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Purification of Dihydroxyacetone Phosphate Acyltransferase from Guinea Pig Liver Peroxisomes

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Dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42), a peroxisomal enzyme which initiates the biosynthesis of glycerolipids (especially the ether-linked glycerolipids) in higher eukaryotes, has been purified by over 3250-fold from guinea pig liver. Initial stages of purification entailed isolation of liver peroxisomes by a combination of differential and density-gradient centrifugation. Dihydroxyacetone phosphate acyltransferase was solubilized from peroxisomal membranes with 3-[3-cholamidopropyl)dimethylammonio]-1-propane sulfonate at moderate ionic strength (0.15 M NaCl). The solubilized enzyme was further purified by a regimen of size-exclusion chromatography, cation-exchange chromatography, and hydroxylapatite chromatography. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of different fractions during the purification of the enzyme, a 69-kDa protein band copurified with the enzyme activity, indicating that the monomeric enzyme may have a M_r of 69,000. This was verified by further purifying the enzyme by chromatofocusing, when a single 69-kDa band was observed on SDS–PAGE. The M_r of dihydroxyacetone phosphate acyltransferase determined by gel filtration is 90 kDa. The V_{max} of the purified enzyme was ~4 μmol acyldihydroxyacetone phosphate (acylDHAP) formed per minute per milligram protein and the $K_{m(DHAP)}$ is ~70 μM when assayed at saturating concentrations of palmitoyl-CoA. Free coenzyme A inhibits the acyltransferase reaction with an inhibition constant (K_i) of approximately 0.76 mM. To date, this is the most highly purified DHAP acyltransferase (>3200-fold) of mammalian origin. © 1993 Academic Press, Inc.

It is now well established that animals can biosynthesize phosphatidic acid by two distinct pathways. One path begins with the fatty-acylation of dihydroxyacetone phosphate (DHAP)[3] catalyzed by acyl-coenzyme A:dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42; DHAPAT) followed by reduction of the keto group by NADPH and acylation of the resulting lysophosphatidic acid (1). The other route is via a similar fatty acylation; however, in this case the acyl acceptor is glycerol 3-phosphate (GP) and the catalyst is acyl coenzyme A:glycerol 3-phosphate acyltransferase (EC 2.3.1.15; GPAT) (2). There are important distinctions between these two pathways with regard to both cell biology and lipid metabolism.

The pathway initiated by GP acyltransferase leads only to the diacylglycerophospholipids and triacylglycerol; however, the product of the DHAP acyltransferase-catalyzed reaction (acyl DHAP) can additionally act as precursor to the glycerolether lipids (e.g., platelet-activating factor, the plasmalogens, and membrane–protein anchors). It has also been shown that these two enzymes differ significantly with regard to their subcellular distributions. GP acyltransferase activity (isoenzymes) occurs in both the mitochondrial outer membrane and the endoplasmic reticulum (microsomal fraction) (3, 4). DHAP acyltransferase activity, on the other hand, is located mainly on the luminal side of the peroxisomal membrane (small amounts of activity are also found in the microsomal fraction) (5, 6). The subcellular site of DHAP acyltransferase activity is clinically relevant; tissues and cultured cells from patients with peroxisomal disorders (e.g., Zellweger cerebrohepatorenal syndrome (7–11), neonatal adrenoleukodystrophy (12), rhizomelic chondrodysplasia

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[3] Abbreviations used: DHAP, dihydroxyacetone phosphate; GP, glycerol phosphate; DHAPAT, DHAP acyltransferase; GPAT, GP acyltransferase; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tes, 2-[[tris-(hydroxymethyl)methyl]amino]ethanesulfonic acid; CMC, critical micellar concentration; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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punctata (13), and infantile Refsum disease (14) have been shown to be deficient in DHAP acyltransferase. These patients are also deficient in tissue plasmalogens. Kinetic analysis of the DHAP acyltransferase in homogenates of skin fibroblasts from Zellweger patients indicates that the deficiency is due to a decrease in the amount of enzyme rather than an alteration in the enzyme's intrinsic catalytic potential (15). The deficiency of DHAP acyltransferase is now routinely employed as a tool to diagnose these diseases (8, 11, 16).

There has been some uncertainty about whether the microsomal DHAPAT activity is actually due to a lack of substrate specificity of the GP acyltransferase present in the endoplasmic reticulum. A number of kinetic and inhibition studies have provided evidence that the microsomal DHAPAT and GPAT activities are dual catalytic functions of a single enzyme (17–20). Conversely, others have shown that the properties of these two acyltransferase activities are quite different (21–25). The reason for such radically different results between laboratories is unclear, but may be due to differences in the methods used to assay these enzyme activities.

GPAT and DHAP acyltransferase are membrane-bound enzymes and hence have been notoriously difficult to isolate (due to loss of enzyme activity during purification). The bacterial GPAT has been isolated from a mutant strain of *Escherichia coli* that overproduced the enzyme by 10-fold (26). The mammalian GPAT has yet to be isolated; however, the enzyme from rat liver mitochondria has recently been solubilized and purified 20-fold (27). With regard to DHAP acyltransferase, earlier work in this laboratory resulted in a 240-fold purification of enzymatically active DHAPAT from guinea pig liver (28). Although this was a significant accomplishment, the final product contained a complex mixture of proteins, none of which could be unequivocally associated with the enzymatic activity. Recently, the second enzyme of the acyl-DHAP pathway, acyl/alkyl-DHAP reductase, has been isolated from rat liver by Datta *et al.* (29). Here, we report the optimization of DHAP acyltransferase solubilization from peroxisomal membranes followed by the chromatographic purification of the enzyme by over 3200-fold relative to the liver homogenate. In addition, the kinetic characterization of the solubilized enzyme is described. A summary of the partial purification of the enzyme has been published (30).

