Kinetic Mechanism of Lecithin Retinol Acyl Transferase†

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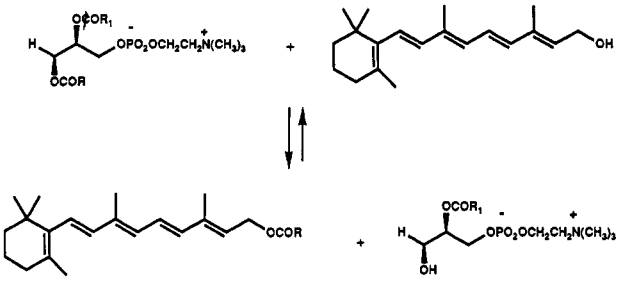
Received August 5, 1992; Revised Manuscript Received November 10, 1992

ABSTRACT: Lecithin retinol acyl transferase transfers acyl groups regiospecifically from the 1-position of lecithins to *all-trans*-retinol (vitamin A) and similar retinoids. LRAT is essential for the biosynthesis of 11-*cis*-retinal, the visual pigment chromophore, and is also required for the general dietary mobilization of vitamin A. The kinetic mechanism of this enzyme is described here, K_M and V_{max} values were determined for the substrates dipalmitoylphosphatidylcholine (DPPC) [1.38 μM and 0.17 μM/(min·mg), respectively] and for *all-trans*-retinol [0.243 μM and 0.199 μM/(min·mg), respectively]. In order to distinguish between a ping-pong bi-bi mechanism and a rapid equilibrium random or ordered bi-bi mechanism, the velocity of product formation as a function of one of the substrates at different fixed concentrations of the other substrate was measured. The parallel lines generated are entirely consistent with a ping-pong bi-bi mechanism in which DPPC first binds to LRAT and acylates it and rule out both simple random binding and ordered kinetic mechanisms. Further evidence for a ping-pong bi-bi mechanism comes from partial exchange reaction studies which show that LRAT can catalyze acyl group interchange between two different lecithin derivatives. Finally, the ping-pong reaction was established as being ordered, using the potent and reversible dead-end inhibitor 13-desmethyl-13,14-dihydro-*all-trans*-retinyl trifluoroacetate. This compound proved to be competitive with respect to DPPC, with a K_I = 11.4 μM, and uncompetitive with respect to *all-trans*-retinol.

Lecithin retinol acyl transferase (LRAT)[1] is a membrane-bound enzyme which catalyzes the reversible acylation of *all-trans*-retinol (vitamin A) by phospholipids (Scheme I) (MacDonald & Ong, 1987, Barry et al., 1989; Saari & Bredberg, 1989). This reaction has so far been determined to occur in the intestine, liver, and retinal pigment epithelium (RPE) (Ong et al., 1991; MacDonald & Ong, 1987; Barry et al., 1989; Saari & Bredberg, 1989). The enzyme is implicated in the mobilization of vitamin A in the liver and intestine. LRAT also plays an essential role in visual pigment regeneration, since *all-trans*-retinyl esters are the "high-energy" precursors for the isomerohydrolase which generates 11-*cis*-retinol in the RPE (Deigner et al., 1989). Oxidation of 11-*cis*-retinol in the RPE produces the visual chromophore 11-*cis*-retinal. LRAT links the chemical potential of phospholipids to the formation of the thermodynamically unstable visual chromophore (Rando, 1991).

LRAT has been solubilized and partially purified (Barry et al., 1989). The enzyme is specific for phosphatidylcholine (lecithin) and only transesterifies retinol and retinol analogs (Cañada et al., 1990). It does not esterify cholesterol or *n*-dodecanol, for example (Cañada et al., 1990). The enzyme is regiospecific for the *sn*-1 position of the lecithin but is not highly specific with respect to the structure of the fatty acid moiety (Cañada et al., 1990). The regioselectivity for the *sn*-1 acyl group makes LRAT thus far unique and possibly a member of a new class of acyl transferases.

Scheme I: Transesterification of Vitamin A Catalyzed by LRAT



The enzyme has not been characterized mechanistically thus far. To begin to characterize the mechanism of action of LRAT, its kinetic mechanism must first be determined. In a bisubstrate reaction of the type catalyzed by LRAT, several (often not mutually exclusive) alternatives present themselves, including ping-pong bi-bi mechanisms, rapid equilibrium random mechanisms, and ordered bi-bi mechanisms. It is shown here that the kinetic mechanism involves an ordered ping-pong bi-bi mechanism. The enzyme appears to be first acetylated by the lecithin to generate an acyl enzyme intermediate and a 1-lysophosphatidylcholine. The vitamin A binds and is then esterified to generate the retinyl ester and the free enzyme.

MATERIALS AND METHODS

Materials

Frozen bovine eye cups were obtained from W. L. Lawson Co. (Lincoln, NE). Sodium boro[^{3}H]hydride (0.1 M NaOH, 13.6 Ci/mmol) and [11,12-^{3}H,^{3}H]*all-trans*-retinol (47.9 Ci/mmol) were from Amersham Corp. L-α-Dipamitoyl[choline methyl-^{3}H]phosphatidylcholine was from Du Pont–New England Nuclear. *all-trans*-Retinal, bovine serum albumin (BSA), dithiothreitol (DTT), L-α-divaleroylphosphatidyl-

† This work was supported by the National Institutes of Health U.S. Public Health Service Grant EY-04096. I.H. was supported by a postdoctoral fellowship from the Swiss National Science Foundation.

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[1] Abbreviations: LRAT, lecithin retinol acyl transferase; DPPC, dipalmitoylphosphatidylcholine; DVPC, divaleroylphosphatidylcholine; VPPC, 1-valeroyl-2-palmitoylphosphatidylcholine; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; RPE, retinal pigment epithelium.

