Published in final edited form as:

Biochemistry. 2006 September 5; 45(35): 10710–10718. doi:10.1021/bi060897y.

The Palmitoyl Transferase Activity of Lecithin Retinol Acyl Transferase

Linlong Xue, Wan Jin Jahng, Deviprasad Gollapalli, and Robert R. Rando *

Department of Biological Chemistry and Molecular Pharmacology Harvard Medical School, 45 Shattuck Street, Boston, MA 02115

Abstract

Lecithin retinol acyl transferase (LRAT) has the essential role of catalyzing the transfer of an acyl group from the *sn*-1 position of lecithin to vitamin A to generate all-*trans*-retinyl esters (tREs). *In vitro* studies had previously shown that LRAT can also exchange palmitoyl groups between RPE65, a tRE binding protein essential for vision, and tREs. This exchange is likely to be of regulatory significance in the operation of the visual cycle.

In the current study, the substrate specificity of LRAT is explored with palmitoylated amino acids and dipeptides as RPE65 surrogates. Both O and S-substituted palmitoylated analogs are excellent substrates for tLRAT, a readily expressed and readily purified form of LRAT. Using vitamin A as the palmitoyl acceptor, tREs are readily formed. The cognate of these reactions occurs in crude retinal pigment epithelial (RPE) membranes as well. RPE membranes containing LRAT transfer palmitoyl groups from radiolabeled DP*PC to RPE65. Palmitoyl transfer is abolished by preincubation with a specific LRAT antagonist both in membranes and with purified tLRAT. These experiments are consistent with an expanded role for LRAT function as a protein palmitoyl transferase.

Keywords

Lecithin Retinol Acyl Transferase; Acyl Donor; Peptide Substrates; Protein Palmitoylation

The phototransduction cascade in vision is initiated by the *cis*→*trans* photoisomerization of the protonated 11-*cis*-retinal Schiff base chromophore of rhodopsin into its all-*trans* congener (1,2). The regeneration of rhodopsin after bleaching requires a series of biochemical reactions to resynthesize the 11-*cis*-retinal chromophore as shown in Scheme 1 (3). The critical *trans*→*cis* isomerohydrolase (IMH) reaction minimally requires two known components- lecithin retinol acyl transferase (LRAT) and RPE65. LRAT is necessary and sufficient for the biosynthesis of all-*trans*-retinyl esters, the substrates for the isomerization reaction (4-6). RPE65 has been determined to be an all-*trans*-retinyl ester (tRE) binding protein *in vitro* when purified (7,8). Based on indirect expression experiments some believe that RPE65 is also an enzyme responsible for the critical *trans*→*cis* isomerohydrolase reaction of the visual cycle (9-11). However, this conclusion seems to be in conflict with the fact that purified RPE65 is chemically inert, and thus unable to convert tREs into 11-*cis*-retinol (8). It is likely that other, possibly unidentified, proteins are essential for biologically relevant isomerization.

Whatever its precise function, RPE65 is certainly a protein of central importance in visual cycle function (12). It was first discovered as a major protein concentrated in the retinal pigment epithelium (13). Knockout studies of this protein of unknown function in mice revealed it to

*To whom correspondence should be addressed. 617-432-1794 (tel), 617-432-0471 (Fax); rando@hms.harvard.edu (email).

be necessary for the biosynthesis of 11-*cis*-retinoids (12). The membrane-associated form of RPE65 (mRPE65) was later shown to stereoselectively bind hydrophobic all-*trans* retinyl esters with high affinity (7,14). mRPE65 has been found to be palmitoylated at three cysteine residues: C231, C329, and C330 (15). C330 is completely conserved in the known RPE65 homologs, and C231 is largely conserved except for the highly conservative S/C substitution in zebra fish (16). The hydrophobic palmitoylation modification allows the protein to associate with RPE membranes where isomerization ensues. Whether all mRPE65 is completely triply palmitoylated is unknown. The soluble form of RPE65 (sRPE65) is not triply palmitoylated and binds all-*trans*-retinol (vitamin A) with a substantially higher affinity than it binds to tREs (15). Importantly, m and sRPE65 are interconverted by LRAT (15). In other words, they are substrates for this enzyme. This surprising result immediately suggests a regulatory loop in which the visual cycle is under the control of the palmitoylation state of RPE65 (15). This can readily be understood when considering what transpires when 11-*cis*-retinol, the product of IMH processing, builds up. The 11-*cis*-retinol is esterified by LRAT, using mRPE65 as the palmitoyl donor. Since only mRPE65 is competent to bind tREs so that they can be engaged in the isomerohydrolase reaction, the conversion of mRPE65 to sRPE65 has the net effect of blocking isomerization, and hence regulating the system (15). This regulatory mechanism also requires that RPE65 function is rate limiting in operation of the visual cycle. Independent evidence supports this view. Specific antagonists of mRPE65 inhibit rates of rhodopsin regeneration *in vivo* in rodents (17) and rhodopsin regeneration rates in mice are dependent on RPE65 levels (18).