MATERIALS AND METHODS

Dihydroxyacetone phosphate, palmitoyl-CoA, coenzyme A, leupeptin, pepstatin, phenylmethanesulfonyl fluoride (PMSF), and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO). Nycodenz is a product of Nyegaard & Co. (Oslo, Norway). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate was purchased from Boehringer-Mannheim (Indianapolis, IN). Asolectin, a soybean phospholipid concentrate, was from Associated Concentrates (Woodside, NY). A 2% stock solution of asolectin micelles was prepared in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA by sonication followed by centrifugation at 100,000*g* for 30 min to remove nondispersed material. Sephacryl S-200 Superfine, Mono-S FPLC column, and Mono-P FPLC column are products of Pharmacia Fine Chemicals (Piscataway, NJ). The Bio-Gel HPHT hydroxylapatite column is a product of Bio-Rad (Richmond, CA). The TSK-3000 HPLC size-exclusion column is a product of LKB-Pharmacia (Piscataway, NJ). All other compounds were reagent grade or better. $DHA^{32}P$ was prepared as described previously (31).

Assay of DHAP acyltransferase. The assessment of DHAP acyltransferase activity was based on the formation of acidic, lipophilic, radioactive product (i.e., acyl-$DHA^{32}P$) from $DHA^{32}P$ and palmitoyl-CoA in the presence of an enzyme sample (32). The standard reaction mixture contained 75 mM Tris-HCl (pH 7.4) or Mes (pH 5.5), 83 μM palmitoyl-CoA, 1.67 mg/ml bovine serum albumin, 8.3 mM NaF, 8.3 mM $MgCl_2$, and 0.42 mM $DHA^{32}P$ (5000 cpm per nanomole) in a total volume of 0.6 ml. After incubation at 37°C for 15 min, the reaction was stopped by the addition of 2.25 ml of chloroform/methanol (1/2), followed by 0.75 ml of chloroform and 0.75 ml of KCl/H_3PO_4 (2 M/0.2 M) to develop separate phases. The lower layer was washed twice with 2.5 ml of chloroform/methanol/water (1/12/12) (33). A portion (1 ml) of the final lower layer was dried and the amount of radioactive product determined by liquid scintillation counting. At all stages of enzyme purification the radioactive lipid product comigrated with synthetic palmitoyl-DHAP when analyzed by thin-layer chromatography using bisulfite-containing solvent (1). Asolectin was added to the assays of solubilized DHAP acyltransferase to a final concentration of 0.17%.

Subcellular fractionation. Guinea pigs (250–300 g) were ether-anesthetized and decapitated prior to removing their liver. The livers were minced, homogenized, and centrifugally fractionated at 4°C as described by de Duve *et al.* (34) with minor modifications developed by Jones *et al.* (35) and Webber (36) to produce a light-mitochondrial fraction (L-fraction) sedimenting between 33,000*g*-min and 250,000*g*-min which is enriched in peroxisomes. The medium for homogenization and fractionation was 0.25 M sucrose, 10 mM Tes, pH 7.5, 1 mM EDTA, containing 0.1% ethanol. Peroxisomes were isolated from the L-fraction by a slight modification of the method described by Ghosh and Hajra (37). The L-fraction was resuspended in homogenization buffer to a volume equivalent to $\frac{3}{4}$ of the original liver weight. Two milliliters of resuspended L-fraction was layered onto 15 ml of a solution containing 30% Nycodenz (w/v), 10 mM Tes, pH 7.5, and 1 mM EDTA in a 25-ml polycarbonate centrifuge tube. After centrifugation at 75,000*g* for 45 min, the resultant pellet was resuspended in homogenization buffer to a volume equivalent to 20% of the original liver weight.

Preparation of peroxisomal membranes. Peroxisomes were ruptured osmotically by 10-fold dilution with 10 mM sodium pyrophosphate, pH 9.0, containing 1 μM leupeptin, 1 μM pepstatin, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM EDTA. The diluted suspension was stirred on ice for 1 h followed by centrifugation at 100,000*g* for 30 min to separate the peroxisomal membranes (pellet) from the soluble matrix proteins. The membranes were resuspended in 25 mM Tes, pH 7.0, to a protein concentration of approximately 10 mg/ml.

Other procedures. The protein content of samples at each step was assessed by the method of Bensadoun and Weinstein (38). SDS–PAGE was performed according to Laemmli (39) in a Mini-Gel apparatus from Bio-Rad (Richmond, CA). The method for silver staining of SDS–PAGE gels was essentially that of Oakley *et al.* (40). Concentration of samples by ultrafiltration was performed in an Amicon (Danvers, MA) stirred cell (10,000-M_r cutoff) under pressure with nitrogen.

RESULTS

The Activity-vs-pH Shift

Jones and Hajra (41) found that peroxisomal DHAP acyltransferase had highest activity when assayed at pH 5.5. However, upon solubilization of the enzyme with sodium cholate the pH optimum shifted to pH 7.4. Since

cholate has limited solubility at slightly acidic pHs, it is possible that the loss of enzyme activity at low pH may be due to aggregation of the cholate micelles at the lower pH values, rather than to changes in the protein itself. To test this hypothesis, samples of membrane-bound, cholate-soluble, and Triton X-100-soluble DHAP acyltransferase were assayed at regular pH intervals between pH 4.5 and 8.5. Both the cholate-solubilized and the Triton X-100-solubilized DHAP acyltransferase showed a loss of enzyme activity when assayed at pH 5.5 (data not shown), indicating that this phenomenon is not due to aggregation of the detergent micelles.

A number of different nonionic and zwitterionic detergents were tested for their ability to solubilize DHAPAT without destroying its catalytic activity (see under Discussion). Of the detergents screened (including Triton X-100, Tween 20, octylglucoside, Chaps, and various Zwittergents (Calbiochem, San Diego, CA)), Chaps provided the highest percentage of solubilization as well as the highest specific activity of solubilized enzyme (data not shown).

Solubility of DHAP Acyltransferase vs CHAPS Concentration

As seen in Fig. 1, the solubility of DHAP acyltransferase increases sigmoidally with the log of the Chaps concentration. The point of inflection of this graph corresponds well with the reported value of 1.4 mM for the critical micellar concentration (CMC) of Chaps (42). Furthermore, DHAP acyltransferase is not inhibited by Chaps concentrations as high as 15 mM. As previously observed with cholate (41), the solubilization of the enzyme increased its activity by 2.5- to 3-fold. From the results of this experiment, a concentration of 15 mM Chaps was chosen for further experiments.