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choline (DVPC), L-α-dipalmitoylphosphatidylcholine (DPPC), and valeric anhydride were from Sigma Inc. Triton X-100 was from Calbiochem Corp. Disodium ethylenediaminetetraacetate (EDTA·2Na) was from Fisher Scientific Co. Lipase from *Rhizopus arrhizus* and tris(hydroxymethyl)-aminomethane were from Boehringer Mannheim, Inc. Dimethylaminopyridine, trifluoroacetic anhydride, and lithium bis(trimethylsilyl)amide were from the Aldrich Chemical Co. Triphenylphosphonium bromide was from Alfa Products, Inc. Tetrabutylammonium fluoride, β-ionylideneacetate, and pyridine were from Fluka, Inc.

Methods

Syntheses. 13-Desmethyl-13,14-dihydro-all-trans-retinyltrifluoroacetate. (a) (4-Hydroxybutyl)triphenylphosphonium Bromide. A solution of 12 g (35 mmol) of triphenylphosphonium bromide in 30 mL of acetonitrile and 40 mL of tetrahydrofuran was refluxed overnight. The white precipitate formed was collected, washed with a few milliliters of cold acetonitrile, and dried under high vacuum to yield 5.8 g of 40% of the phosphonium salt (Maryanoff et al., 1985). The ^{1}H NMR spectrum is entirely consistent with the assigned structure: (500 MHz, DMSO-d_6) δ 1.55 (4 H, m), 3.4 (2 H, t, J = 5.5 Hz), 3.55 (2 H, m), 7.7–7.9 (15 H, 2 m).

(b) 13-Desmethyl-13,14-dihydroretinol Trismethylsilyl Ether. To an ice-cooled suspension of 9.23 g (2.23 mmol) of (4-hydroxybutyl)triphenylphosphonium bromide in 4 mL of dry tetrahydrofuran, 4.7 mL (4.7 mmol, 2.1 equiv) of a 1 M solution of lithium bis(trimethylsilyl)amide was added under argon. After 30 min of stirring at the same temperature, 485 mg of β-ionilidenealdehyde (Wendler et al., 1951) dissolved in 2 mL of dry tetrahydrofuran was slowly added to the bright red homogeneous solution of the ylide. Stirring was continued for 1 h at 0 °C and then for 2 h at room temperature. After addition of 200 mL of diethyl ether, the reaction mixture was washed three times with 200 mL of saturated NaCl solution. The organic layer was dried over $MgSO_4$ and concentrated to provide a yellow oil. Chromatography on silica (hexanes) afforded the product as a slightly yellow oil in 70% yield (1.1 g, 3.18 mmol) (Maryanoff et al., 1985). The NMR and IR spectra are entirely consistent with the assigned structure. IR (CCl_4) cm^{-1}: 2950 (s), 2900 (m), 1240 (m), 1080 (m), 960 (m), 830 (s). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.1–0.115 (9 H, 2 s), 1.0–1.02 (6 H, 2 s), 1.46 (2 H, m), 1.6 (2 H, m), 1.62 (2 H, p, J = 6.5), 1.68–1.72 (3 H, 2 s), 1.88–1.91 (3 H, 2 s), 2.0 (2 H, q, J = 5), 2.19 (1 H, q, 6.5), 2.27 (1 H, q, J = 6.5), 3.59 (2 H, td, J_1 = 6.5, J_2 = 4), 5.4–6.7 (5 H).

(c) 13-Desmethyl-13,14-dihydroretinol. A solution of 1.1 g of 13-desmethyl-13,14-dihydroretinol trimethylsilyl ether (3.18 mmol, 1 equiv) in 20 mL of dry diethyl ether was treated with 1.5 equiv of tetrabutylammonium fluoride (4.8 mL of a 1 M solution in tetrahydrofuran) at room temperature under argon. After 20 min, the reaction mixture was diluted with 200 mL of diethyl, ether washed three times with 100 mL of brine, dried over $MgSO_4$, filtered, and concentrated to afford 828 mg (3.02 mmol) of product as light yellow oil in 95% yield. The NMR and IR spectra are entirely consistent with the assigned structure. IR (CCl_4) cm^{-1}: 2950 (s), 2900 (sh), 1440 (w), 1240 (m), 1200 (w), 960 (m). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.9–1.2 (6 H, 2 s), 1.46 (2 H, m), 1.6 (2 H, m), 1.7 (5 H, m), 1.88–1.92 (3 H, 2 s), 2.0 (2 H, m), 2.24–2.33 (2 H, 2 q, J = 7.5), 3.7 (2 H, t, J = 5.5), 5.5–6.5 (5 H).

(d) 13-Desmethyl-13,14-dihydro-all-trans-retinyl Trifluoroacetate. An excess of 10 equiv (500 μL, 3.6 mmol) of trifluoroacetic anhydride and of 20 equiv (580 μL, 7.2 mmol) of pyridine was added to an ice-cooled solution of 100 mg (0.36 mmol) of 13-desmethyl-13,14-dihydroretinol in 10 mL of dry diethyl ether. The reaction mixture was stirred at 0 °C under argon for 30 min, diluted with 80 mL of diethyl ether, washed three times with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residual yellow oil was purified using silica preparative thin-layer chromatography (hexanes/Et_2O, 9:1) to afford the trans/cis mixture of esters in 50% yield. The isomers could be separated by HPLC using a LiChrosorb Si 60 (5 μm) column and eluting with 0.5% ethyl ether in hexane at a flow rate of 0.5 mL/min. Under these conditions, the 11-cis isomer had a retention time of 15.3 min, and the trans isomer had a retention time of 16.2 min. The ratio of the two isomers is cis/trans = 16.9/83.1. Only the trans isomer is a potent inhibitor of LRAT. The NMR, UV, and IR spectra are entirely consistent with the assigned structure. IR (CCl_4) cm^{-1} of the cis/trans mixture: 2950 (s), 2900 (sh), 1780 (s), 1420 (m), 1390 (w), 1350 (m), 1340 (m), 1210 (s), 1160 (s), 1140 (s), 960 (m). UV (*n*-hexanes) of the cis/trans mixture: λ_{max} = 287; ε = 28 980. ^{19}F NMR of the *all-trans* isomer ($CDCl_3$; ref, α,α,α-trifluorotoluene): 12.733 (s). ^{1}H NMR (500 MHz, $CDCl_3$) of the *all-trans* isomer ($CDCl_3$): δ 1.02 (6 H, s); 1.46 (2 H, m); 1.61 (2 H, m); 1.69 (3 H, s); 1.87 (2 H, m); 1.90 (3 H, s); 2.0 (2 H, t, J = 8 Hz); 2.25 (2 H, q, J = 6.5 Hz); 4.37 (2 H, t, J = 7 Hz); 5.66 (H, q, J_1 = 15.5 Hz, J_2 = 6.5 Hz); 6.06 (3 H, m); 6.44 (H, q, J = 15.3 Hz).

