Solubilization and partial characterization of lecithin-retinol acyltransferase from rat liver

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Lecithin-retinol acyltransferase (LRAT) is a microsomal enzyme that can esterify retinol bound to either cellular retinol binding protein or cellular retinol binding protein type II, utilizing phosphatidylcholine as the acyl source. Here, we report the solubilization of rat liver microsomal LRAT by 1.0% Brij-35, and partial purification via Phenyl Sepharose CL-4B chromatography. Kinetic properties of the solubilized enzyme were determined for retinol bound to cellular retinol binding protein (Km of 2.4 μM), retinol bound to cellular retinol binding protein type II (Km of 1.3 μM) and dilauroylphosphatidylcholine (Km of 2.5 μM). The solubilized enzyme was very selective for acyl donor in that phosphatidylethanolamine and phosphatidic acid were not utilized. With phosphatidylcholine as the acyl source, only the acyl group at the sn-*1 position was transferred to retinol. The reaction catalyzed by LRAT is reminiscent to that catalyzed by lecithin-cholesterol acyltransferase (LCAT); both enzymes transfer acyl groups from phosphatidylcholine to lipid alcohols to form esters. The catalytic mechanism of the two enzymes may also be similar. LCAT utilizes a serine for the phospholipase-like cleavage of phosphatidylcholine and one (or both) of two vicinal sulfhydryls as the intramolecular acceptor for the fatty acyl group, prior to transfer to cholesterol. Similar to LCAT, LRAT was inhibited by N-ethylmaleimide at concentrations as low as 15 μM, and completely inhibited by 1.0 mM p-aminophenylarsineoxide, a reagent that requires vicinal sulfhydryls in order to form a stable covalent complex. But unexpectedly, LRAT retained over 50% of its activity after treatment with 20 mM diisopropylfluorophosphate, diethyl* p-*nitrophenyl phosphate, or* m-*aminophenyl boronic acid, reagents that have been shown to inhibit LCAT as well as other enzymes utilizing serine residues for hydrolysis. Solubilized LRAT was inhibited by 5.0 mM phenylmethylsulfonylfluoride, and this inhibition was reversible within seconds of dithiothreitol addition to the inactive enzyme. Similar results have been reported for the sulfhydryl-protease papain. These inhibitor studies suggest that the active residue in LRAT necessary for the phospholipase cleavage step is a cysteine rather than the serine employed by LCAT.*

Keywords: retinol; retinyl esters; lecithin-retinol acyltransferase; lecithin-cholesterol acyltransferase; phosphatidylcholine

Introduction

The esterification of retinol with long chain fatty acids is utilized for storage of vitamin A in the liver and other organs,[1] for the export of ingested vitamin A from the small intestine,[1] and is apparently a required step for the isomerization of all-*trans*-retinol to 11-*cis*-retinol in the retinal pigment epithelium.[2] Such organs or tissues contain a microsomal enzyme activity which can utilize exogenous phosphatidylcholine or an endogenous acyl pool in the microsomes as a source of fatty acid for the esterification of retinol.[3-6] Importantly, these activities will esterify retinol when it is bound to the intracellular carrier protein for retinol in the intestine, cellular retinol binding protein type II,[7] or, in other organs, cellular retinol binding protein.[8-10] Retinol bound to these carrier proteins is restricted

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from esterification by a second, distinct enzyme activity, acyl CoA-retinol:acyltransferase, which is also present in both intestine[11] and liver.[12]

The ability to use phosphatidylcholine as substrate and the described sensitivity to various inhibitors[3] has suggested that these retinol esterifying enzymes may be similar to the well-characterized enzyme lecithin-cholesterol acyltransferase (LCAT), a plasma enzyme which catalyzes the esterification of cholesterol with the *sn*-2 fatty acid of phosphatidylcholine. This similarity has led to the use of the name lecithin-retinol acyltransferase (LRAT) to describe the retinol esterifying activity.[3,5] An interesting difference is that LRAT selectively transfers the *sn*-1 fatty acid of phosphatidylcholine, rather than the *sn*-2 acyl group utilized by LCAT.[3]

Study of microsomal LRAT has been complicated by its ability to use the endogenous pool of phospholipids in the microsomes as its acyl source. Here, we report the solubilization and separation of liver LRAT from endogenous acyl donors, which has facilitated analysis. Importantly, the solubilized and partially purified hepatic LRAT demonstrates many of the same rigid specificities that the membrane-bound enzyme exhibits. In addition, by extending inhibitor studies, we report an interesting mechanistic difference from LCAT: the apparent use of a cysteine residue, rather than a serine, in the catalytic process.

Materials and methods

Materials

[^{3}H]Retinol-CRBP(II) was prepared as described previously.[7] The *p*-aminophenylarsineoxide (*p*-APAO[1]) was synthesized as described,[13] and the resulting product confirmed by fast atom bombardment mass spectrometry. This material was stable only when stored at 4° C or below. 1-Lauroyl-2-myristoyl-phosphatidylcholine and 1-myristoyl-2-lauroyl-phosphatidylcholine were synthesized as before.[3] Brij-35, *p*-arsanilic acid, DTT, DFP, diethyl *p*-nitrophenyl phosphate, sodium glycocholate, NEM, *m*-aminophenylboronic acid, β-mercaptoethanol, PMSF, papain, sodium taurocholate, Tween-20 and all diacyl- and lysophospholipids were from Sigma. Me_2SO, 2,3 dimercapto-1-propane-sulfonic acid and aluminum oxide (activated, neutral, Brockmann I) were from Aldrich. CHAPS, CHAPSO, deoxyBigCHAP, Lubrol PX, octyl β-glucoside, Triton X-100 and Extracti-Gel were obtained from Pierce Chemical Co. Casein and Tris Ultra Pure were from ICN Biochemicals. Phenyl-sepharose CL-4B was purchased from Pharmacia, and DE-52 was obtained from Whatman. Sodium deoxycholate was from Calbiochem. Zwittergent 3-14 was from Boehringer Manneheim. All solvents were HLPC-grade and were obtained from Burdick and Jackson Laboratories.