Solubility vs Ionic Strength

With the Chaps concentration held constant at 15 mM and the salt concentration varied from 0 to 250 mM, it was found that the optimum solubilization occurred at 150 mM KCl (data not shown). At salt concentrations above 150 mM, the fraction of the recovered DHAP acyltransferase activity found in the supernatant did not increase significantly, and the specific activity of the soluble fraction was lower.

Solubility vs Protein Concentration

The protein-to-detergent ratio was found to have no significant effect upon solubilization of the enzyme. When the membrane protein concentration was varied from 3 to 10 mg/ml at constant Chaps concentration (15 mM), there was very little difference in the amount or specific activity of the enzyme solubilized. In all cases, >85% of the DHAP acyltransferase activity was solubilized (data not shown).

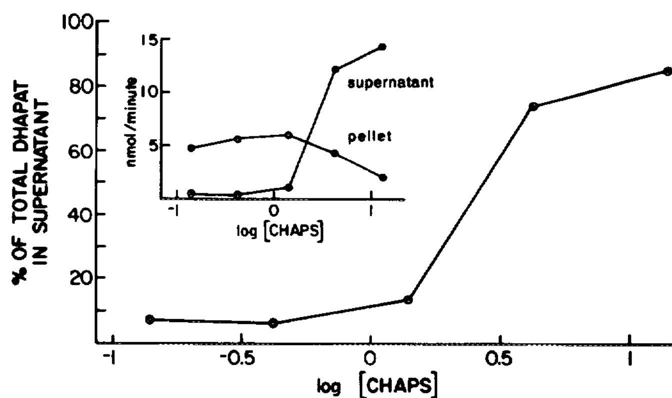

FIG. 1. Solubilization of DHAPAT vs Chaps concentration. Seventy-five-microliter aliquots (0.3 mg protein) of peroxisomal membranes suspended in 0.15 M KCl, 50 mM Tris-HCl, pH 7.4, were placed in Airfuge Tubes (Beckman Instruments) on ice. An equal volume of 0.15 M KCl/ 50 mM Tris-HCl containing increasing concentrations of Chaps was added to each tube. After centrifugation at 150,000*g* for 15 min, the supernatants and resuspended pellets were assayed for DHAPAT activity at pH 7.5. The main graph shows the percentage of the total activity (supernatant + pellet) recovered in the supernatant. The inset gives the actual activity in the supernatants and pellets.

Solubility vs pH

Although the CMC of Chaps is relatively independent of hydrogen ion concentration, the pH of the solubilizing media can have a significant effect on the solubility of a polyionic macromolecule such as a protein. When the Chaps concentration was 15 mM and the salt concentration was 150 mM, the solubilization of DHAP acyltransferase increased ~12% with increasing proton concentration between pH 6 and 8. The optimum pH was ~6.5 (data not shown).

Standard Solubilization Protocol

The experiments described above led to the following standard solubilization protocol. The pelleted peroxisomal membranes (from osmotically shocked peroxisomes) were suspended in 25 mM Tes (pH 7.0) to a protein concentration of between 10 and 20 mg/ml. This membrane suspension was mixed with an equal volume of 300 mM NaCl, 30 mM Chaps, 20 mM Mes (pH 6.5), 2 mM dithiothreitol, 2 μM leupeptin, 2 μM pepstatin, 2 mM EDTA, and 0.4 mM PMSF. After mixing gently on ice with a magnetic stirrer for 15 min, the insoluble material was sedimented at 100,000*g* for 60 min. This procedure solubilized 80–90% of the available DHAP acyltransferase with a specific activity of 65–85 mU DHAP acyltransferase activity/mg protein.

Chromatographic Purification of DHAP Acyltransferase

Low-pressure size-exclusion chromatography. The solubilized enzyme was first purified by size-exclusion chro-

matography on a large (1.6 × 95 cm; 180 ml) column of Sephacryl S-200. The profile of enzyme activity and 280-nm absorbance of the effluent is shown in Fig. 2. In this case, 94 mg of solubilized peroxisomal membrane protein was loaded onto the column as described in the legend to Fig. 2. There is only one major peak of 280-nm absorbance; however, this peak has a shoulder on the high molecular weight side and a smaller fused peak on the low molecular weight side. The main peak is centered at a position equivalent to a M_r of 166 kDa, but spans a range from 200 kDa to ~50 kDa. Analysis of the proteins in this peak by SDS-PAGE reveals a multitude of different protein species and yet almost all of the proteins have molecular weights between 30 and 70 kDa.

DHAP acyltransferase is found in the shoulder fractions on the low molecular weight side of the main peak. DHAP acyltransferase elutes from this column with a K_{av} = 0.186, which corresponds to an estimated M_r of 127 kDa based on standardization of the column with proteins of known molecular weight. The recovery of DHAP acyltransferase activity in fractions 12 through 15 is approximately 60% of the loaded enzyme. The purification factor is about 1.8, giving an average specific activity in these fractions of nearly 120 mU/mg protein. The peak fractions (fraction 12 through fraction 15) were pooled for further purification.

Cation-exchange chromatography. The pooled fractions from the Sephacryl S-200 column were diluted 3-fold to lower the ionic strength and then passed over a Mono-S cation-exchange column. The adherent proteins were eluted with a salt gradient from 0 to 0.5 M NaCl (see Fig. 3). DHAP acyltransferase elutes in a sharp peak between 100 and 150 mM salt in a region of relatively low uv absorbance. The purification factor for DHAP acyltransferase in the four peak fractions (12–15) is about 12-fold in this case and the recovery of activity is about 38% (relative to the load). These four fractions were pooled for further purification by hydroxylapatite chromatography.

Hydroxylapatite chromatography. The pooled fractions from the Mono-S column were loaded directly onto the hydroxylapatite column without prior dilution or dialysis. The proteins which remained bound to the column were eluted with a biphasic potassium phosphate gradient as described in the legend to Fig. 4. DHAP acyltransferase eluted as a single peak of activity between 130 and 210 mM phosphate (see Fig. 4). The three fractions with the highest DHAP acyltransferase activity (fractions 12–14) were pooled for further purification by high pressure size-exclusion chromatography.