Synthesis of 1-Valeroyl-2-palmitoylphosphatidylcholine (VPPC). A mixture of 480 μL of ethyl ether and 1480 μL of buffer (sodium borate, pH 6.5, [Ca^{2+}] = 5.3 μM) was prepared and to it was added 40 mg of DPPC and 20 μL of lipase (from *R. arrhizus*, 50 000 units/mL). The mixture was stirred at room temperature. After 8 h another 20 μL of lipase was added, and the mixture was stirred for an additional 8 h. Thin-layer chromatography on silica ($CHCl_3$/MeOH/H_2O, 65:25:4) showed that hydrolysis was incomplete. However, the reaction was terminated anyway to prevent 1,2-acyl migration. The solution was extracted with $CHCl_3$/MeOH (7:3, v/v) three times. The extracts were combined and evaporated at room temperature. The residue was washed three times with cold ether, and the supernatants were discarded after each washing. The final dried residue weighed 27 mg and contained mostly 2-palmitoyllysophosphatidylcholine, along with a small amount of DPPC. The R_f for the lyso analog was 0.05 ($CHCl_3$/MeOH/H_2O, 65:25:4) (Slotboom et al., 1970).

2-Palmitoyllysophosphatidylcholine (12.4 mg) was added to 300 μL of dry chloroform. Valeric anhydride (67.8 mg) and 5.24 mg of dimethylaminopyridine were then added, and the solution was stirred at room temperature under nitrogen for 12 h. After being washed with 300 μL of 0.1 N HCl, the water phase was extracted twice with 200 μL of chloroform. The combined chloroform extracts were dried under nitrogen, and the residue was washed with cold ether and centrifuged. After the supernatant was discarded, the white solid was dissolved in chloroform and purified on a Dynamax-300A column (C_{18}, 2500 × 4.6 mm), eluting with 95% MeOH/5% buffer (1 mM potassium phosphate, pH 7.4) at 0.5 mL/min. VPPC (8.5 mg) was obtained. The yield was 58.5%, and R_f was 0.11 ($CHCl_3$/MeOH/H_2O, 65:25:4). The NMR spectrum is entirely consistent with the assigned structure. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (6 H, m), 1.24 (4 H, s), 2.28 (26 H, m), 3.35 (9 H, s), 3.7–5.2 (m,

methylene group of glycerol backbone and choline moiety) (Mason et al., 1981).

Partial Purification of Lecithin Retinol Acyltransferase (LRAT). The method used for the partial purification of LRAT was substantially the same as previously reported (Barry et al., 1989). Bovine pigment epithelium membranes were solubilized in 20 mM Tris-HCl, pH 9.0, 2 mM DTT, 1 mM EDTA·2Na, 1% Triton X-100, and 1 mg/mL DPPC. After thorough mixing for 1 h at 4 °C, the solubilized material was centrifuged at 4 °C at 10500*g* for 1 h. The supernatant was applied to a Mono-Q column on a LKB-Pharmacia Biotechnology Inc. Superose 6 LCC-500 FPLC system at 5 °C and eluted using a linear gradient of buffers A and B (A, 20 mM Tris-HCl, pH 9.0, 2 mM DTT, 1 mM EDTA·2Na, 0.1% Triton X-100, and 0.1 mg/mL DPPC; B, buffer A plus 1 M NaCl). The collected fractions were assayed as previously published (Barry et al., 1989). The fractions with high activity were mixed, applied to a reactive Green-5 agarose column, and eluted with a linear gradient of buffer A and C (C, buffer A plus 2 M NaCl). The fractions were assayed and stored at −70 °C separately [specific activity = 7.1 μmol/(min·mg)]. Enzyme partially purified in this manner is stable for months at −20 °C. The enzyme used is approximately 20-fold purified over the solubilized material. Protein concentrations were determined by the Peterson modification of the Lowry method (Peterson, 1977).

Kinetic Measurements. Solutions of DPPC in 2.5% BSA (10, 25, 100, 250, and 1000 μM, respectively) were prepared and sonicated for 30 min. Varying amounts of [11,12-^{3}H,^{3}H]-*all-trans*-retinol in hexane solution were dried under nitrogen in a tube (along with varying concentrations of **1** when inhibition studies were being performed). Twenty microliters of DPPC stock solution in BSA was added, and the mixture was agitated by vortexing for 1 min before 60 μL of Tris-HCl buffer (pH 9.0) was added. The mixture was preequilibrated for 10 min at 23 °C, and 20 μL of proteins containing LRAT was added. The final concentrations in the assay solution were as follows: Tris-HCl, 0.1 M (pH 9); DTT, 2 mM; partially purified LRAT, 0.54 μg/mL; DPPC concentrations were 2.27, 5.27, 20.27, 50.27, and 200.27 μM, and, for these DPPC values, [11,12-^{3}H,^{3}H]*all-trans*-retinol concentrations were 0.2, 0.3, 0.5, 0.75, 1.0, and 1.35 μM, respectively. After 10 min of incubation, 500 μL of methanol was added to stop the reaction. After vortexing, 100 μL of water and 400 μL of hexane were added. The mixture was again agitated by vortexing for 1 min and centrifuged for 5 min at 15 °C. Finally, 200-μL aliquots of the hexane layer were removed, and the amount of retinyl ester was determined on a Dynamax-60A (250 mm × 4.6 mm) column connected to an on-line Berthold radioactivity monitor as previously described (Barry et al., 1989). Elution was with *n*-hexane/ethyl acetate/isopropanol (90:10:1, v/v/v).