Solubilization of LRAT

Rat liver microsomes were prepared as before[8] with the resulting microsomal pellets resuspended to a concentration of 40–50 mg protein per ml in 0.2 M KH_2PO_4, pH 7.2, containing 1 mM DTT. Solubilization of LRAT activity was examined using a series of commercially available detergents. All procedures followed suggested guidelines.[14] Microsomal protein was suspended in 1 ml of 0.2 M KH_2PO_4, 1.0 mM DTT, and 1.0% detergent (wt/vol) at a concentration of 5.0 mg protein per ml. The solution was stirred at 4° C for 1 hr and then centrifuged in a Beckman TL-100 Ultracentrifuge using the TLA 100.3 rotor at 170,000 × *g* for 30 min at 4° C. The supernatant was removed and the pellet was resuspended in 1 ml 0.2 M KH_2PO_4 buffer containing 1 mM DTT and 1.0% detergent (wt/vol). For control values, microsomal protein was stirred and centrifuged in a similar manner in 0.2 M KH_2PO_4 buffer containing only 1 mM DTT. Ten μl of both the supernatant and resuspended pellet fractions were assayed for LRAT activity as described below.

Esterification assay

Retinyl ester production was assayed in a final incubation volume of 0.5 ml for HPLC analysis or 120 μl for radioactive studies as previously described.[3] DLPC was prepared as a 0.4 mM stock solution in Me_2SO and added to incubations to a final concentration of 20–40 μM. In all radioactive assays, except where indicated, the final concentration of Me_2SO was kept at 10% (vol/vol); this level of Me_2SO did not inhibit LRAT activity. All assays, except the indicated inhibition studies, included 1.0 mM DTT. Reactions were initiated with the addition of [^{3}H]-retinol-CRBP or [^{3}H]-retinol-CRBP(II) and then incubated in a 37° C stationary water bath for ten min. The reaction was stopped by the addition of four volumes of ice-cold ethanol containing 100 μg per ml BHT, and the esters were extracted into sixteen volumes hexane. [^{3}H]-Retinyl esters were quantitated by batch analysis on deactivated alumina columns as described elsewhere.[8] Unlabeled retinyl esters were quantitated by HPLC analysis.[3] Each experiment was repeated at least one time, and the values shown are an average of at least two determinations.

Chromatography of LRAT

All procedures were carried out at 4° C. The Phenyl Sepharose column was prepared in 0.2 M KH_2PO_4, pH 7.2, containing 20% glycerol and 1.0 mM DTT. The final column dimensions were 2.6 cm diameter with an average length of 30 cm (160 ml in volume). Brij-35 solubilized microsomal protein was loaded onto the column at a flow rate of 1 ml per min and 6.5 min fractions collected. After loading, the column was rinsed with 1.5 column volumes of 0.2 M KH_2PO_4 buffer, pH 7.2, containing 20% glycerol, 1.0 mM DTT, and 0.04% Brij-35, then with 2 column volumes of 0.01 M Tris-acetate buffer, pH 8.2, containing 20% glycerol, 1.0 mM DTT and 0.04% Brij-35. Following this washing procedure, LRAT activity was eluted with 2 column volumes of 0.01 M Tris-acetate buffer, pH 8.2, containing 20% glycerol, 1.0 mM DTT and 1.0%

Brij-35. Ten μl of selected fractions were assayed for LRAT activity as described above. The fractions containing LRAT activity were pooled and stored at −70° C.

A DEAE-cellulose column (2.6 × 18 cm) was equilibrated in 0.01 M Tris-acetate, pH 8.2, containing 20% glycerol. 1.0 mM DTT, and 0.2% Brij-35. The pooled fractions from Phenyl Sepharose chromatography were applied to the DEAE-cellulose column at a flow rate of 1.0 ml per min and 7 min fractions collected. The column was then washed with 2.5 volumes of the above 0.01 M Tris-acetate buffer. After washing, a gradient from 0.01 M Tris-acetate, pH 8.2, to 0.26 M Tris-acetate, pH 8.2, both with 20% glycerol, 1.0 mM DTT, and 0.2% Brij-35, was applied to the column in a total of 5 column volumes. Selected fractions were assayed for LRAT activity and fractions containing activity were pooled and stored at −70° C.

Lipid specificity studies

Phospholipid substrate preparations were added as sonicated dispersions in 0.2 M KH_2PO_4, pH 7.2, at a final concentration of 40 μM in a 0.5 ml assay volume.[3] Material from DEAE-cellulose chromatography was exchanged into the 0.2 M KH_2PO_4 assay buffer via an Extracti-Gel D column and 4.75 μg protein was assayed. Reactions were initiated by addition of 1.5 nmol retinol-CRBP(II). Following incubation at 37° C, the esters were extracted into hexane and analyzed by methods previously described.[3]

Inhibitor studies

LRAT (2μg), partially purified by Phenyl Sepharose chromatography, was preincubated with increasing concentrations of either NEM, *p*-aminophenylarsineoxide, PMSF, DFP, diethyl-*p*-nitrophenylphosphate, or *m*-aminophenylboronic acid. The assay was initiated by the addition of 2.4 nmol DLPC and 240 pmol [^{3}H]-reinol-CRBP(II) (final volume of 120 μl) and incubated for 10 min at 37° C prior to extraction of the ester products and analysis by alumina columns.[8] All inhibitors were introduced to the assay in 2 μl Me_2SO except m-aminophenylboronic acid, which was dissolved in 0.2 M KH_2PO_4 buffer. When appropriate, the controls contained 2 μl Me_2SO without inhibitor. Inhibition studies involving p-aminophenylarsineoxide, NEM, and PMSF were done in the absence of DTT. Each data point is the average of two determinations.