High-pressure size-exclusion chromatography. The three fractions from the hydroxylapatite column with the highest DHAP acyltransferase activity were pooled, concentrated by ultrafiltration, and passed over a TSK-3000 HPLC size-exclusion column (8 × 300 mm). Figure 5

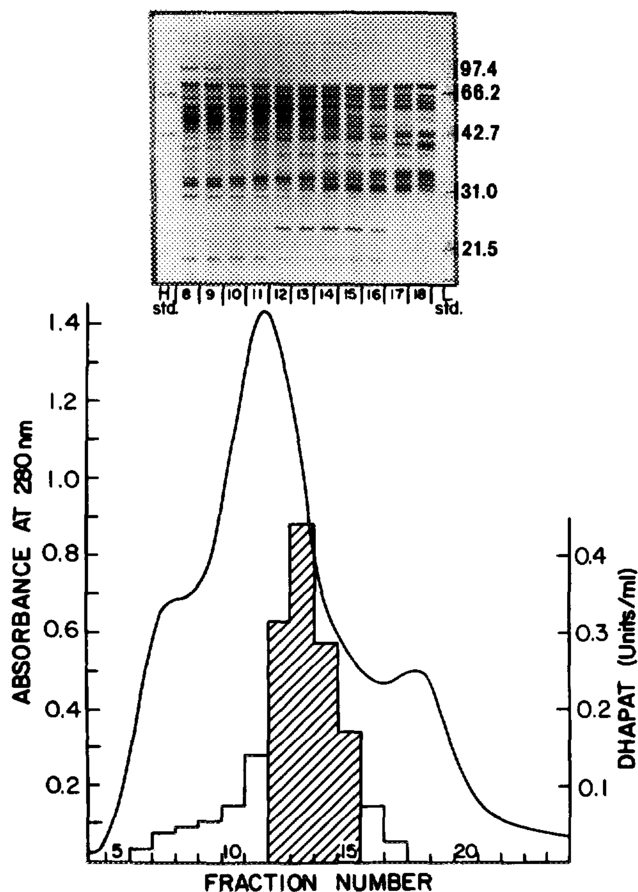

FIG. 2. Low-pressure size-exclusion chromatography. Nine milliliters of solubilized peroxisomal membrane proteins (10.5 mg/ml) was passed over a 1.6 × 95-cm (180 ml) column of Sephacryl S-200 Superfine at 6 ml/h. The mobile phase was 150 mM NaCl, 5 mg/ml Chaps, 10 mM Mes, pH 6.5, 1 mM DTT, 0.02% NaN_3 at 4°C. Fraction size = 3 ml. The bar graph represents the DHAPAT activity in each fraction. Fractions pooled for further purification are indicated by the shaded bars. SDS-PAGE: Each sample lane contains 1 µl of the corresponding fraction. The protein in each lane is as follows: 8, 1.8 µg; 9, 2.2 µg; 10, 3.0 µg; 11, 3.6 µg; 12, 3.6 µg; 13, 2.8 µg; 14, 2.1 µg; 15, 1.8 µg; 16, 1.6 µg; 17, 1.7 µg; 18, 1.9 µg.

shows the profile of uv absorbance and DHAP acyltransferase activity from this column, as well as the SDS-PAGE analysis of the major DHAP acyltransferase-containing fractions. The majority (~$\frac{2}{3}$) of the DHAP acyltransferase activity elutes in fraction 4, as does most of the uv absorbing material. Essentially all of the remaining DHAP acyltransferase is found in fraction 5. The total recovery of DHAP acyltransferase from this column is about 96% of the loaded enzyme.

Chromatofocusing. The enzyme was further purified by chromatofocusing on a Mono-P column (5 × 200 mm; Pharmacia). The starting buffer was 25 mM Bis-Tris propane, pH 7.3, 200 mM betaine, 5 mg/ml Chaps. The elution buffer was 10% Polybuffer 74 (Pharmacia), pH 4.5, 200 mM betaine, 5 mg/ml Chaps. DHAP acyltransferase elutes between pH 5.4 and 5.1 as a trailing peak (data not

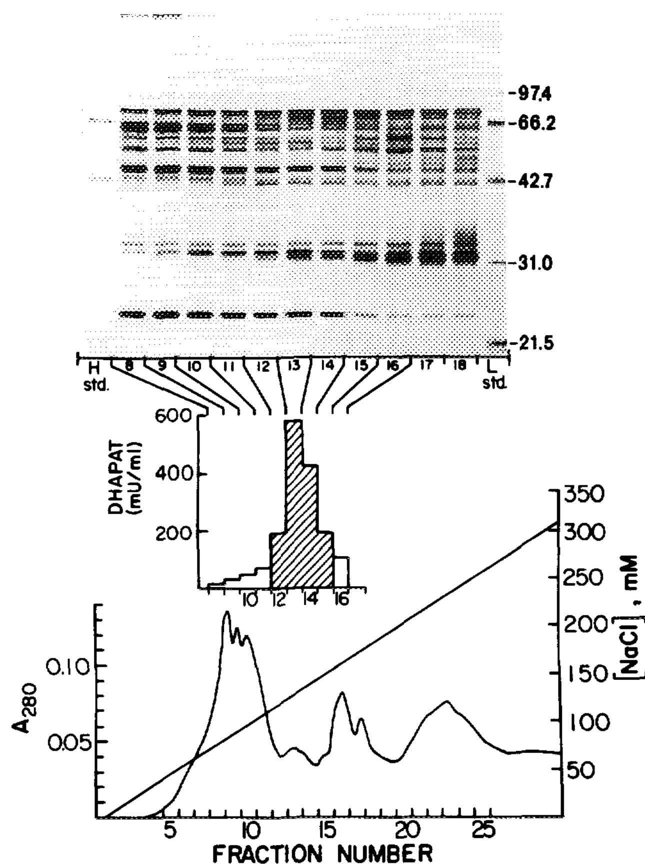

FIG. 3. Cation-exchange chromatography. Fractions 12 through 15 from the Sephacryl S-200 column (2.8 ml each; 29 mg protein total) were combined, diluted threefold with 25 mM Mes, 5 mg/ml Chaps, 1 mM DTT, pH 6.5, at 4°C and loaded at 0.5 ml/min onto a 0.5 × 5.0-cm (1.0 ml) column of Mono-S cation-exchange media (preequilibrated with the diluent buffer). Proteins were eluted with a NaCl gradient from 0 to 400 mM in diluent buffer. The flow rate was 0.5 ml/min with a total gradient volume of 40 ml and a fraction size of 1.0 ml. The bar graph shows the DHAPAT activity in the highest-activity fractions (shaded bars indicate fractions that were pooled for further purification). SDS-PAGE: Each lane contains 5 μl of its corresponding fraction. The protein in each lane is as follows: lane 8, 2.0 μg; 9, 2.6 μg; 10, 2.4 μg; 11, 1.7 μg; 12, 1.3 μg; 13, 1.4 μg; 14, 1.0 μg; 15, 1.3 μg; 16, 2.0 μg; 17, 2.0 μg; 18, 1.6 μg.

shown). The protein and enzyme activity recovered from this step are so low that the specific activity could not be accurately determined; however, a sample of this chromatofocusing-purified DHAP acyltransferase can be seen in lane 7 of Fig. 6.