There are several outstanding questions concerning the biochemical basis for the respective roles of LRAT and RPE65 in the regulatory switch mechanism. In this mechanism, a palmitoyl thioester of mRPE65 is transferred to vitamin A, suggesting that the latter is a peptidomimetic of lecithin, the standard acyl ester donor for LRAT. In the current studies, we address the substrate specificity of tLRAT, a truncated and readily expressible variant of LRAT (19), with respect to acyl donor specificity. We report that simple acyl cysteine and serine-containing molecules are excellent substrates for tLRAT. Therefore, the enzyme processes both ester and thioester moieties found in simple amino acids and dipeptides. It is also important to establish that LRAT mediated palmitoyl transfer occurs in the biological context of RPE membranes, in addition to occurring with purified proteins. We also provide evidence for the LRAT-mediated transfer of palmitoyl groups to RPE65 in crude membranes derived from bovine RPE. Thus LRAT functions as a protein palmitoyl transferase both in the purified form and in crude membranes.

Materials and Methods

Materials

Frozen bovine eye cups were obtained from W. L. Lawson Co. (Lincoln, NE). Gel code blue (Coomassie staining) was from Pierce. The silver staining kit, polyvinylidene fluoride membrane, anti-rabbit IG conjugated horseradish peroxidase and ECL-Western blotting kit were from GE Healthcare. Precast 2D gels (8–16%, 9 cm × 13 cm) for SDS-PAGE, two-color prestained protein markers (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa), semi dry Western blot transfer kit and avidin conjugated horseradish peroxidase were from Bio-Rad. All other reagents were of analytical grade. [11,12-³H₂] all-*trans*-retinol was obtained from NEN Life Sciences. [1-¹⁴C]-L- α -dipalmitoyl diphosphatidylcholine in DMSO (114 mCi/mmol) is a product of Dupont. L- α -Dipalmitoylphosphatidylcholine (DPPC), bovine serum albumin (BSA), dithiothreitol (DTT), DMSO and THF (containing BHT) were from Sigma. All other chemicals were from Aldrich Sigma.

Syntheses of Palmitoylated Amino Acids and Peptides

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-hexadecanoylsulfanyl-propionic acid

(FCP): Palmitoyl Chloride (0.123g, 0.435 mmol, 1.5 eq) was added to *N*- α -fmoc-cysteine (0.1g, 0.29 mmol) in distilled, ice-cold trifluoro acetic acid (TFA) (5 mL) and the mixture was stirred at room temperature. After 1 h, 25 mL of ice water was added to this mixture. The white precipitate was filtered and washed with 25 mL of H₂O. The crude product was purified by silica column chromatography with 80/20 hexane/ethyl acetate as eluant.

¹H NMR (CDCl₃, 200 MHz, δ in ppm): 7.86 (d, J_{HH} = 7.4, 2H), 7.72 (d, J_{HH} = 6.6, 2H), 7.42 (t, J_{HH} = 7.6, 2H), 7.32 (t, J_{HH} = 7.3, 2H), 6.81 (d, J_{HH} = 8.6, 2H), 4.53–4.22 (m, 5H), 3.59 (dd, J_{HH} = 7.6, 2H), 2.60 (t, J_{HH} = 7.2, 2H), 1.64 (t, J_{HH} = 6.6, 2H), 1.26 (s, 24H) and 0.88 (t, J_{HH} = 6.2, 3H). ESI-MS $[M-H]^+$ 582.3254 (obsd), 582.3175 (calcd).

Hexadecanoic acid 2-carboxy-2-(9H-fluoren-9-ylmethoxycarbonylamino)-ethyl ester

(FSP): FSP was synthesized by the procedure described above for the synthesis of FCP. The crude product was purified by silica column chromatography using 80/20 n-hexane/ethyl acetate as eluant.