Reversibility of PMSF inhibition

To quantitate reversibility of PMSF inhibition, liver microsomes were preincubated at a concentration of 3.9 mg protein per ml for 10 min at 37° C in the presence or absence of 1 mM PMSF. Then, 12 μl aliquots were removed and added to 0.2 M KH_2PO_4, pH 7.2 containing DLPC. Reactions were initiated by addition of a solution containing DTT and [^{3}H]-retinol-CRBP(II) so that the 120 μl assay contained 46 μg protein, 2.4 nmol DLPC, 240 pmol [^{3}H]-retinol-CRBP(II), and increasing amounts of DTT. The reaction was incubated for 10 min at 37° C and the ester products were analyzed by batch analysis over alumina columns. For the time course study, the assay was initiated by addition of a solution containing DLPC, [^{3}H]-retinol-CRBP(II), and DTT such that the final 120 μl contained 46 μg protein, 2.4 nmol DLPC, 240 pmol [^{3}H]-retinol-CRBP(II), and 12 μmol DTT. These assays were incubated at 37° C for varying amounts of time and were stopped by transfer of 100 μl into four volumes of ice-cold ethanol containing 100 μg per ml BHT. Each experiment was repeated at least two times, and is reported as the average.

Papain assay and inhibition

The assay for papain activity was as previously described.[15] Briefly, 0.6 ml distilled, deionized H_2O containing 30 μg papain was added to 0.2 ml of freshly prepared activating agent, 0.05 M cysteine and 0.02 M EDTA, pH 8.0. Tris-HCl, 0.05 M, pH 8.0, was added to obtain a final volume of 1 ml. One ml aliquots of a 1.0% casein solution, brought to 37° C, were added to each tube and allowed to incubate at 37° C for 10 min with shaking. The reaction was terminated by the addition of 3 ml of a 5.0% TCA solution and the precipitates allowed to form by standing at least 1 hr at 25° C. The tubes were centrifuged at 1000*g* for 20 min. The A_{280} of the supernatant liquid was determined as the measure of papain activity. Inhibition was examined by adding increasing concentration of the inhibitors, DFP, and diethyl *p*-nitrophenyl phosphate, in 2 μl Me_2SO to the 0.6 ml papain solution (30 μg). The Me_2SO did not interfere with the assay. The 1.0% casein solution, warmed to 37° C, was added and incubation and analysis proceeded as above. Assays of 30 μg papain with 2 μl Me_2SO only were included for control values of protease activity, and heat inactivated papain was used as blank values.

Results and discussion

Solubilization and partial purification of LRAT activity

Members of several different classes of detergents were examined for their ability to solubilize the LRAT activity in rat liver microsomal preparations (*Table 1*). All detergents were examined at 1.0% (wt/vol). This study did not correct for possible detergent inhibition of LRAT activity. An extensive detergent comparison was not undertaken as the results in *Table 1* revealed several detergents capable of solubilizing active LRAT. LRAT proved to be particulary stable when solubilized by the polyoxyethylene Brij-35, and Brij-35 was chosen for use in the ensuing studies. Interestingly, Brij-35 resulted in solubilized preparations that exhibited an increase of activity over control. This enhanced enzyme activity may be related to the different lipid environment of the enzyme after detergent treatment. We have observed that the fatty acid composition of the microsomes influences LRAT activity (un-

Table 1 Detergent solubilization of retinol esterification activity

Detergent	Supernatant activity	Pellet activity
	% Control	% Control
Sodium deoxycholate	36	5
Sodium taurocholate	79	58
Sodium glycocholate	100	69
CHAPS	56	23
CHAPSO	58	30
DeoxyBigCHAP	28	6
Octyl glucoside	32	10
Tween 20	5	3
Lubrol PX	15	2
BRIJ-35	196	14
TRITON X-100	7	3
Zwittergent 3-14	9	19

Note: Liver microsomes (5 mg) were solubilized with indicated detergents as described under **Methods.** The percentages given are in comparison to the amount of esterification obtained from a microsomal sample which did not undergo detergent treatment. In all assays, a final concentration of 0.1% detergent was maintained. See **Results and discussion** for further details.

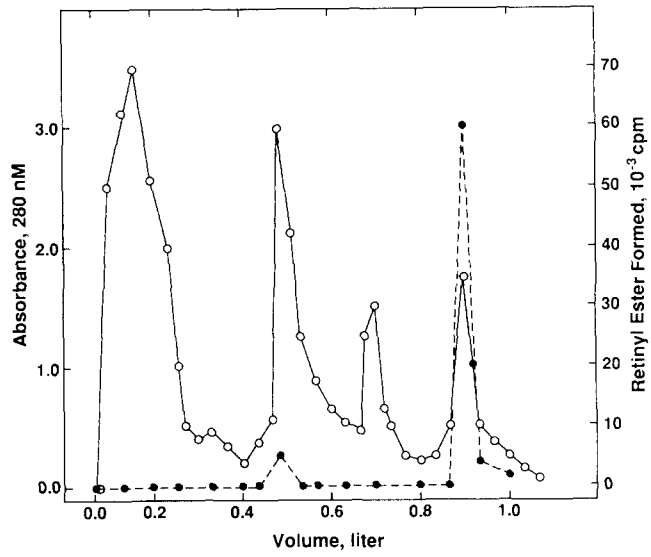

Figure 1 Chromatography of solubilized LRAT on Phenyl Sepharose CL-4B. Approximately 100 ml of solubilized material was applied to the Phenyl Sepharose CL-4B column, 160 ml in volume, at a flow rate of 1 ml per min with collection of 6.5 ml fractions. The column was washed with 240 ml of 0.2 M KH_2PO_4 buffer, pH 7.2, containing 20% glycerol, 1.0 mM DTT, and 0.04% Brij-35, then with 480 ml of buffer containing 0.01 M Tris-acetate, 20% glycerol, 1.0 mM DTT, and 0.04% Brij-35. LRAT was then eluted with 0.01 M Tris-acetate, 20% glycerol, 1.0 mM DTT, and 1.0% Brij-35. Absorbance was monitored at 280 nm (○) and selected fractions were assayed for activity as described under **Methods** (●). Fractions corresponding to 0.84–0.94 liters above were pooled and stored at −70° C.