Summary of purification. The results of the purification process from two independent experiments are summarized in Table I. This is a balance sheet for the entire procedure (from postnuclear supernatant to nearly pure DHAP acyltransferase as obtained from the size-exclusion HPLC). Figure 6 shows an electrophoretogram of the proteins present in the primary DHAPAT-containing fractions at each step of the purification process from solubilization through the chromatofocusing step as described in the text.

Characterization of DHAP Acyltransferase

Kinetics. Due to the severe loss of activity during chromatofocusing of DHAP acyltransferase, the enzyme from fraction 4 of the TSK-3000 column was used for analysis of the steady-state kinetics of the reaction. The reaction rate data were collected for DHAP concentrations ranging from 20 through 500 μM at three different concentrations of palmitoyl-CoA. The reaction was run at pH 7.5 with two different levels of DHAP acyltransferase (40 and 80 ng protein). All other parameters of the

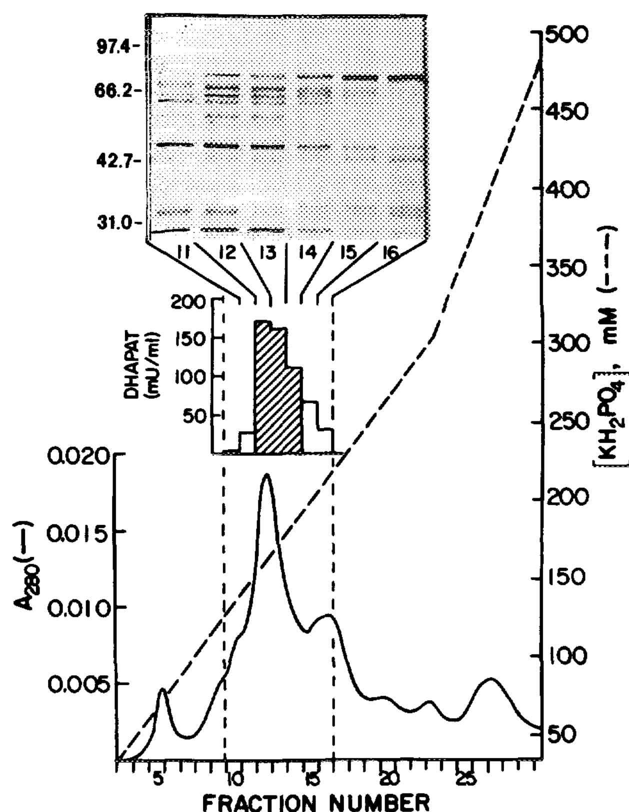

FIG. 4. Hydroxylapatite chromatography. Fractions 12 through 15 from the Mono-S column (0.95 ml each; 0.93 mg protein total) were pooled and loaded directly onto a 0.5 × 4.8-cm HPHT column of hydroxylapatite (Bio-Rad) preequilibrated with 10 mM K-phosphate, 0.3 mM $CaCl_2$, 5 mg/ml Chaps, 50 mM NaCl, 1 mM DTT, 0.05% NaN_3, pH 6.8. The column was washed with equilibration buffer until the A_{280} returned to baseline. The adherent proteins were eluted with a linear phosphate gradient starting with 100% equilibration buffer and ending with 40% equilibration buffer/60% 0.5 M K-phosphate, 0.01 mM $CaCl_2$, 5 mg/ml Chaps, 1 mM DTT, 0.05% NaN_3, pH 6.8, with a flow rate of 0.5 ml/minute. Fraction size was 1.0 ml. The bar graph shows the DHAPAT activity in the highest-activity fractions (shaded bars indicate fractions that were pooled for further purification). SDS-PAGE: Each sample lane contains 15 μl of its corresponding fraction. The protein content of each lane is as follows: lane 11, 1.0 μg; 12, 1.3 μg; 13, 0.9 μg; 14, 0.6 μg; 15, 0.6 μg; 16, 0.7 μg.

assay system were held constant and are as described in the Materials and Methods section. Figure 7 shows Hanes–Woolfe plots (43) of the experimental results. This method of plotting was chosen because it does not accentuate the rate values at low substrate concentrations as does the double-reciprocal plot of Lineweaver and Burk (44). The slope and Y-intercept of the plot are $1/V_{max}$ and $K_{M(DHAP)}/V_{max}$, respectively. The values for $K_{M(DHAP)}$ and V_{max} derived from each set of data are presented in Table II.

At a subsaturating concentration of palmitoyl-CoA (8.3 μM), the apparent K_M for DHAP is 36 μM and V_{max} is 1060 mU/mg protein. However, when the palmitoyl CoA concentration is above 25 μM, the apparent K_M for DHAP is approximately doubled to between 61 and 78 μM. The enzyme is probably saturated with palmitoyl-CoA at concentrations just above 25 μM since V_{max} values at both 25 and 83 μM palmitoyl-CoA are nearly identical (3830 and 4170 mU/mg protein, respectively).