¹H NMR (CDCl₃, 200 MHz, δ in ppm): 7.81 (d, J_{HH} = 7.0, 2H), 7.65 (d, J_{HH} = 7.0, 2H), 7.36 (p, J_{HH} = 7.8, 4H), 5.43 (dd, J_{HH} = 4.0, 1H), 4.86–4.25 (m, 5H), 2.53 (t, J_{HH} = 6.4, 2H), 2.17 (t, J_{HH} = 7.8, 2H), 1.28 (s, 24H) and 0.88 (t, J_{HH} = 6.4, 3H). ESI-MS $[M-H]^+$ 566.3476 (obsd), 566.3403 (calcd).

2-{2-[(9H-Fluoren-9-ylmethoxycarbonyl)-methyl-amino]-3-hexadecanoylsulfanyl-propionylamino}-3-hexadecanoylsulfanyl-propionic acid (FC2P-OH):

(a) 2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-tritylsulfanyl-propionic acid t-butyl ester (FCTB) was synthesized by a reported procedure (20).

(b) 2-Amino-3-tritylsulfanyl-propionic acid t-butyl ester (NCTB): To FCTB (100 mg, 0.155 mmol) in 10 mL anhydrous CH_2Cl_2 , 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (0.24 mL, 1.55 mmol) was added and the solution was stirred at room temperature for 1 h. The reaction mixture was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography with 35:65 ethyl acetate:n-hexane as eluant.

^1H NMR (CDCl_3 , 200 MHz, δ in ppm): 7.46–7.19 (m, 15H), 3.06 (m, 3H) and 1.05 (s, 9H).

(c) 2-[2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-tritylsulfanyl-propionylamino]-3-tritylsulfanyl-propionic acid tert-butyl ester (FC2TB): To FCTB (100 mg, 0.155 mmol) in 30 mL anhydrous CH_2Cl_2 ,

1-Hydroxybenzotriazole (HOBt) (21 mg, 0.155 mmol) and 4-dimethylaminopyridine (4-DMAP) (38 mg, 0.31 mmol) was added. To the stirring solution *N,N'*-Diisopropylcarbodiimide (DIPCI) (29 μL , 0.18 mmol) was added and the stirring was continued for an h. To the reaction mixture after 1 h NCTB (57 mg, 0.155 mmol) was added. The reaction mixture was stirred at room temperature for a further 12 h. The solvent was removed under reduced pressure, the crude reaction mixture was dissolved in 20 mL of CH_2Cl_2 and washed with 2×20 mL 0.1 M HCL followed by 3×20 mL H_2O , followed by 2×20 mL saturated NaHCO_3 . The solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with 15:85 ethyl acetate:n-hexane as eluant.

^1H NMR (CDCl_3 , 200 MHz, δ in ppm): 7.73 (t, $J_{\text{HH}} = 6.2$, 2H), 7.56 (d, $J_{\text{HH}} = 6.2$, 2H), 7.43–7.14 (m, 34H), 6.43 (d, $J_{\text{HH}} = 7.8$, 1H), 4.36 (m, 3H), 3.83 (q, $J_{\text{HH}} = 7.0$, 2H), 3.65 (d, $J_{\text{HH}} = 7.2$, 2H), 3.50 (d, $J_{\text{HH}} = 5.0$, 2H) and 1.38 (s, 9H).

(d) **2-{2-[(9H-Fluoren-9-ylmethoxycarbonyl)-methyl-amino]-3-mercaptopropionylamino}-3-mercaptopropionic acid (FC2A)**: To FC2TB (100mg, 0.1 mmol) in 20 mL CH_2Cl_2 /TFA maintained at -20°C , was added triethyl silane (TES) (0.61 mmol, 96 μL). The reaction was stirred at -20°C for 30 min and the reaction was allowed to warm to room temperature. The solvent was removed under reduced pressure and purified by column chromatography with 40:60 ethyl acetate:n-hexane as eluant.

(e) **2-{2-[(9H-Fluoren-9-ylmethoxycarbonyl)-methyl-amino]-3-hexadecanoylsulfanylpropionylamino}-3-hexadecanoylsulfanylpropionic acid (FC2P-OH)**: Was synthesized by the same method used in the preparation of FCP.