published observation) and that may be due to a similar effect of changing lipid environment. Because Brij-35 concentrations greater than 0.3% in the assay caused significant inhibition, assays were performed at final concentrations of 0.02–0.08% detergent, remaining above the critical micelle concentration of the detergent.[16]

Barry et al.[6] recently reported solubilization of a phosphatidylcholine-dependent retinol acyltransferase from bovine pigment epithelium that may be similar to the activities we have previously reported for rat intestine[3] and rat and human liver.[4] Here, several differences were noted in the solubilization properties of the two enzymes. The most effective detergent for solubilizing the ester synthetase from bovine pigment epithelium is 0.1% zwittergent 3-14.[6] Similar conditions applied to the liver micromsomes resulted in less than half the recovered activity in the supernatant fraction. In addition, the ester synthetase for pigment epithelium is not solubilized by 1.0% Brij-35[6] in contrast to the effective solubilization of liver LRAT observed here. These solubility properties suggest the enzymes of these two tissues may be different.

The hepatic LRAT activity was purified partially by chromatography on Phenyl Sepharose CL-4B. The retained activity required a stringent elution procedure. Changes in ionic strength frequently effective for elution in hydrophobic interaction chromatography did not elute LRAT but did elute substantial other material, as shown by the elution profile in *Figure 1*. Elution of LRAT required saturation of the column with Brij-35. The combined material in the peak contained 80 mg protein of the 580 mg applied. The specific activity increased about four-fold to 930 pmol retinyl ester formed/min/mg protein, compared to 225 pmol retinyl ester formed/min/mg protein in the microsomal starting material, with a yield of 57% of applied activity. Factors affecting this yield of activity may be loss of activity due to proteolysis, decrease in activity due to removal of lipid components normally associated with LRAT, or both. In further studies, LRAT was not retained on CM-cellulose, octyl sepharose CL-4B, lentil-lectin sepharose-4B, or hydroxyapatite under the various conditions examined. The activity was retained by DEAE cellulose but elution did not result in significant additional purification (not shown). However, after the Phenyl Sepharose step, LRAT had been separated successfully from endogenous acyl donors to the extent that activity was now wholly dependent on the addition of exogenous lipid substrate, and allowed study of the activity free of competition by endogenous lipids. Characterization was done primarily with material recovered from the Phenyl Sepharose column unless otherwise indicated. The enzyme was stored at −70° C in 0.01 M Tris acetate buffer, pH 8.2, containing 20% glycerol, 1.0 mM DTT, and 1.0% Brij-35. When thawed, the enzyme was stable at 4° C for at least 7 days.

Characterization of substrate preferences for partially purified LRAT

Basic kinetic properties of the solubilized, partially purified LRAT were determined and compared to those previously established for LRAT in intact microsomes. *Figure 2* illustrates the saturable production of [^{3}H]-retinyl esters with increasing amounts of [^{3}H]-

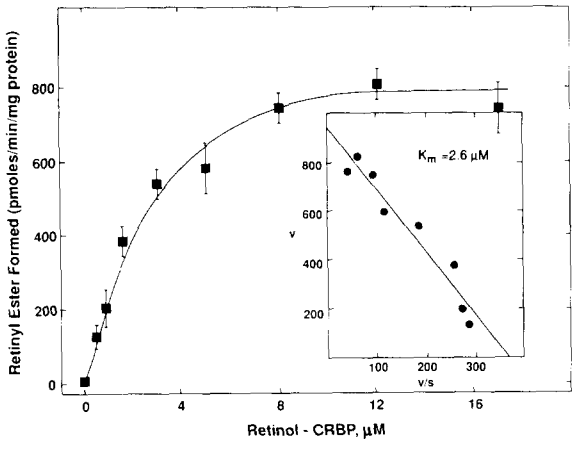

Figure 2 Concentration dependence of retinol-CRBP esterification by partially purified LRAT. Increasing concentrations of [^{3}H]-retinol-CRBP were incubated with 2 μg of material from Phenyl Sepharose CL-4B chromatography. The assays were performed as in **Methods** in a final volume of 120 μl containing 1.0 mM DTT, 20 μM DLPC, and 10% Me_2SO (vol/vol). The reactions were analyzed by chromatography on alumina columns as described. The average of four determinations (± SEM) are shown. Inset is an Eadie Hofstee transformation of the data used to calculate Km and Vmax.

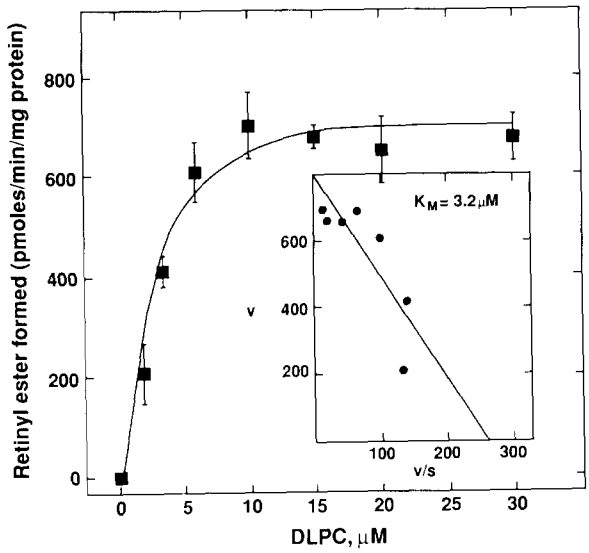

Figure 3 Concentration dependence of DLPC on retinol-CRBP(II) esterification by partially purified LRAT. Increasing concentrations of DLPC were incubated with 2 μg of material from Phenyl Sepharose CL-48 chromatography. The assays were performed as in **Methods** in a final volume of 120 μl containing 1.0 mM DTT. The DLPC was introduced in Me_2SO and all incubations were adjusted to 10% Me_2SO (vol/vol). The reaction was initiated by 2 μM [^{3}H]-retinol-CRBP(II) and were analyzed by chromatography on alumina columns as described. The average of 6 determinations (± SEM) are shown. The inset is the Eadie-Hofstee linear transformation of the data used to calculate Km and Vmax.