Inhibition by coenzyme A. The enzyme is inhibited by the product CoASH. In order to approximate the product-inhibition constant (K_I) for the inhibition of DHAP acyltransferase by coenzyme A, a sample of Chaps-solubilized enzyme was assayed in the presence of coenzyme A at concentrations ranging from 50 to 500 μM. The experiment was performed at two concentrations of palmitoyl-CoA (42 and 83 μM). The results from these experiments are illustrated in the graphs of Fig. 8. The method of data analysis is that described by Stinson and Holbrook (45) and allows the direct determination of the dissociation constant for coenzyme A if one assumes that the fractional occupancy (α) is equivalent to the percentage of inhibition by coenzyme A. The results are quite similar at both palmitoyl-CoA concentrations, indicating that the binding site for this substrate is probably saturated at these con-

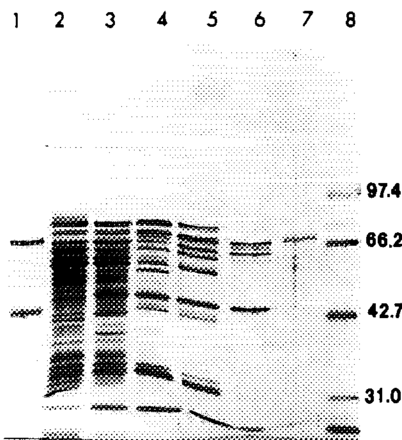

FIG. 6. Summary SDS-PAGE gel. This gel shows the protein constituents of the main DHAPAT-containing fractions from each step in the purification process from solubilization to chromatofocusing. The sample and protein content of each lane is as follows: lane 1, 0.6 μg of high MW standards; 2, 7.9 μg of Chaps-solubilized peroxisomal membrane proteins; 3, 2.1 μg of fraction 14 from the Sephacryl S-200 column; 4, 1.4 μg of fraction 13 from the Mono-S ion-exchange column; 5, 1.3 μg of fraction 12 from the hydroxylapatite column; 6, 0.8 μg of fraction 4 from the TSK-3000 column; 7, 30 μl of chromatofocused DHAPAT; 8, 0.7 μg of low MW standards.

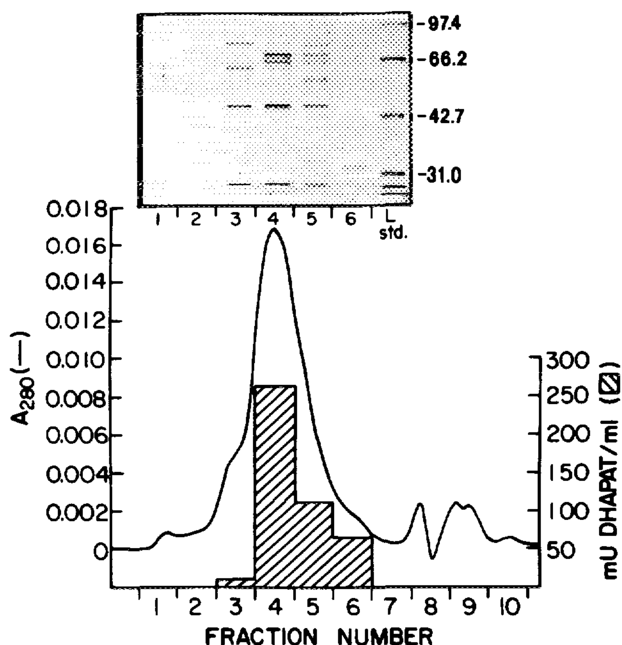

FIG. 5. High-pressure size-exclusion chromatography. Fractions 12–15 (0.95 μl each; 213 μg protein) from the hydroxylapatite column were pooled, concentrated to 150 μl in a centrifugal microconcentrator, and passed over a TSK-3000 HPLC column. The mobile phase is 150 mM NaCl, 5 mg/ml Chaps, 10 mM Bis-Tris propane (Sigma Chemical Co.), pH 7.5, 1 mM DTT, and 0.02% NaN_3 flowing at 0.5 ml/min. Fraction size = 1.0 ml. SDS-PAGE: Each sample lane contains 10 μl of its corresponding fraction. The protein content of each lane is as follows: lane 1, 0.3 μg; 2, 0.2 μg; 3, 0.4 μg; 4, 0.8 μg; 5, 0.5 μg; 6, 0.3 μg.

TABLE I

Purification Table

Fraction	Protein (mg)	mU DHAPAT per mg	RSA	Total mU	% Recovery
S1	31000	0.76	1	23600	100%
L	2670	3.07	4.0	8200	35%
Peroxisomes	566	12.2	16.1	6920	29%
SOL	186	73.8	97	13720	58%
S-200	75.3	118.5	156	8920	38%
Mono-S	2.4	1056	1390	2520	11%
Hydroxylapatite	0.84	1357	1790	1140	5%
TSK-3000	0.19	2470	3250	470	3%

Note. The starting material was 210 g of guinea pig liver (seven animals). The values given in the table are averages for two independent purification experiments. S1, the post-10,000g-min supernatant. L, the 250,000g-min pellet. SOL, the solubilized peroxisomal membrane proteins. RSA, relative specific activity.

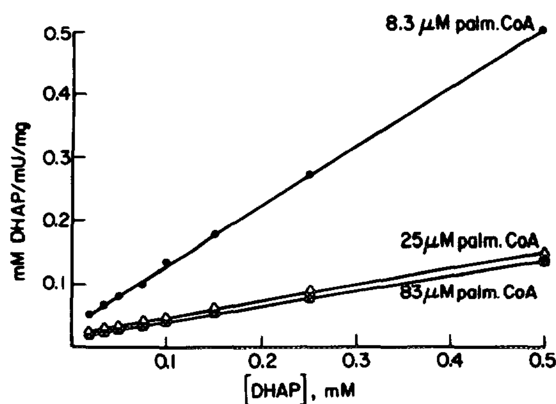

FIG. 7. Steady-state kinetics of DHAPAT. A sample of fraction 4 from the TSK-3000 column was diluted 50-fold with a solution containing 150 mM NaCl, 15 mM Chaps, 10 mM Tes, pH 6.5, 1 mM DTT, and 0.2% NaN_3. Aliquots were assayed for DHAPAT activity at pH 7.5 by the standard assay procedure except that the DHAP concentration ranged between 20 and 500 μM and the palmitoyl-CoA concentration ranged between 8.3 and 83 μM. Each data point is the average of the enzyme activity at two protein levels (40 and 80 ng).