^1H NMR (CDCl_3 , 200 MHz, δ in ppm): 7.77 (d, $J_{\text{HH}} = 7.8$, 2H), 7.59 (d, $J_{\text{HH}} = 7.0$, 2H), 7.41 (t, $J_{\text{HH}} = 7.2$, 2H), 7.32 (t, $J_{\text{HH}} = 7.6$, 2H), 6.93 (d, $J_{\text{HH}} = 7.0$, 1H), 4.68–4.20 (m, 5H), 3.75 (t, $J_{\text{HH}} = 7.0$, 3H), 2.51 (t, $J_{\text{HH}} = 7.2$, 4H), 1.66 (t, $J_{\text{HH}} = 6.6$, 4H), 1.25 (s, 48H) and 0.88 (t, $J_{\text{HH}} = 6.2$, 6H). ESI-MS $[\text{M}-\text{H}]^+$ 923.5640 (obsd), 923.5563 (calcd).

Hexadecanoic acid 2-(1-carboxy-2-hexadecanoyloxy-ethylcarbamoyl)-2-[(9H-fluoren-9-ylmethoxycarbonyl)-methyl-amino]-ethyl ester (FS2P-OH): The structures of molecules described below are the serine cognates of the cysteine analogs described above are therefore omitted.

(a) 2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-trityloxy-propionic acid tert-butyl ester (FSTB): FSTB was synthesized by a reported procedure (20).

^1H NMR (CDCl_3 , 200 MHz, δ in ppm): 7.76 (d, $J_{\text{HH}} = 7.8$, 2H), 7.63 (d, $J_{\text{HH}} = 6.2$, 2H), 7.43–7.19 (m, 19H), 5.80 (d, $J_{\text{HH}} = 9.4$, 1H), 4.36 (m, 3H), 4.24 (t, $J_{\text{HH}} = 7.2$, 1H) 3.48 (s, 2H) and 1.45 (s, 9H).

(b) 2-Amino-3-trityloxy-propionic acid tert-butyl ester (NSTB): To FSTB (100 mg, 0.159 mmol) in 10 mL anhydrous CH_2Cl_2 , 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (0.25 mL, 1.59 mmol) was added and the solution was stirred at room temperature for 1 h. The reaction mixture was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography with 35:65 ethyl acetate:n-hexane as eluant.

^1H NMR (CDCl_3 , 200 MHz, δ in ppm): 7.44–7.14 (m, 15H), 3.38 (m, 3H) and 1.43 (s, 9H).

(c) 2-[2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-trityloxy-propionylamino]-3-trityloxy-propionic acid (FS2TB): To FSTB (100 mg, 0.176 mmol) in 30 mL anhydrous CH_2Cl_2 , HOBT (24 mg, 0.176 mmol) and 4-DMAP (43 mg, 0.35 mmol) was added. To the stirring solution DIPCI (33 μL , 0.21 mmol) was added and the stirring was continued for an h. To the reaction mixture after 1 h NSTB (61 mg, 0.176 mmol) was added. The reaction mixture was stirred at room temperature for a further 12 h. The solvent was removed under reduced pressure, the crude reaction mixture was dissolved in 20 mL of CH_2Cl_2 and washed with 2×20 mL 0.1 M HCL followed by 3×20 mL water, followed by 2×20 mL saturated NaHCO_3 . The solvent was

removed under reduced pressure and the crude product was purified by silica gel column chromatography with 15:85 ethyl acetate:n-hexane as eluant.

^1H NMR (CDCl_3 , 200 MHz, δ in ppm): 7.75 (d, $J_{\text{HH}} = 7.4$, 2H), 7.52 (d, $J_{\text{HH}} = 7.0$, 2H), 7.44–7.19 (m, 34H), 5.39 (d, $J_{\text{HH}} = 7.2$, 1H), 4.54 (d, $J_{\text{HH}} = 7.2$, 1H), 4.43–4.07 (m, 5H), 3.53–3.33 (m, 2H) and 1.41 (s, 9H).

(d) 2-{2-[(9H-Fluoren-9-ylmethoxycarbonyl)-methyl-amino]-3-hydroxy-propionylamino}-3-hydroxy-propionic acid (FS2A): To FS2TB (100mg, 0.1 mmol) in 20 mL CH_2Cl_2 /TFA maintained at -20°C , was added Triethyl silane (TES) (0.61 mmol, 96 μL). The reaction was stirred at -20°C for 30 min and the reaction was allowed to warm to room temperature. The solvent was removed under reduced pressure and purified by column chromatography with 40:60 ethyl acetate:n-hexane as eluant.