retinol-CRBP. The Eadie Hofstee plot (*Figure 2*, inset) resulted in a calculated Km value of 2.6 μM and a Vmax of 940 pmol/min/min/mg protein. The average of four determinations was 2.4 ± 0.20 (SEM) μM and the average Vmax 920 ± 70 (SEM) pmol retinyl ester formed/min/mg protein. The Km for retinol-CRBP previously determined for microsomal preparations is 1.0 μM.[8] Retinol-CRBP(II) was also examined as substrate carrier because CRBP(II) is transiently expressed in liver during the perinatal period.[8] It was also an effective substrate for esterification by solubilized LRAT, with a calculated Km of 1.3 ± 0.2 (SEM) μM from 6 determinations, and a Vmax of 970 ± 230 (SEM) pmol retinyl ester formed/min/mg protein. Becasue of the greater abundance and ease of purification of CRBP(II), [^{3}H]-retinol-CRBP(II) was used as substrate carrier for the remainder of the studies on partially purified LRAT.

Solubilization and partial purification of LRAT allowed the utilization of exogenous lipids as the acyl donor source without the complication of competition from endogenous acyl donors. Dilauroylphosphatidylcholine (DLPC) was chosen as a model substrate based on its solubility and proven ability to donate a lauryl group for retinol esterification.[3] The DLPC concentration dependence of esterification of [^{3}H]-retinol-CRBP(II) in the presence of Brij-35 is shown in *Figure 3*. The Eadie Hofstee transformation (*Figure 3*, inset) resulted in a Km of 3.2 μM and Vmax of 800 pmol retinyl ester formed/min/mg protein. The average of six determinations was 2.5 ± 0.7 (SEM) μM and a Vmax of 1000 ± 200 (SEM) pmol retinyl ester formed/min/mg protein. In all trials, DLPC concentrations greater than 30 μM resulted in a depression of activity. Data from the higher concentrations were excluded from the calculation of kinetic parameters. A systematic study of possible change in kinetic parameters with detergent was not undertaken. For the above studies, detergent levels were maintained at 0.08% (wt/vol) in the assay.

Previously, dilaurylphosphatidylethanolamine (DLPE) and dilaurylphosphatidic acid (DLPA) had been ineffective acyl donors when provided to liver microsomes.[3] In that case, however, the exogenous lipid necessarily was competing with endogenous phosphatidylcholines. Thus, the ability of soluble LRAT to utilize either of these compounds in the absence of any endogenous phosphatidylcholines was examined. LRAT recovered after both Phenyl Sepharose CL-4B and DEAE-cellulose chromatography was used for these studies. As shown in *Figure 4*, at the concentrations examined, only DLPC was an effective substrate for the production of retinyl laurate; in particular LRAT showed essentially no ability to use phosphatidylethanolamine, which would be protonated and similar in charge to phosphatidylcholine under the conditions examined. Thus, the rigid specificity for phosphatidylcholine observed for membranous LRAT was maintained when the enzyme was solubilized.

Liver microsomal LRAT has been shown previously to display positional specificity for the *sn*-1 position of phosphatidylcholine.[4] The solubilized and partially purified enzyme retained this specificity as shown when LRAT was provided with positionally distinct phosphatidylcholine. When 1-lauroyl-2-myristoyl-phosphatidylcholine was presented as exogenous substrate, retinyl laurate was the sole ester