Exchange Reaction between DPPC and DVPC in the Presence of LRAT. Dipalmitoyl-[choline methyl-^{3}H]phosphatidylcholine (1.04 μL) (31 Ci/mmol, 0.032 mmol/mL) and 6.38 μL of DVPC (4.7 mM) were dried under nitrogen. Twenty microliters of 2.5% BSA in distilled water was added, and the solution was sonicated for 20 min. Sixty microliters of Tris-HCL buffer (pH 9.0) and 20 μL of partially purified LRAT were then added. The final concentrations in the incubation mixture were DPPC, 0.6 μM (0.27 μM unlabeled DPPC in enzyme and 0.33 μM labeled DPPC); DVPC, 300 μM; BSA, 0.5%; DTT, 2 mM; Tris-HCl, 0.1 M; and LRAT, 0.054 μg/mL. The mixture was incubated for 2 h at 23 °C. A control experiment minus LRAT was also performed. The reaction was stopped by adding 200 μL of methanol. After 100 μL of water and 200 μL of chloroform were added, the mixture was vortexed for 1 min and centrifuged for 20 min at 15 °C. The chloroform layer was dried under nitrogen and the samples were analyzed on a Microsorb C_{18} column (2500 × 4.6 mm), eluting with 95% MeOH/5% buffer (1 mM potassium phosphate, pH 7.4) at 1 mL/min. The HPLC was connected to an on-line Berthold radioactivity monitor for radioactivity determinations.

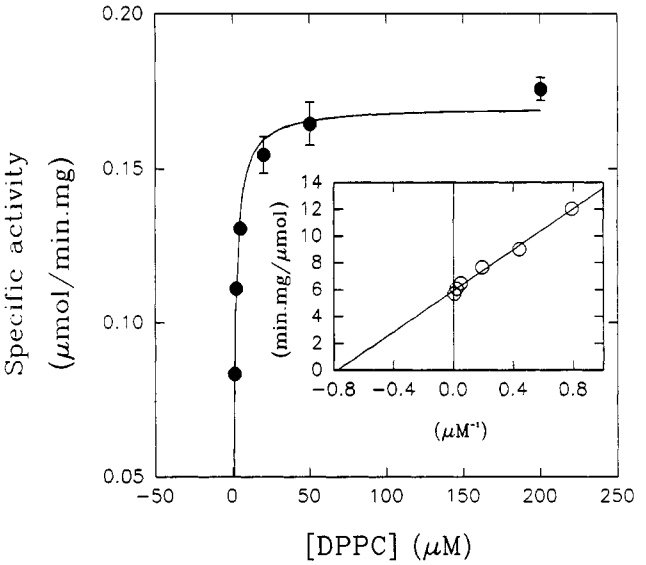


FIGURE 1: Retinyl ester formation as a function of varying dipalmitoylphosphatidylcholine concentrations. The Michaelis–Menten plot and the Lineweaver–Burk plot (inset) of the [11,12-^{3}H]retinyl ester formation as a function of DPPC at fixed [retinol] = 1 μM are shown. The symbols represent the mean value of duplicate determinations, and error bars represent the standard deviations. K_M = 1.38 μM and V_{max} = 0.17 μmol/(min·mg). The kinetics were determined as described under Methods.

RESULTS

Kinetic Studies on LRAT. Solubilized and partially purified LRAT was first studied with respect to measuring K_M and V_{max} values for the two substrates under consideration here, *all-trans*-retinol and DPPC. As shown in Figures 1 and 2, Michaelis–Menten and Lineweaver–Burk plots were constructed for both substrates. The K_M and V_{max} values for DPPC are 1.38 μM and 0.17 μM/(min·mg), respectively, and for *all-trans*-retinol they are 0.243 μM and 0.199 μM/(min·mg), respectively.

In the next set of experiments, it was of interest to distinguish between a ping-pong bi-bi mechanism and a rapid equilibrium random or ordered bi-bi mechanism. This can sometimes be accomplished by measuring the velocity of product formation as a function of one of the substrates at different fixed concentrations of the other substrate. In a ping-pong bi-bi mechanism, plots of $1/v$ versus 1/[substrate (A)] at different fixed substrate (B) concentrations should yield a family of parallel lines whose slopes equal K_M/V_{max} (Segel, 1975a). On the other hand, both rapid equilibrium random and ordered mechanisms yield lines which intersect at the point where the 1/[substrate (A)] value is negative and the $1/v$ value is positive, negative, or zero (Segel, 1975b). Therefore, kinetic studies of this kind are informative with respect to whether a ping-pong bi-bi mechanism is operative or not. Rates of *all-trans*-retinyl palmitate formation were determined by varying DPPC concentrations at different fixed *all-trans*-retinol concentrations (Figure 3) and by varying the *all-trans*-retinol concen-