(e) Hexadecanoic acid 2-(1-carboxy-2-hexadecanoyloxy-ethylcarbamoyl)-2-[(9H-fluoren-9-ylmethoxycarbonyl)-methyl-amino]-ethyl ester (FS2P-OH): Was synthesized by the method described for FCP.

^1H NMR (CDCl_3 , 200 MHz, δ in ppm): 7.79 (d, $J_{\text{HH}} = 7.0$, 2H), 7.62 (d, $J_{\text{HH}} = 7.0$, 2H), 7.36 (p, $J_{\text{HH}} = 7.8$, 4H), 5.43 (dd, $J_{\text{HH}} = 4.0$, 1H), 4.86–4.25 (m, 9H), 2.53 (t, $J_{\text{HH}} = 6.4$, 4H), 2.17 (t, $J_{\text{HH}} = 7.8$, 4H), 1.28 (s, 48H) and 0.88 (t, $J_{\text{HH}} = 6.4$, 6H). ESI-MS $[\text{M}-\text{H}]^+ 891.6093$ (obsd), 891.6020 (calcd).

Methods

Preparation of the Bovine Pigment Epithelium (RPE) Membranes—The procedure for the preparation of bovine RPE membranes is described elsewhere (21). Prior to use, the membranes were irradiated with UV light (365 nm) on ice for 5 min in order to destroy endogenous retinoids. RPE stock solution contains 1–2 mg/mL protein determined by the Bradford assay. RPE membranes were solubilized in 10 mM Tris-HCl (pH 8.0), 2 mM DTT, 1 mM EDTA, and 1% CHAPSO. After thorough mixing for 1 h at 4°C , the preparation was centrifuged at $10,500 \times g$ for 1 h. Dialysis was performed at 4°C using Tris-HCl (10 mM, pH 8.0), EDTA (1 mM), DTT (2 mM), and CHAPSO (0.1%) after concentrating the sample by Centriprep.

Preparation of tLRAT and Enzymatic Assays—The expression and purification of tLRAT were carried out as described (19). The acyl donors were dissolved in DMSO/THF (50:50, v/v). In kinetic studies, the reaction mixture (100 μL) contained 100mM Tris-HCl buffer (pH 8.4), 1mM DTT, 1 mM EDTA, 1% CHAPS, 10 μM tLRAT and varying concentration of acyl donor (4 μM , 10 μM , 50 μM , 100 μM , 150 μM , 200 μM , 250 μM , 300 μM , 350 μM , and 400 μM). The reactions were initiated by adding all-*trans*-retinol (final concentration 10 μM). After incubation at room temperature for 2 to 10 min, the reaction was quenched by adding 500 μL methanol followed by addition of 500 μL hexane for the purpose of retinoid extraction. The extract was subject to normal phase HPLC (NP-HPLC) analysis as previously described (22).

Specific RPE protein labeling by dipalmitoyl phosphatidylcholine (DPPC)—All labeling experiments were performed in the dark room under dim red light. Generally, 100 μL of RPE (200–400 μg of total proteins) membranes were incubated with $[1-^{14}\text{C}]\text{-L-}\alpha\text{-dipalmitoyl diphosphatidylcholine}$ in DMSO (114 mCi/mmol, 0.4 nM – 1 μM) for 2 h at 4°C to perform the palmitoyl transfer reaction. Incubations were also performed using purified mRPE65 (0.04 μM) and tLRAT (2 μM). In the inhibition experiments, the known LRAT inhibitor, all-*trans*-retinyl bromoacetate (tRBA, 5 μM) was used (21). After incubation,

proteins were precipitated with cold acetone at -20°C for 30 min, followed by centrifugation. The labeled RPE proteins were dissolved in 2% SDS and subjected to SDS-PAGE. Tris-Glycine polyacrylamide gel (4–20%) electrophoresis was carried out using Tris (25 mM), glycine (192 mM), and SDS (0.1%) buffer system described by Laemmli (23). Protein denaturation was performed by heating the samples (100°C , 2 min) in sample buffer containing SDS (4%), 2-mercaptoethanol (10%), glycerol (20%), bromophenol blue (0.004%) and Tris-HCl (125 mM, pH 6.8). Proteins were visualized by Coomassie staining or silver staining. For mini gels and medium size gels ($8\text{ cm} \times 8\text{ cm} \times 1\text{ mm}$ and $9 \times 13\text{ cm} \times 1\text{ mm}$), 20 μL of the protein solution (20–40 μg total protein) were loaded for each well, and for 2-D gel ($9 \times 13\text{ cm} \times 1\text{ mm}$), 200 μL of sample (100 μL RPE + 100 μL rehydration buffer) were loaded along the molecular weight markers.