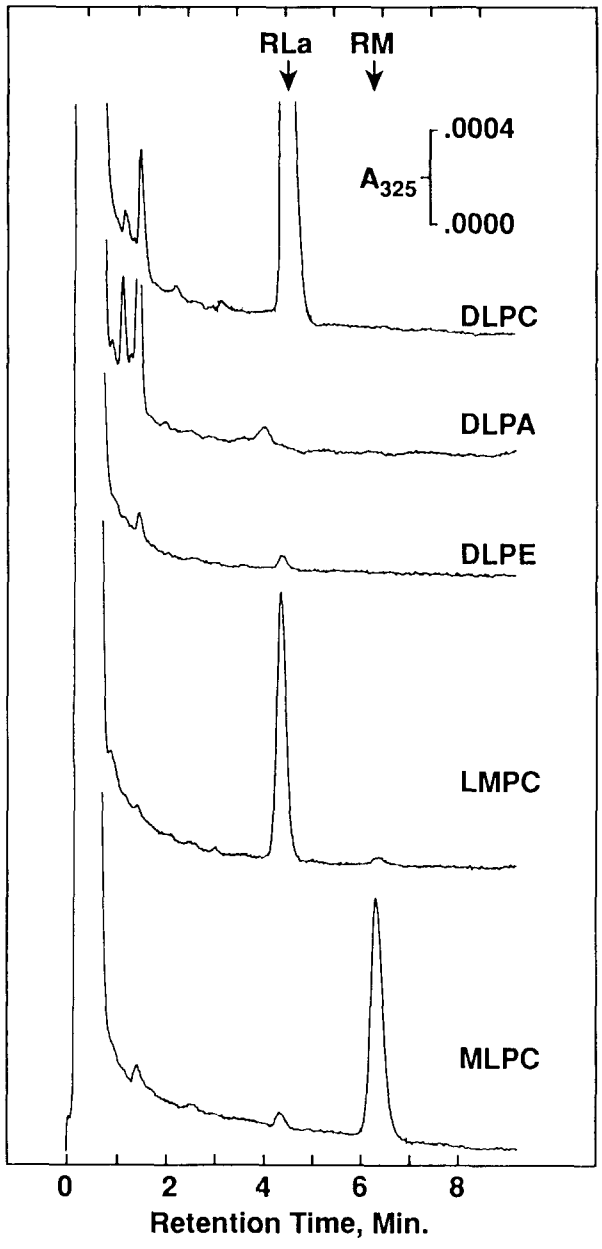

Figure 4 Substrate specificity and positional selectivity of partially purified LRAT. Material after Phenyl Sepharose CL-4B and DEAE-cellulose chromatography (5 μg) was incubated in 0.2 M KH_2PO_4 buffer, pH 7.2, containing 1 mM DTT and 20 nmol of the indicated lipid. Esterification reactions were initiated by the introduction of 1.5 nmol retinol-CRBP(II) (final volume 0.5 ml). The reactions were incubated for 10 min at 37° C and extracted and analyzed by reverse-phase HPLC using a Vydac C18 column with a Supelguard LC-18 guard column from Supelco. The tracings shown are representative of two separate experiments. *DLPC*, dilauroylphosphatidylcholine, *DLPA*, dilauroylphosphatidic acid, *DLPE*, dilauroylphosphatidylethanolamine, *LMPC*, 1-lauroyl-2-myristoylphosphatidylcholine, *MLPC*, 1-myristoyl-2-lauroyl phosphatidylcholine. *RLa*, retinyl laurate, *RM*, retinyl myristate.

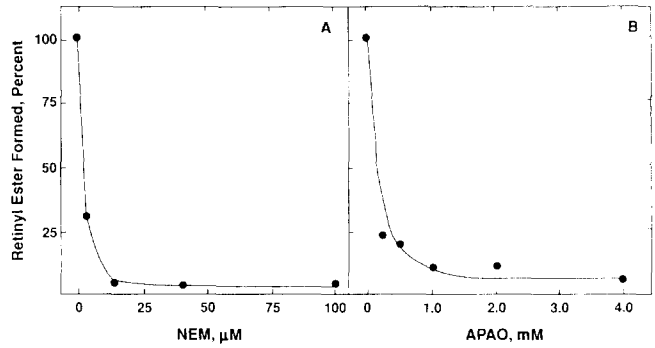

Figure 5 Effect of sulfhydryl-directed reagents on the esterification of retinol-CRBP(II) by partially purified LRAT. Protein from Phenyl Sepharose CL-4B chromatography, 2 μg, was preincubated at 37° C for 10 min in the presence of increasing amounts of NEM (panel A), or *p*-APAO (panel B) in 0.2 M KH_2PO_4, pH 7.2. Addition of [^{3}H]-retinol CRBP(II) and DLPC (2 μM and 20 μM, respectively, final volume of 120 μl) initiated the reaction. The reactions were analyzed by alumina chromatography as described. The results are expressed as the percentage of activity remaining when compared to untreated protein.

product. Conversely, when 1-myristoyl-2-lauroylphosphatidylcholine was provided as substrate, retinyl myristate was the only observed ester product (*Figure 4*). These studies indicated that soluble, partially purified LRAT retained important properties of membranous LRAT and prompted additional, mechanistic studies of LRAT.

Inhibitor studies on solubilized LRAT

As mentioned, esterification of retinol by LRAT has features similar to the esterification of cholesterol catalyzed by LCAT. Here, various inhibitors were utilized in order to probe possible similarities in mechanism between the two enzymes. Inhibitor studies on LCAT have shown that transacylation occurs in a sequential manner. First is a phospholipase cleavage of the *sn*-2 position of lecithin by a serine/histidine pair followed by an intramolecular transfer of the fatty acyl moiety from the serine to one (or either) of two sulfhydryls, prior to the ultimate transfer to the 3-hydroxyl of cholesterol.[17] Consequently, LCAT is inhibited by both antiesterases and sulfhydryl reagents.[17] LRAT activity was also quite sensitive to sulfhydryl reagents. For example, solubilized LRAT was completely inhibited by NEM at concentrations as low as 15 μM (*Figure 5A*), apparently even more sensitive than LCAT.[18] That LCAT has vicinal sulfhydryls in the active site was demonstrated by blocking acyltransferase activity with trivalent organoarsenical compounds specific for such vicinal thiols.[19] The inhibition is reversible by 2,3-dimercapto-1-propanesulfonic acid.[19] Interestingly, LRAT behaved similarly. Preincubation of solubilized LRAT with various concentrations of *p*-aminophenylarsineoxide (*p*-APAO) caused inhibition comparable to that seen for LCAT (*Figure 5B*). Incubation with 25 mM 2,3-dimercapto-1-propanesulfonic acid for one hour at 25° C led to re-

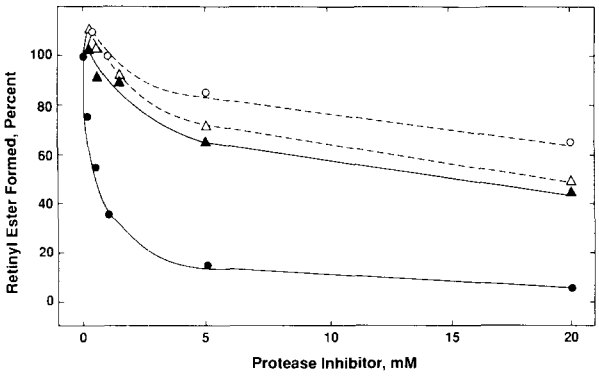

Figure 6 Effect of serine-directed reagents on esterification of retinol-CRBP(II) by partially purified LRAT. Protein from Phenyl Sepharose CL-4B chromatography (2 μg) was preincubated with increasing concentrations of PMSF (●), DFP (▲), diethyl *p*-nitrophenyl phosphate (△) or *m*-aminophenylboronic acid (○) for 10 min at 37° C. The boronate compound was added as a solution in 0.2 M KH_2PO_4 buffer; all others were introduced in 2 μl Me_2SO. The 120 μl total assay volume contained 20 μM DLPC in all cases, and 1 mM DTT, except in the case of PMSF. [^{3}H]-Retinol-CRBP(II) (2 μM) initiated the reaction. Following a 10-min incubation at 37° C, the reactions were analyzed by chromatography on alumina as described. The results are expressed as the percentage of activity remaining when compared to untreated protein.