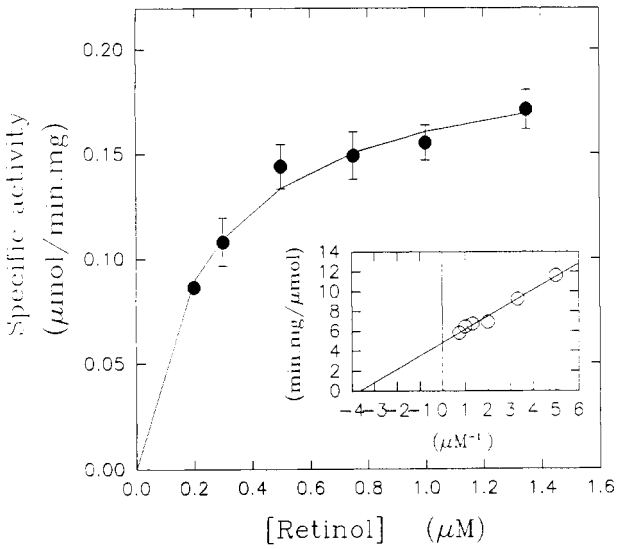


FIGURE 2: Retinyl ester formation as a function of varying *all-trans*-retinol concentrations. The Michaelis–Menten plot and the Lineweaver–Burk plot (inset) of [11,12-^{3}H]*all-trans*-retinyl ester formation as a function of [11,12-^{3}H]-*all-trans*-retinol at fixed [DPPC] = 200 μM are shown. The symbols represent the mean value of duplicate determinations, and error bars represent the standard deviations. K_M = 0.243 μM and V_{max} = 0.199 μmol/(min·mg). The kinetics were determined as described under Methods.

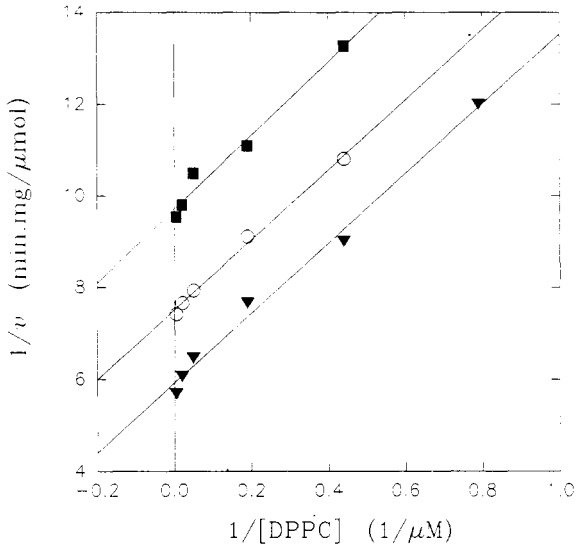


FIGURE 3: Lineweaver–Burk plots for the formation of retinyl ester at fixed *all-trans*-retinol concentrations. The Lineweaver–Burk plots for the acylation of [11,12-^{3}H]-*all-trans*-retinol with fixed amounts, (■) 0.2, (○) 0.5, and (▼) 1 μM, of [11,12-^{3}H]*all-trans*-retinol and varied [DPPC] are shown. The symbols represent the average values of duplicate experiments. The average standard deviation of these values is less than 4%. Kinetic measurements were made as indicated under Methods.

trations at different fixed DPPC concentrations (Figure 4). The curves in Figures 3 and 4 are entirely consistent with a ping-pong bi-bi mechanism, because they form parallel series of lines. Since the families of lines do not converge, they appear to rule out either simple random binding or ordered kinetic mechanisms.

Inhibition Studies with 13-Desmethyl-13,14-dihydro-all-trans-retinyl Trifluoroacetate 1. Further kinetic analysis using product inhibitors, such as retinyl esters, is necessary to further dissect the kinetic mechanism with respect to the order of substrate binding. Unfortunately, none of the simple aliphatic retinyl esters tested as product inhibitors was potent enough to carry out these studies. Moreover, the product

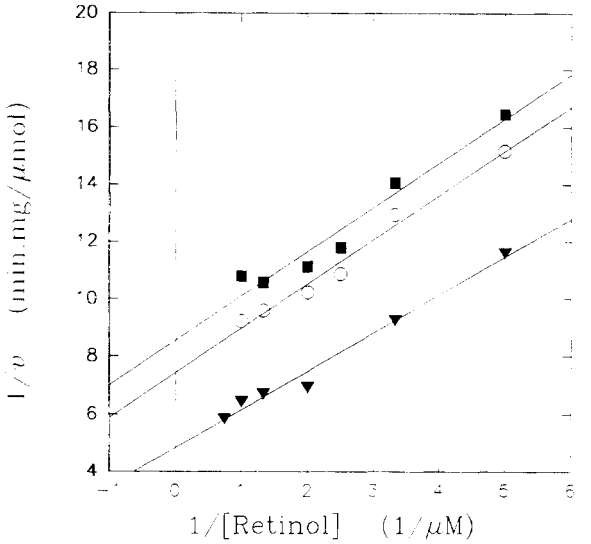
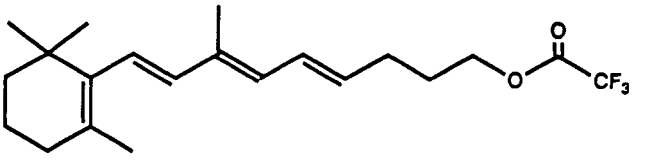


FIGURE 4: Lineweaver–Burk plots for the formation of retinyl ester at fixed DPPC concentrations. The Lineweaver–Burk plots for the acylation of retinol with fixed amounts, (■) 2, (○) 20, and (▼) 200 μM, of DPPC and varied amounts of [11,12-^{3}H]*all-trans*-retinol are shown. The symbols represent the average values of duplicate experiments. The average standard deviation of these values is less than 3.9%. Kinetic measurements were made as indicated under Methods.

1-lysophosphatidylcholines nonspecifically inhibit the detergent solubilized LRAT, precluding the use of these derivatives in kinetic studies as well.

Therefore, a novel and potent product-like LRAT inhibitor was sought: 13-desmethyl-13,14-dihydro-*all-trans*-retinyl trifluoroacetate **1** was prepared, and is shown here to be a



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potent and reversible inhibitor of LRAT. To begin with, it was shown that preincubation of LRAT with 6 μM **1** for 30 min did not lead to the irreversible inhibition of the enzyme, allowing for the use of **1** to determine the kinetic mechanism of LRAT.