2D SDS-PAGE analysis—2D gel electrophoresis was performed by isoelectric focusing (IEF) using Immobiline Dry Strips with a pH gradient of 3–10, (11 cm, GE Healthcare) in the first dimension and 8–16% ($9\text{ cm} \times 13\text{ cm}$, Bio-Rad) SDS-PAGE in the second dimension. Before re-hydration of the immobilized pH gradient (IPG) strip, 200 μL of rehydration buffer (7 M urea, 2 M thiourea, 0.2% Triton X-100, 2% CHAPS, 0.5% IPG buffer, a few grains of bromophenol blue, 0.7 mg DTT in double distilled water) were mixed with the sample. Isoelectrofocusing in the IPGphor system (GE Healthcare) was conducted at a step gradient voltage of 500 V (3 h), 1000 V (3 h), 4000 V (1 h), 6000 V (1 h), 8000 V (11 h) with very low currents (less than 50 μA /IPG strip). After IEF, strips were equilibrated with 10 mL of SDS equilibration buffer (50 mM Tris-HCl, 6 M urea, 30 % glycerol, 2% SDS, 100 mg DTT, a few grains of bromophenol blue). Proteins were visualized by Coomassie staining and by Western blotting.

Western Blot Analysis—The preparation of polyclonal anti-LRAT peptide antibodies was reported previously (19). An anti-RPE65 peptide (NFITKVNPELETIK) antibody was obtained from Genemed (San Francisco, CA). After protein separation by SDS-PAGE, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane for 30 min at 15 V using transfer buffer (25 mM Tris, 192 mM glycine and 20% ethanol) by semi-dry transfer apparatus (Bio-Rad). The membrane was blocked with 5% non-fat dried milk or super block blocking buffer (Pierce, 3% BSA) for 2 h at room temperature. Anti-RPE65 antibody (1:4000 dilution, 2 h), anti-rabbit Ig linked horseradish peroxidase (1:8000 dilution, 1 h) from donkey, and the enhanced chemiluminescence (ECL) system were used to detect the RPE65 band or spot.

Analysis of the radiolabeled proteins—The approximately 65 kDa protein was removed from the gel by cutting and dehydrated in CH_3CN for 10 min. The dried gel band was incubated with trypsin (12.5 $\text{ng}/\mu\text{L}$, 5 $\mu\text{L}/\text{mm}^2$ gel, overnight) at 37°C . The gel pieces were centrifuged and the supernatant was collected. Peptides were further extracted by one change of 20 mM NH_4HCO_3 and three changes of 50% CH_3CN (20 min between changes) at 25°C . The collected peptide solution was dried and re-dissolved in 50% CH_3CN (50 μL) and counted by Beckman LS 6500 scintillation counter using Ultima Gold scintillation cocktail (Perkin-Elmer).

Results

Palmitoylated Peptide Substrates of tLRAT

The three palmitoylated cysteine residues of mRPE65 are at positions C231, C329 and C330 (15). In order to determine whether simplified palmitoylated cysteine/serine derived amino acids and dipeptides can serve as substrates for purified tLRAT, the analogs shown in Scheme 2 were prepared and tested for substrate activity. Saturation kinetics were observed with the four analogs as shown in Figures 1A & B and 2A & B. Figure 1 A&B shows the data for the

palmitoylated serine analogs (**FSP** and **FS2P-OH**) and Figure 2 A&B shows the data for the cognate cysteine analogs (**FCP** and **FC2P-OH**). The enzymatic assays were performed with saturating concentrations of vitamin A as the second substrate, and the time-dependent formation of all-*trans*-retinyl palmitate is determined. It is clear that the analogs are all excellent substrates for tLRAT. Table 1 shows a tabulation of the kinetically determined $V_{\max}$ and K_M values for the analogs compared to DPPC (24) and mRPE65 (15). While the K_M values for the amino acid analogs and dipeptides are higher than for DPPC, and considerably higher than for mRPE65, their $V_{\max}$ values as substrates are equal to or better than for the biological substrates.