covery of 92% of the LRAT activity. This suggests that LRAT may also employ vicinal sulhydryls in its catalytic mechanism and supported our previous suggestion of mechanistic similarities between LRAT and LCAT.[3] However, additional studies below suggested an important difference.

As mentioned, the phospholipase cleavage step of LCAT is mediated by an active site serine which can be blocked with serine protease inhibitors.[17] For example, 3 mM PMSF reduces LCAT activity to about 30%.[17] Similarly, 5 mM PMSF reduced solubilized LRAT activity to 18% (*Figure 6*). However, when the study was extended to other serine protease inhibitors, an unexpected difference between LRAT and LCAT was observed. Inhibition by DFP is a common diagnostic test for the existence of an active site serine[20] and 1 mM is more than sufficient for complete inhibition of LCAT.[21,22] However, little inhibition of LRAT was observed at 1 mM DFP and 50% inhibition of activity was achieved only when the concentration was increased to 20 mM DFP (*Figure 6*). Another serine-directed reagent, diethyl *p*-nitrophenyl phosphate, has enhanced ability to enter hydrophobic binding pockets of enzymes[23] and is a complete inhibitor of LCAT at 100 μM.[24] However, this reagent was also an ineffective inhibitor of LRAT even at a concentration of 20 mM. Boronates inhibit serine proteases via the formation of a tetrahedral boronate adduct and LCAT is inhibited by phenylboronic acid and *m*-aminophenylboronic acid, with Ki values of 1 to 3 mM.[25] LRAT was much less sensitive to *m*-aminophenylboronic acid; 20 mM was required before significant inhibition was observed. In addition, we found LRAT was not retained on an immobilized boronate column (data not shown), in contrast to LCAT.[25] These results were incompatible with serine being the residue utilized for catalysis by LRAT and suggested an important distinction from LCAT.

Similarities of LRAT and papain inhibition

The inhibition of LRAT by PMSF, but lack of inhibition by other serine-directed inhibitors suggested that the phosphatidylcholine cleavage by LRAT may utilize cysteine rather than serine. If cysteine were the active residue, LRAT might be compared to the sulfhydryl protease papain, which is also inhibited by PMSF.[26] To examine similarities, papain was preincubated with DFP or with diethyl *p*-nitrophenyl phosphate in the range from 25 μM to 20 mM for 10 min at 25° C prior to assaying protease activity. Both proved to be ineffective inhibitors. For DFP, 100% activity was recovered even at 20 mM inhibitor. Diethyl *p*-nitrophenyl phosphate treatment resulted in some inhibition, but over 40% of papain activity remained even with 20 mM inhibitor (data not shown). Inhibition at high diethyl *p*-nitrophenyl phosphate concentrations was not suprising as elevated levels of inhibitor can react with other than activated residues and decrease activity. This could also explain the decrease in LRAT activity observed with high concentrations of these compounds. These results with papain suggested that DFP and diethyl *p*-nitrophenyl phosphate will not react with a cysteine to produce a stable trisubstituted phosphorothioate capable of blocking enzyme activity and were consistent with the hypothesis that LRAT utilizes a cysteine rather than a serine.

The product of the reaction of PMSF (an activated sulfonate) with an active cysteine is a stable sulfonate thioester which can be reversed by the addition of thiol compounds, as has been shown for papain.[26] In contrast, the product with a serine is a sulfonate ester which is not reversed by thiols.[27] Consequently, PMSF inhibition of partially purified LRAT should be reversible if cysteine was the active residue modified. Indeed, 10 mM DTT and 100 mM β-mercaptoethanol reversed PMSF inhibition of solubilized LRAT but enzyme instability under the conditions employed in reversal made quantitation difficult. Liver microsomal preparations proved more stable under the conditions required and were used to quantitate reversal. The concentration dependence for DTT reversal of PMSF inhibition is shown in *Figure 7*; 75% recovery of activity was observed with 100 mM DTT.

PMSF-treated papain recovers activity rapidly, with a half life of twenty seconds, when treated with 5 mM DTT.[26] As shown in *Figure 8*, reversal of LRAT inhibition also occurred rapidly, in less than one min of incubation with 100 mM DTT, consistent with the time course of papain reversal. In this experiment, reversal was more complete than observed in *Figure 7*. Consequently, the ability to recover activity by treatment of PMSF-inactivated LRAT with thiols sup-