The use of a dead-end inhibitor is essential to determine kinetic order in a ping-pong mechanism. If the inhibitor can bind to both free enzyme and the enzyme–substrate complex, it will show mixed-type inhibition (Segel, 1975c). The Lineweaver–Burk plots will have different slopes and intercepts and will converge in the second quadrant. If the inhibitor only binds to one form of the enzyme, it will be competitive to the substrate which binds to the same form of the enzyme as it does and will be uncompetitive with the other substrate (Segel, 1975c). If competitive inhibition should arise, the resultant Lineweaver–Burk plots will have different slopes and the same intercepts. For uncompetitive inhibition, the lines will have the same slopes and different intercepts. As shown in Figure 5, in which the concentrations of DPPC and **1** are varied at a fixed vitamin A concentration, the inhibitor **1** proved to be competitive with respect to DPPC. The measured K_I for **1** was 11.4 ± 2.8 μM. In Figure 6 a similar analysis is performed, but with varying vitamin A concentrations and a fixed concentration of DPPC. In this case the inhibitor **1** proved to be uncompetitive with respect to vitamin

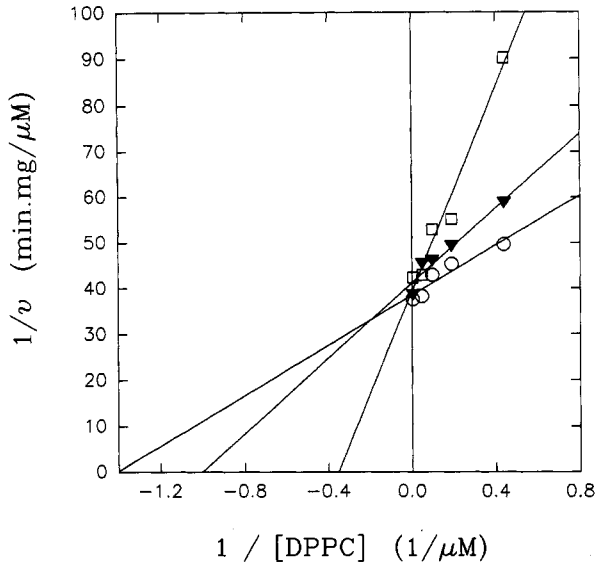


FIGURE 5: Lineweaver–Burk plots for the formation of retinyl ester at fixed *all-trans*-retinol concentrations in the presence of varying concentrations of inhibitor. Lineweaver–Burk plots for the acylation of [11,12-^{3}H]-*all-trans*-retinol at fixed [[11,12-^{3}H]*all-trans*-retinol] = 0.3 μM, (○) 0, (▼) 20, and (□) 33.3 μM of the inhibitor 13-desmethyl-13,14-dihydro-*all-trans*-retinyl trifluoroacetate **1** and varied [DPPC] are shown. The symbols represent the average values of duplicate experiments. The average standard deviation of these values is less than 4.6%. K_I = 11.4 ± 2.8 μM.

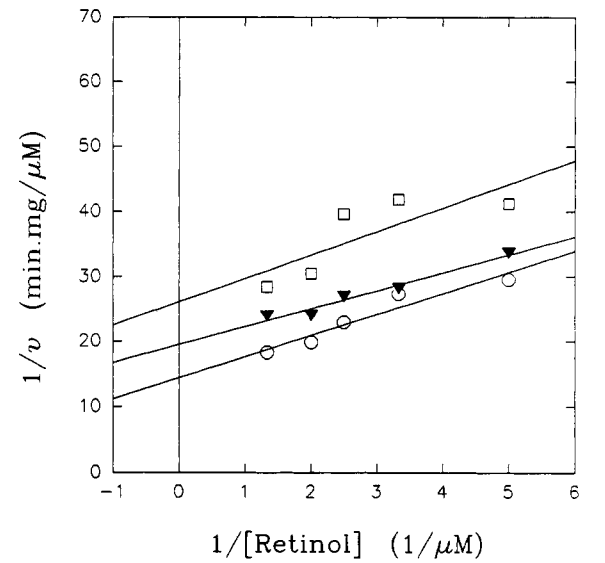


FIGURE 6: Lineweaver–Burk plots for the formation of retinyl ester at fixed DPPC concentrations in the presence of varying concentrations of inhibitor. The Lineweaver–Burk plots for the acylation of [11,-12-^{3}H]*all-trans*-retinol with fixed [DPPC] = 20.3 μM, (○) 0, (▼) 5, and (□) 10 μM of the inhibitor 13-desmethyl-13,14-dihydro-*all-trans*-retinyl trifluoroacetate **1**, and varied amounts of [11,12-^{3}H]-*all-trans*-retinol] are shown. The symbols represent the average values of duplicate experiments. The average standard deviation of these values is less than 3.7%.

A. Therefore these results demonstrate an ordered ping-pong mechanism in which **1** binds to the same form of the enzyme as does DPPC.