The studies confirm that tLRAT accepts both oxygen and thio esters as substrates (15). Thus the analogs listed in Scheme 2 may be considered as peptide mimics of lecithin.

Membrane Associated LRAT Accepts RPE65 as an Acyl Donor

The studies carried out thus far on mRPE65 as an acyl donor have involved the use of purified tLRAT and RPE65 in solution (15). To further address the biological relevance of the observations made, it is important to determine whether full length LRAT and RPE65 in their natural membrane environments interact in the expected ways. This can be readily probed in RPE membranes. To address this issue, we studied whether DPPC acts as an acyl donor for RPE65 in bovine RPE membranes, and whether this process is LRAT mediated. The mechanism of LRAT action involves its S-acylation at C161 in a ping-pong kinetic process (25,26). Thus added DPPC will palmitoylate endogenous LRAT, which is then able to transfer an *sn*-1 palmitoyl group to RPE65 or tROL (Scheme 3). The transfer of a labeled palmitoyl moiety of DPPC to RPE65 in RPE membranes is taken as evidence that RPE65 is reversibly palmitoylated by LRAT.

RPE65 labeling by DPPC

In the experiments described here, RPE membranes are incubated with DP*PC and the fate of palmitoyl transfer is determined. The expectation is that the radiolabeled palmitate group will be transferred to RPE65 associated with the RPE membranes. Figure 3A shows a 1-D SDS-PAGE gel of labeled and control samples (identical amounts of protein in both samples) visualized either by Coomassie staining or by Western blot using an anti-RPE65 antibody. The RPE65 band in the labeled lane was excised and the level of radioactivity present was determined by scintillation counting (Figure 3B). That the radioactivity associated with the RPE65 band is labeled RPE65 is confirmed by 2D SDS-PAGE (Figure 3C). The use of 2D SDS-PAGE insures that it is indeed RPE65 that is labeled during the incubation process. After incubation with DP*PC and RPE membranes, the proteins were separated by pI (1st dimension) and molecular weight (2nd dimension). After 2D SDS-PAGE analysis, labeled proteins were analyzed by Western blotting and scintillation counting. As shown in Figure 3C, two distinct RPE65 spots (pI=6 and 9) were labeled by DP*PC and the band at pI 9 RPE65 was more highly labeled (Figure 3D). The two bands at pI=6 and 9 are typically observed on 2D gels by Western blotting (unpublished experiments) with anti-RPE65 antibodies and may be related to the large number of cysteine residues of the protein, some of which are protected by palmitoylation (15).

LRAT Catalyzes the Palmitoylation of RPE65 in RPE Membranes

The experiments reported above show that DPPC functions as a palmitoyl group donor for RPE65 either directly or indirectly. It was of interest to determine whether LRAT is directly involved in the acyl transfer process. The involvement of LRAT in the transfer of a palmitoyl group from DP*PC to RPE65 was probed by using the known specific affinity-labeling agent of LRAT, all-*trans*-retinyl bromoacetate (tRBA) (27). In these experiments, RPE membranes either served as a control sample (Figure 4- lane 1) or were pre-incubated with tRBA (Figure

4-lane 2) to inhibit LRAT. Samples (lanes 1 and 2) were then incubated with DP*PC, while Figure 4 lane 3 was not incubated with the DP*PC. SDS-PAGE was performed on the samples and the extent of radioactivity incorporation into RPE65 was determined after excision of the RPE65 band followed by scintillation counting. As can be seen here, tRBA had a pronounced effect on limiting ^{14}C -palmitoyl incorporation into RPE65. These experiments strongly suggest that LRAT is responsible for the transfer of a palmitoyl group to RPE65 and, of course, that transfer is from the *sn*-1 position of the DPPC. To further establish this, tLRAT, a truncated form of LRAT that can be purified (19), was studied. Here the transfer reaction can be directly studied in the absence of possible interfering proteins.

These experiments were performed in a manner similar to the experiments described above. In Figure 5, lane 1, RPE65 is labeled by purified tLRAT using DP*PC as the acyl donor. Preincubation with tRBA substantially blocks the incorporation of radioactivity into RPE65 (Figure 5, lane 2). This inhibition is almost complete because the extent of radioactivity incorporation observed in lane 2 is essentially the same as when RPE65 is incubated with DP*PC in the absence of tLRAT (Figure 5, lane 3). Lane 4 in Figure 5 is the background control in which DP*PC is absent from the incubation mixture. These experiments leave no doubt that LRAT reversibly catalyzes the transfer of palmitoyl groups between DP*PC and RPE65 and that the enzyme is a palmitoyl transferase for proteins.