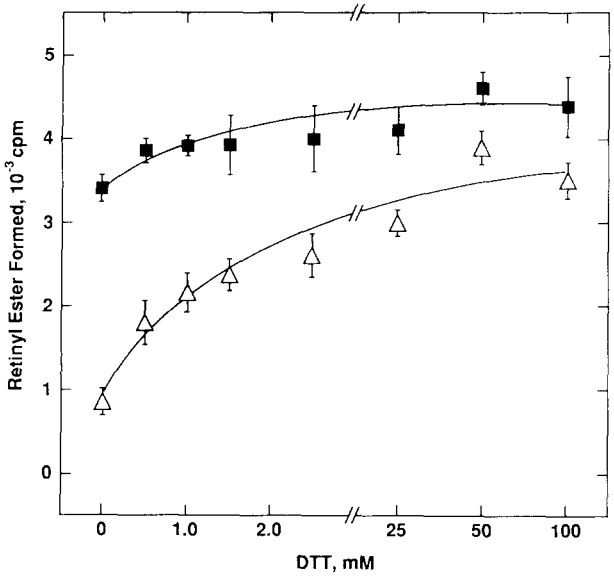

Figure 7 Effect of DTT concentration on reversal of PMSF inhibition. Liver microsomes, 3.90 mg/ml, were preincubated for 10 min at 37° C either in the presence (△) or absence (■) of 1 mM PMSF in 0.2 M KH_2PO_4 buffer, pH 7.2. (total volume 1 ml). Aliquots (12 μl) were then added to an assay mixture containing 0.2 M KH_2PO_4 and 2.4 nmol DLPC. The reaction was initiated by addition of a solution containing 240 pmol [^{3}H]-retinol-CRBP(II) and increasing amounts of DTT. The final volume was 120 μl. The reaction was extracted and analyzed by chromatography on alumina columns as described.

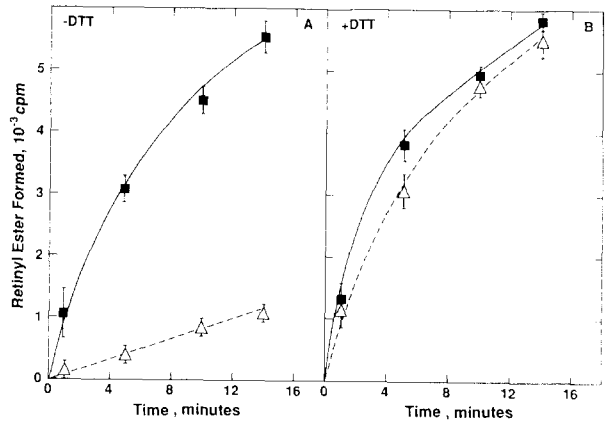

Figure 8 Time dependence of DTT effect on reversal of PMSF inhibition. Liver microsomes, 3.90 mg/ml were preincubated for 10 min at 37° C either in the presence (△) or absence (■) of 1 mM PMSF in 0.2 M KH_2PO_4 buffer, pH 7.2. (total volume 1 ml). Aliquots, 12 μl, were then added to 0.2 M KH_2PO_4, pH 7.2., for a final assay volume 120 μl. The reaction was initiated by addition of a solution containing 2.4 nmol DLPC, 240 pmol [^{3}H]-retinol-CRBP(II), plus (panel B) or minus (panel A) 12 μmol DTT. The assays were allowed to incubate for increasing amounts of time at 37° C prior to analysis by alumina chromatography as described.

ports the hypothesis that a cysteine, rather than serine, is utilized by LRAT.

Examination of the number of active-site cysteines

The fact that LCAT contains a serine in addition to two cysteines in its active site led us to examine whether LRAT had a simple substitution of cysteine for serine, leading to three cysteines in its active site, or only had two, the minimum for inhibition by *p*-APAO. In the three cysteine model, one cysteine would be involved in phospholipase cleavage and either of the other two might serve as the second acyl acceptor from the first cysteine; in the two cysteine model, only one cysteine is available to act as the second acyl group acceptor. To examine this question, LRAT was reacted with *p*-APAO and then exposed to NEM. Subsequent treatment with 2,3-dimercapto-1-propane sulfonic acid would remove *p*-APAO but would not be able to reverse NEM inhibition. If only two sulfhydryls are present in the active site, one would expect that *p*-APAO would completely protect LRAT from inactivation by NEM. If three sulfhydryls are present, it is possible that *p*-APAO could interact with any two of the three cysteines, depending on the particular geometry present. In this case, protection would depend on which two of the three cysteines reacted. If the cysteine involved in phospholipase cleavage had reacted with *p*-APAO, protection of activity could occur. If that cysteine had not been involved in the *p*-APAO interaction, complete loss of activity would be expected because NEM could then react irreversibly with that cysteine which would prevent the phospholipase cleavage portion of the mechanism. The results obtained were as follows. After 1 mM *p*-APAO treatment (reducing activity to 9.0% of control), but no NEM treatment, 70% activity was recovered after incubation with 25 mM 2,3-dimercapto-1-propane sulfonic acid for 10 min at 37° C. In contrast, when *p*-APAO treatment was followed by incubation with 40 μM NEM, exposure to 2,3-dimercapto-1-propane sulfonic acid as above led to recovery of only 47% activity, suggesting the presence of a cysteine unprotected by *p*-APAO. As control, NEM was incubated with LRAT prior to *p*-APAO treatment; after reversal by 2,3-dimercapto-1-propane sulfonic acid 8.0% of activity was recovered. Repeat experiments yielded similar results. The partial protection against NEM inhibition accomplished by pretreatment with *p*-APAO is consistent with three cysteines in the active site of LRAT.

Although additional work with pure enzyme is required, we suggest that LRAT differs from LCAT in utilizing a cysteine rather than a serine for the phospholipase cleavage step of the catalytic process. However, the various similarities already observed between these two enzymes suggest a possible evolutionary relationship between them. For example, it has been suggested that cysteine is the evolutionary precursor for active-site essential serines[28] and, in some cases, such precursors appear to be still extant (for examples, see reference 28). These acyltransferase enzymes may be an additional example.

Abbreviations

p-APAO *p*-aminophenylarsineoxide
BHT butylated hydroxytoluene
CRBP cellular retinol binding protein type I
CRBP(II) cellular retinol binding protein type II
DENP diethyl-*p*-nitrophenyl phosphate
DLPC dilauroylphosphatidylcholine
DTT dithiothreitol
HPLC high performance liquid chromatography
LCAT lecithin-cholesterol acyltransferase
LRAT lecithin-retinol acyltransferase
NEM N-ethylmaleimide
PMSF phenylmethylsulfonyl fluoride

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