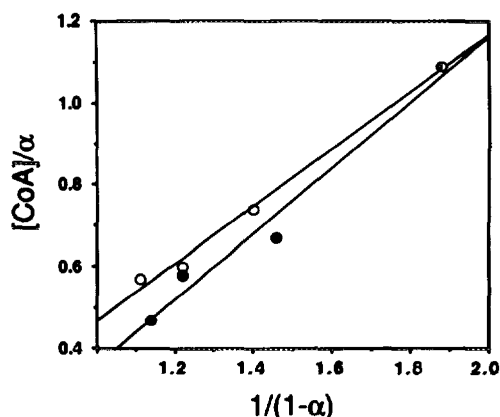

FIG. 8. Inhibition of DHAPAT by coenzyme A. Samples of Chaps-solubilized peroxisomal membrane proteins were assayed for DHAPAT activity in the presence of free coenzyme A at concentrations ranging from 0 to 500 μM. Assays were performed at two concentrations of palmitoyl-CoA, 42 μM (●) and 83 μM (○). The method of data analysis is from Stinson and Holbrook (45). α = the percentage inhibition of enzyme activity at each given CoA concentration and is assumed to be equivalent to the fractional saturation of the active site with CoA.

centrations of acyl-CoA and bovine serum albumin. The K_I can be extracted from the graphs as simply the slope of the line. By this method, the K_I for coenzyme A is determined to be approximately 0.74 to 0.80 mM.

Stimulation by phosphatidylcholines. Jones and Hajra (28) showed that the partially purified DHAPAT is stimulated by a number of phospholipids, especially by egg phosphatidylcholine and soybean lipids (asolectin). Therefore, stimulation of the enzyme activity by selected species of phosphatidylcholine was investigated (see Fig. 9). Of the species tested, including didecanoyl-, dilauroyl-, dimyristoyl-, and dipalmitoyl-PC, all stimulated the enzymatic activity of the Chaps-solubilized DHAP acyltransferase. Dilauryl-PC gave the best stimulation (70–87% increase in activity), while chain lengths longer or shorter than C12 were significantly less effective.

DISCUSSION

In order to further our understanding of the biosynthesis of the glycerophospholipids, especially the ether-linked members of this family of compounds, it is necessary to characterize the enzymes catalyzing the reactions involved. DHAP acyltransferase, which initiates glycerolipid biosynthesis from DHAP, was partially purified from guinea pig liver by Jones and Hajra (28) using a mixture of 0.2% sodium cholate, 1 M KCl, 10 mM Tris–HCl, 1 mM DTT, pH 7.5, to solubilize DHAP acyltrans-

TABLE II

Steady-State Kinetic Constants

[Palmitoyl-CoA] (μM)	K_m (μM)	V_{max} (mU/mg)
8.3	36	1060
25	78	3830
83	61	4170

Note. The assay conditions were standard as described in the Materials and Methods section (Tris-buffered at pH 7.5 plus 0.17% asolectin suspension) except that the DHAP and palmitoyl-CoA concentrations were as given in the text.

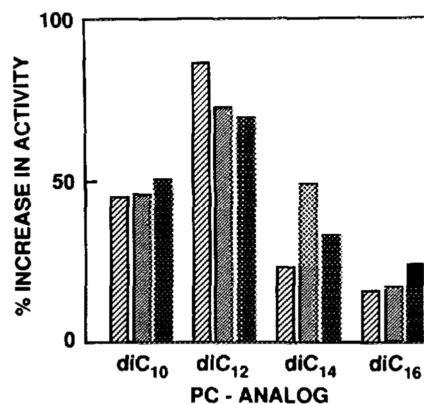

FIG. 9. Effect of phosphatidylcholines on solubilized DHAPAT. Chaps-solubilized DHAPAT was assayed at pH 7.5 as described in the Materials and Methods section except that the reaction mixture contained either didecanoyl phosphatidylcholine (didecanoyl PC), dilauroyl-PC, dimyristoyl-PC, or dipalmitoyl-PC. Each PC analog was added as sonicated suspension in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5, prior to starting the assay reaction. Each analog was tested at three different levels in the assay systems, i.e., 240–340 μM (▨), 1200–1680 μM (▩), and 2280–3370 μM (■). The actual concentrations of the PC analogs in the assay mixtures were as follows: didecanoyl-PC, 310, 1540, and 3080 μM; dilauroyl-PC, 280, 1380, and 2770 μM; dimyristoyl-PC, 250, 1260, and 2520 μM; and dipalmitoyl-PC, 240, 1190, and 2380 μM.

ferase. This mixture removes the enzyme from the membrane in active form; however, the high ionic strength of this solution precludes the use of some purification methods (e.g., ion-exchange chromatography). In addition, the aggregation number of cholate micelles varies dramatically with small changes in pH (46) which can make the purification process difficult to standardize. In order to avoid these difficulties, an array of nonionic and zwitterionic detergents were screened for their ability to solubilize DHAP acyltransferase (36). These detergents included Triton X-100, Tween-20, octylglucoside, various Zwittergents (Calbiochem), and Chaps. Of the detergents tested, only Chaps provided efficient, stable solubilization of the peroxisomal DHAP acyltransferase activity. Chaps, like cholate, is a member of the bile-acid class of detergents; however, it is zwitterionic and therefore relatively unaffected by changes in pH (42).

Jones and Hajra (41) showed that, upon detergent solubilization of peroxisomal membranes, the pH at which DHAP acyltransferase is most active shifts from pH 5.5 to 7.4. This pH shift may be due to an alteration of the active-site geometry, a latency restriction which is relieved by solubilization of the peroxisomal membrane, or to the existence of two DHAP acyltransferases (i.e., inhibition of one which is active at low pH and stimulation of another which is active at neutral pH). On the other hand, the shift in pH optimum may be due to two independent factors, for example, a combination of a broadening of the pH range of enzyme activity masked by an artifactual loss of activity resulting from aggregation of cholate/enzyme micelles at low pH.

If the loss of pH 5.5 enzyme activity was due to changes in the protonation state of cholate, then Triton X-100-solubilized enzyme should show much less inhibition at this pH (as Triton is a nonionic detergent). The results from this experiment indicated that the choice of detergent has very little effect on the loss of enzyme activity at low assay pH. In fact, the curve for the Triton X-100-solubilized enzyme lies about $\frac{1}{2}$ pH unit higher than that for the cholate-solubilized sample (36). Therefore, it can be concluded that the weakly acidic character of cholate is not the primary factor responsible for the loss of activity at acidic pH.