Exchange Reactions between Different Lecithins. An ordered ping-pong mechanism generally requires that there be a transfer of a moiety from the substrate to the enzyme followed by the release of the first product and the subsequent binding of the second substrate. Transfer of the enzyme-bound moiety to the second substrate completes the enzymatic reaction. In the case of LRAT, it is likely that the DPPC binds first and transfers an acyl group to the enzyme, followed by the binding of the vitamin A and transfer of the acyl group to the vitamin. If it is assumed that DPPC binds first to the enzyme, it would be predicted that the enzyme should be able to carry out exchange reactions between the two different lecithins in the absence of vitamin A. To carry out this experiment, DPPC ([^{3}H]choline methyl) and nonradioactive DVPC were incubated together with LRAT as shown in Figure 7. A small amount of the lysolecithin derivatives of DPPC and DVPC are generated both enzymatically and nonenzymatically upon incubation. In Figure 7A is shown the HPLC tracing of the synthetic mixed phospholipid 1-valeroyl-2-palmitoylphosphatidylcholine (VPPC). In Figure 7B, a radiochromatogram is shown which demonstrates the enzymatic formation of radioactive VPPC and the formation of a lysolecithin derivative. Approximately 1.5% of the total added radioactivity was found as the mixed phospholipid VPPC. Extended periods of incubation did not markedly increase this amount. Importantly, the nonenzymatic control exhibited in Figure 7C does not show the formation of VPPC. It is concluded that LRAT can catalyze the partial exchange reaction between lecithin derivatives, and hence the kinetic mechanism of LRAT involves the initial transfer of an acyl group from a lecithin to the enzyme.

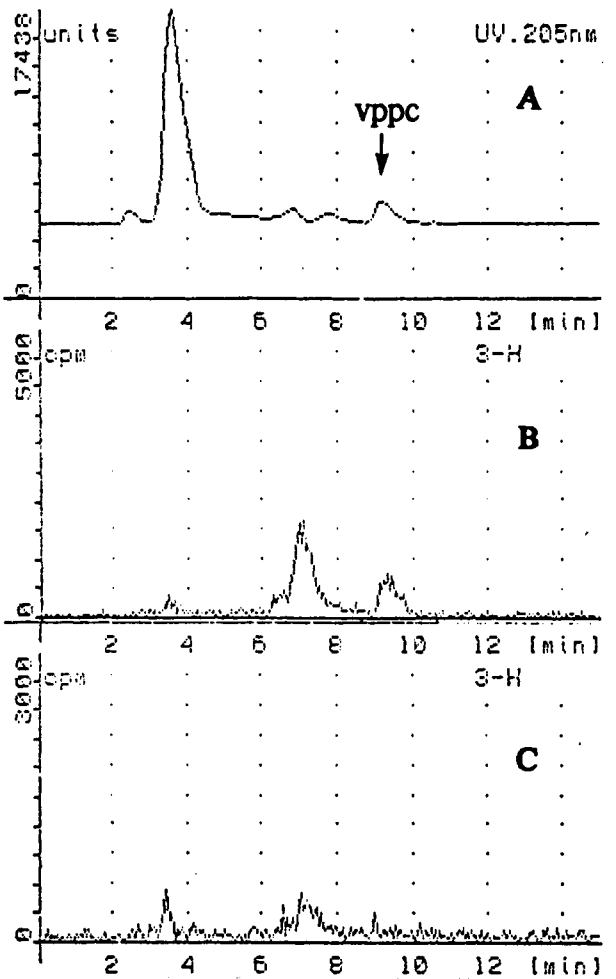
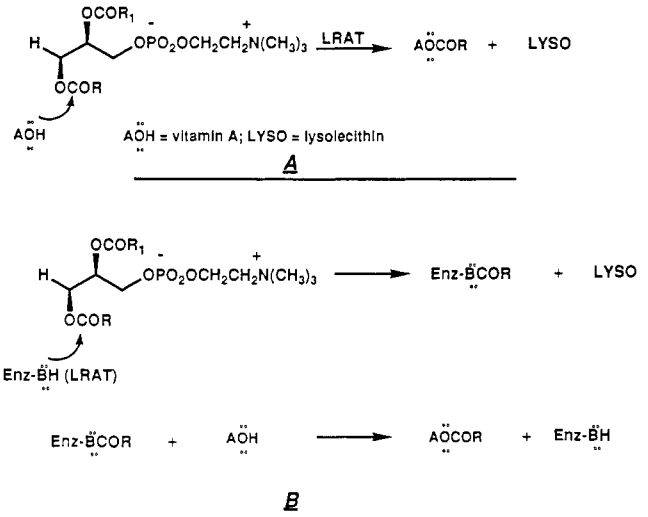


FIGURE 7: Exchange reaction between DVPC and [choline methyl-^{3}H]DPPC. This experiment was performed as described under Methods. (A) UV detection was at 205 nm. Synthetic VPPC had a retention time of 8.3 min. (B) Exchange in the presence of LRAT. The radiochromatograph shows the formation of labeled VPPC. The second peak with a retention time of 6.4 min is lysolecithin. This compound is formed spontaneously. (C) Control experiment in the absence of LRAT. VPPC is not formed here. The peak for DPPC, which has a retention time of 80 min, is not shown in these graphs.

Scheme II: Possible Transesterification Mechanisms



DISCUSSION

The experiments described here were designed to determine the kinetic mechanism of LRAT action. Without considering the order of substrate binding, two general mechanisms of LRAT action present themselves (Scheme IIA,B). A direct substrate–substrate acyl transfer reaction is shown in Scheme IIA, and a ping-pong mechanism is shown in Scheme IIB. These general mechanisms are distinguishable by kinetic studies. Of the various bisubstrate kinetic mechanisms possible for enzymes, a ping-pong process is most informative mechanistically.

It is possible to distinguish between a ping-pong bi-bi mechanism and a rapid equilibrium random or ordered bi-bi mechanism by measuring the velocity of product formation as a function of one of the substrates at different fixed concentrations of the other substrate. In a ping-pong bi-bi mechanism, plots of $1/v$ versus $1/[\text{substrate (A)}]$ at different fixed substrate (B) concentrations yield a family of parallel lines whose slopes equal K_M/v_{max} (Segel, 1975a). Both rapid equilibrium random and ordered mechanisms yield lines which intersect at the point where the $1/[\text{substrate (A)}]$ value is negative and the $1/v$ value is positive, negative, or zero (Segel, 1975b). Rates of *all-trans*-retinyl palmitate formation were determined by varying DPPC concentrations at different fixed *all-trans*-retinol concentrations (Figure 3) and by varying *all-trans*-retinol concentrations at different fixed DPPC concentrations (Figure 4). The parallel lines generated in the plots shown in Figures 3 and 4 are clear indications that a ping-pong mechanism is operative.

