. Similarly, the activity of phospholipase A$_2$ is not only dependent on the C-2 acyl group of the substrate, but is also dependent on the C-1 acyl group. For example, both phospholipases favor a highly unsaturated acyl group at C-2, but the phospholipase A$_1$ activity prefers a C-1 stearoyl group. On the other hand, phospholipase A$_2$ activity exhibits a higher specificity toward a C-1 palmitoyl group. The specificity of phospholipase A$_2$ for a highly unsaturated acyl group at C-2 is similar to the hamster heart microsomal enzyme (4) and to the enzyme from human platelets (21). It should be noted that the acyl specificities exhibited by the purified enzyme makes it unlikely that this enzyme is the only phosphatidylycholine-hydrolyzing phospholipase A in hamster heart. Our recent studies on the remodeling of newly synthesized phosphatidylycholine in hamster heart clearly demonstrated that
the deacylation of phosphatidylcholine is extensive (7), and this process is definitely not limited to the arachidonyl-containing species. An interesting point worth mentioning is that the combined specific activities of phospholipases A₁ and A₂ on any single molecular species of phosphatidylcholine are somewhat lower than those obtained from phosphatidylinositol-[methyl-³H]choline isolated from heart (mixed molecular species). It is possible that the enzymes may prefer phosphatidylcholine with mixed acyl groups. Alternatively, enzyme activity may be stimulated by one of the molecular species present in cardiac phosphatidylcholine, although that particular molecular species may not be the preferred substrate. With the availability of the purified enzyme, further studies on the modulation of this cardiac phospholipase by different molecular species of phosphatidylcholine and other lipids can be conducted.

Both phospholipase A₁ and A₂ activities from hamster heart cytosol show no absolute requirement for calcium or any divalent metallic cations at physiological pH. Similar calcium-independent phospholipase A₁ and A₂ activities have also been reported recently in the rat (12) and canine (13) heart cytosol. However, the pH profiles of the phospholipases A₁ and A₂ purified in this study differ from those of the rat or canine heart cytosolic enzymes (12, 13, 22). In addition, the Kₘ obtained for hamster heart phospholipases A₁ and A₂ (0.5 mM for both activities) is much higher than those reported for rat heart (0.073 mM) (12) or canine heart (0.003 mM) (13). Although the reason for the discrepancies is not clear, the variation in Kₘ may result from differences in substrates used, assay conditions, and animal species. In order to gain some insight into the physiological roles of phospholipase A in the cardiac tissue, all the assays in this study were conducted at physiological pH.

The specific activity of hamster heart phospholipase A in the cytosol is similar to that obtained in canine heart (13) and in rat heart (12). The specific activity of the purified enzyme is also of the same order of magnitude as purified platelet phospholipase A₂ (23). Although a calcium-dependent phospholipase A in the mitochondria and microsomes of mammalian tissues with a 12,000–14,000 subunits has been reported (24–26), our own marker enzyme studies and work done by other investigators (12, 13) clearly indicate that the present Ca²⁺-independent phospholipase A is a true cytosolic enzyme. The presence of a cytosolic phospholipase A in heart implies that the enzyme may be responsible for the metabolism of the phospholipids in the cytosol. In view that the cytosol contains only a very small amount (2%) of total cardiac phospholipids, the need of a distinct phospholipase A solely for the metabolism of the cytosolic phospholipids is debatable. Hence, it is possible that the true physiological role of this enzyme in the cytosol remains to be defined.

REFERENCES


Additional references are found on p. 18935. Continued on next page.
Hamster Heart Cytosolic Phospholipase A

Preparation of phospholipase A2 - Labeled phospholipase A2 (PLA2) was prepared according to the following procedure. The hamster heart was excised from young Syrian golden hamsters (100 to 150 g) on the day following delivery. The heart was then homogenized using a Polytron homogenizer in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM CaCl2. The homogenate was then centrifuged at 100,000 x g for 1 hour and the supernatant was used as the source of PLA2. The specific activity of the enzyme was determined using the method of Spirin et al. (1984).

Preparation of heart homogenate - The heart homogenates were prepared as follows. The heart was excised from young Syrian golden hamsters (100 to 150 g) on the day following delivery. The heart was then homogenized using a Polytron homogenizer in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM CaCl2. The homogenate was then centrifuged at 100,000 x g for 1 hour and the supernatant was used as the source of PLA2. The specific activity of the enzyme was determined using the method of Spirin et al. (1984).

Preparation of PLA2-depleted homogenate - The PLA2-depleted homogenate was prepared by the following procedure. The hamster heart was excised from young Syrian golden hamsters (100 to 150 g) on the day following delivery. The heart was then homogenized using a Polytron homogenizer in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM CaCl2. The homogenate was then centrifuged at 100,000 x g for 1 hour and the supernatant was used as the source of PLA2. The specific activity of the enzyme was determined using the method of Spirin et al. (1984).

Preparation of heart homogenate - The heart homogenates were prepared as follows. The heart was excised from young Syrian golden hamsters (100 to 150 g) on the day following delivery. The heart was then homogenized using a Polytron homogenizer in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM CaCl2. The homogenate was then centrifuged at 100,000 x g for 1 hour and the supernatant was used as the source of PLA2. The specific activity of the enzyme was determined using the method of Spirin et al. (1984).
Fig. 14. Analysis of fatty acid binding protein activity on enzyme sample by Sephacryl 6B chromatography.

Hamster heart cytosolic phospholipase A was partially purified by column chromatography and the enzyme preparation (10 mg protein) was equilibrated with 1 M NaCl and 60 mM Tris-HCl, pH 8.0. The mixture was applied to a Sephacryl 6B column, and the fractions eluted from the column were analyzed for protein content by Bio-Rad (10). Phospholipase A activity was determined and radiometrically assayed with 1,2-DAG [2-14C].

Table 1

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Cross-linking of phospholipase A activity: Cross-linking of the enzyme phospholipase A was carried out according to the procedure of Carpenter and Harrington with glycerol 3-phosphate [14C] (21). The purified enzyme samples were cross-linked in a solution containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 20% glycerol, and 0.01% Triton X-100 in a solution at 4°C for 40 h. Subsequently, 10 mM sodium acetate buffer, pH 4.0, and 1% of mercaptoethanol were added and the mixture was incubated at 37°C for an additional 3 h. The mixture was frozen at -46°C overnight and an aliquot of the reaction mixture (0.3 ml) was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Other determinations: Protein content was determined by the Bio-Rad method of Bradford (22), using bovine serum albumin as standard. The phospholipase A enzyme activity was measured spectrophotometrically at 296 nm. Lipoxygenase was measured by the procedure described by Tamm (23). Reactivity in each sample was determined with a lipoygenase assay and the resulting activities were calculated by chemical calibration method.

References