We confirmed the previous finding (28) that phosphatidylcholine stimulates the solubilized and purified enzymes. As a follow-up to this work, we have tested an array of phosphatidylcholines differing in the chain length of their constituent fatty acids. Dilauroyl-PC, the most active species tested, gave 87% stimulation of enzyme activity at 275 μM (0.13 mg/ml); higher concentrations were less effective. The previous results of Jones and Hajra (28) showed less stimulation with hen-egg PC at such low concentrations, although the lipid mixture was more effective at higher concentrations (e.g., 2–4 mg/ml) than used in the present study. Didecanoyl-, dimyristoyl-, and dipalmitoyl-PC gave substantially less activation at all concentrations than did dilauroyl-PC, indicating that the effect is not due simply to differential solubility of the PC analogs.

The four-step chromatographic procedure described above produces a highly purified, active DHAP acyltransferase. The first step, low pressure size exclusion chromatography, provides an initial clean-up of the solubilized enzyme solution, i.e., removes excess membrane lipid and any small (<25 kDa) compounds. In some experiments this step was replaced by gel filtration on the TSK-3000 HPLC column; however, no significant increase in the yield or purity of the pooled fractions was found. The absence of a significant peak at the void volume indicates that essentially all proteins are present in small mixed micelles or free in solution and no large aggregates (i.e., >200 kDa) have formed. DHAP acyltransferase elutes with a K_{av} = 0.186 as would a protein of M_r 127 kDa (calculated from molecular weight standards chromatographed in the same buffer). However, if a trace amount of solubilized peroxisomal membrane protein is chromatographed simultaneously with molecular weight standards, then DHAP acyltransferase activity elutes as a 90-kDa protein (data not shown). This discrepancy is probably due to differences in micelle size between the two conditions (in the first instance a much higher level of membrane lipid is present); however, interaction of DHAP acyltransferase with another protein of smaller M_r is also possible.

Cation-exchange chromatography on the Mono-S column gave an average ninefold purification of the enzyme (relative to the combined fractions 12–15 from the Sephacryl S200 column). This step demonstrates that DHAP acyltransferase is a basic protein (positively charged at neutral pH). Comparison of the enzyme activity profile with the SDS–PAGE gel reveals only two bands which copurify with the DHAP acyltransferase activity, one moderately dark band at ~69.3 kDa and one much lighter-staining band at ~67.2 kDa.

Hydroxylapatite chromatography provided only a 1.7-fold purification; however, it reduced the number of proteins in the enzyme sample by 50% (from 17 to 9). Of the nine bands in the SDS–PAGE lanes from the fractions with peak DHAP acyltransferase activity, only one copurifies with DHAP acyltransferase activity; this band corresponds to a molecular weight of ~68 kDa.

High-pressure size-exclusion chromatography removes another six of the contaminating proteins from the enzyme sample. Furthermore, this step confirms that the enzyme activity copurifies with a protein of M_r ~69 kDa as seen in previous steps. The specific activity of the DHAP acyltransferase in fraction 4 from this column is nearly 3400 mU/mg protein in this case (2470 mU/mg average from two preparations) giving a purification factor of 1.4 for this step. The purification factor at this point is 6400-fold relative to the S1-fraction (average is 3250-fold).

The enzyme can be further purified to near homogeneity by chromatofocusing on a Mono-P column (Pharmacia); however, this procedure results in such a drastic reduction in enzyme yield that the specific activity cannot be accurately determined. The loss of activity during chromatofocusing is probably not due to separation of heterologous subunits of the enzyme since fraction mixing did not recover any of the lost activity (data not shown). A sample of this chromatofocusing-purified DHAP acyltransferase can be seen in lane 7 of Fig. 6. It is clear from this figure and the electrophoretograms of the fractions from each chromatographic purification step that the DHAP acyltransferase activity copurifies with a protein of M_r 69,000. Taking into consideration that during gel filtration (where the enzyme is present in a micellar complex) the apparent M_r is about 90 kDa, it may be reasonable to assume that the native enzyme is a monomeric protein of 69 kDa bound to the peroxisomal membrane.

Steady-state kinetic analysis of the 6400-fold-purified DHAP acyltransferase resulted in smooth and linear plots at subsaturating as well as saturating concentrations of palmitoyl-CoA. The values for $K_{M(\mathrm{DHAP})}$ calculated for this highly purified form of DHAP acyltransferase are quite similar to those determined previously for this enzyme from guinea pig and rat liver peroxisomes (28, 41, 47), rat fat cell microsomes (17), and developing rat brain (31). Other sources of the enzyme, including rat liver microsomes (18, 19, 47) and preadipocytes (48) consistently show much higher values for $K_{M(\mathrm{DHAP})}$ (range = 0.16 to 1.6 mM). The similarity between the $K_{M(\mathrm{DHAP})}$ values for the purified enzyme and the membrane-bound peroxisomal enzyme indicate that the tertiary structure of the active site has not been significantly altered during the purification process.

The approximate K_I for inhibition of the enzyme by free coenzyme A (~0.76 mM) is much higher than that which is apparently required to saturate the enzyme with palmitoyl-CoA (<42 μM). These results demonstrate that the acyl-CoA binding site on the enzyme is highly selective for the acylated form of coenzyme A.

The isolation of pure DHAP acyltransferase will be helpful in establishing the nature of the loss of this enzyme activity and the deficiency of ether lipids in various congenital peroxisomal disorders. In some of these diseases, such as Zellweger CHRS and neonatal adrenoleukodystrophy, the DHAP acyltransferase deficiency is probably due to impairment of peroxisomal assembly rather than a defect in the primary structure of the enzyme molecule (15). On the other hand, tissues from patients with diseases such as rhizomelic chondrodysplasia punctata have morphologically normal peroxisomes but still show a deficiency for DHAP acyltransferase (49). It is possible that in such peroxisomal disorders the defect is in the enzyme molecule itself. The availability of specific antibodies against DHAP acyltransferase and also the detailed knowledge of the molecular structure of this membrane-bound enzyme will facilitate the establishment of the specific defects in these diseases.

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