While the kinetic measurements shown in Figures 3 and 4 are certainly indicative of a ping-pong mechanism, they fall short of proving the case and further establishing the order of substrate binding. As illustrated in Scheme IIB, the most likely ping-pong mechanism in the case of LRAT involves initial acylation of the enzyme by the lecithin, generating an acyl–enzyme intermediate. The 2-acyllysophospholipid would leave the active site of the enzyme, followed by the binding of retinol. Finally, the enzyme-bound acyl group is transferred to the retinol to generate the retinyl ester. This mechanism predicts that DPPC binds first to the enzyme in the absence of vitamin A. This suggests that LRAT should be capable of catalyzing the interchange of acyl groups from the 1-position of two different lecithin derivatives.

It had already been demonstrated that LRAT can transfer acyl groups from the 1-position of both DPPC and DVPC to vitamin A (Cañada et al., 1990). When LRAT was incubated with radiolabeled ([^{3}H]choline methyl)DPPC and an excess of nonradioactive DVPC, the formation of a small amount (~1.5% of total) of radiolabeled VPPC was observed. Lysophospholipids were not required to initiate this exchange reaction, since they were generated to a small degree from the phospholipids during the incubations. The observed catalytic interchange, although small, demonstrates that LRAT can carry out a partial reaction. This result is entirely consistent with the occurrence of a ping-pong mechanism.

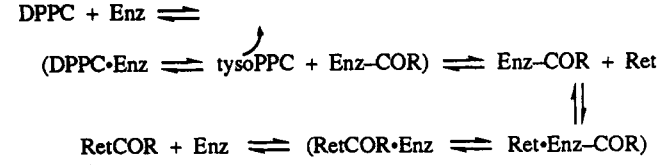
The kinetic experiments described above do not distinguish between a random or an ordered ping-pong mechanism. To address this issue, a dead-end inhibitor is required (Segel, 1975c). None of the simple aliphatic retinyl esters tested as product inhibitors was nearly potent enough to be useful in these studies. In fact, their binding was so weak that we were never able to determine K_M values for retinyl esters, such as *all-trans*-retinyl palmitate or *all-trans*-retinyl valerate. Moreover, the 1-lysophosphatidylcholines are also not useful, because they nonspecifically inhibit the detergent-solubilized LRAT. Thus a potent and specific LRAT antagonist was sought.

Previously, we had shown that *all-trans*-retinyl-α-bromoacetate is a potent inhibitor of LRAT (Trehan et al., 1990). However, this molecule is not immediately useful for kinetic studies, because there is an irreversible component to the observed inhibition (unpublished experiments). We sought an analog of this compound that would show purely reversible inhibition. To these ends, 13-desmethyl-13,14-dihydro-*all-trans*-retinyl trifluoroacetate (**1**) was prepared and is shown here to be a potent and reversible inhibitor of LRAT. Several considerations served as the basis for the design of this molecule. An electron-withdrawing ester functionality was deemed essential in order to obtain the desired potency of action based on our results with *all-trans*-retinyl-α-bromoacetate (Trehan et al., 1990). It is interesting to note that lecithin cholesterol acyl transferase, an enzyme which also possesses lysolecithin acyl transferase activity (Subbaiah et al., 1980), is potently inhibited by *sn*-2-difluoroketone phosphatidylcholine analogs (Jauhiainen et al., 1990). These fluorinated analogs are thought to potently inhibit lecithin cholesterol acyl transferase by mimicking tetrahedral enzyme-bound intermediates and hence behave as transition-state analogs. A similar mechanism probably comes into play with the retinyl ester analog **1**. It is also possible that **1** reversibly acylates LRAT. However, we have no evidence for this, and moreover we have not observed significant enzyme-related cleavage of **1**. Ultimately, **1** was chosen for study since the readily available *all-trans*-retinyl trifluoroacetate proved to be highly unstable and hence unsuitable for use here. Removing the trifluoroacetate from conjugation produced stable molecules. Finally, the 13-desmethyl analog was prepared to remove the possibility of enantiomeric effects.

The kinetic experiments conducted with **1** showed that it is competitive with respect to DPPC and uncompetitive with respect to *all-trans*-retinol. Because DPPC can bind to the free enzyme, so must **1**. Since *all-trans*-retinol is uncompetitive with respect to **1**, retinoid substrates cannot bind to free enzyme, and hence the kinetic mechanism of LRAT must involve an ordered ping-pong process as shown in Scheme III.

No mechanistic work has yet been published on LRAT, so that the nature of the active-site residues engaged in the acyl transfer process are not yet identified. Nevertheless, group-specific chemical reagents afford some clues. LRAT is inactivated by sulfhydryl-directed reagents, including *p*-hydroxymercuribenzoate and the trivalent arsenicals, and by

Scheme III: Kinetic Mechanism for LRAT[a]



[a] DPPC, dipalmitoylphosphatidylcholine; lysoPPC, 2-palmitoyllysophosphatidylcholine; Ret, *all-trans*-retinol; Enz, LRAT; R, $(CH_2)_{14}CH_3$.

serine-directed reagents, such as phenylmethanesulfonyl fluoride (Fulton & Rando, 1987; Herr et al., 1991). These observations suggest the possibility that an active serine or cysteine residue may be essential. The fact that trivalent arsenicals inactivate LRAT requires a vicinal disposition of cysteine residues. It is noteworthy that lecithin cholesterol acyl transferase is also sensitive to trivalent arsenicals, but the affected cysteine residues proved not to be essential for catalysis (Francone & Fielding, 1991). Further studies on LRAT will clearly be essential for determining the nature of its active-site residues and determining its full mechanism of action. The kinetic studies described here provide an important starting point for these further investigations.

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