Discussion

The studies reported extend earlier work reporting that LRAT can transfer palmitoyl groups from palmitoylated RPE65 (mRPE65) to vitamin A (15). The reversible palmitoylation of RPE65 regulates its retinoid binding selectivity (15). One unusual aspect of this work concerns the acyl donor specificity of LRAT. The standard function of this enzyme in vision is to transfer an acyl group from the *sn*-1 of lecithin (largely palmitoyl) to vitamin A to generate lysophospholipids and all-*trans*-retinyl esters, the isomerohydrolase substrates (21). The finding that mRPE65 is also a substrate for tLRAT broadened the profile for acyl donors for LRAT. In the current study, the acyl donor substrate specificity for LRAT was further explored. Studies on the palmitoylated amino acid and dipeptide substrates show that simple analogs are excellent substrates for tLRAT. As expected from our previous studies on RPE65 (15), the enzyme can process both oxygen and thioesters. The trend is for a greater $V_{\max}$ for the thioesters compared to the oxygen esters. This is expected because of the greater kinetic lability of the former compounds. The fact that the enzyme can process thio and oxygen esters shows that the conservative S for C substitution at C231 of LRAT in one instance in the RPE65 homologs is of no consequence mechanistically and thus C231 is functionally conserved. The K_M values for the synthetic substrates proved to be relatively high, especially when compared to mRPE65. Clearly there must be substantial protein-protein contacts in the interactions of LRAT and mRPE65.

The studies discussed above and previously described were performed with tLRAT, an N and C-truncated form of LRAT that is readily expressed and purified from bacteria and insect cells, presumably because of its greater solubility (19). It was then of interest to determine if the palmitoyl transfers occur as effectively with full length LRAT in its natural membraneous environment. To these ends, palmitoyl transfer was studied in bovine RPE membranes which contain native LRAT and membrane associated RPE65 (mRPE65) (15). To follow the transfer of palmitoyl groups, DP*PC was added to the membranes which contain LRAT and RPE65, along with other RPE membrane proteins. Importantly, palmitoylation of membrane associated RPE65 occurred from the added radiolabeled lecithin. It is clear that with the putative turnover of membrane some of the membrane associated RPE65 will have palmitoylation sites available for modification. It is known that mRPE65 can contain up to three palmitoyl groups per enzyme molecule (15). The labeling of RPE65 by LRAT using DP*PC as an acyl donor is readily

understood, as shown in Scheme 3. Since LRAT mechanistically operates through a ping-pong mechanism (22) in which an acyl enzyme intermediate is formed, the palmitoylation of RPE65 after accepting a palmitoyl group from DPPC is unsurprising.

The acylation of RPE65 was shown likely to be mediated by LRAT in membranes because it could be blocked by pre-incubation with tRBA, a known specific affinity-labeling agent of LRAT (21). However, other acylation mechanisms might still be possible, and might occur in a stepwise fashion given the metabolic complexity of RPE membranes. To unambiguously demonstrate that LRAT is the agent of palmitoylation we turned to the use of purified tLRAT. In *in vitro* experiments with tLRAT, it was readily shown that palmitoyl groups are transferred between and DP*PC and purified RPE65, and this incorporation was also inhibited by tRBA. Therefore, there is no doubt that LRAT acts as an acyl transferase for RPE65 both *in vitro* and in crude membrane systems.

In conclusion, it is shown here that cysteine and serine containing palmitoylated peptides are good substrates for tLRAT. Thus the enzyme can process both oxygen and thioester amino acid and dipeptide substrates. These substrates are local mimics of the mono and dipalmitoylation sites in RPE65. It is now established that LRAT can function as a palmitoyl transferase in bovine RPE membranes and mediate palmitoyl transfer between lecithin, RPE65 and vitamin A and its esters. These results further strengthen the case for a regulatory role for the palmitoylation of RPE65. A further important point to be resolved is whether there are additional membrane bound palmitoyl transferases which utilize phospholipids as acyl donors. Thus far, the known palmitoyl transferases are soluble and utilize palmitoyl CoA as the donor (28-29).

Acknowledgements

The work described here from the author's laboratory was supported by the U. S. Public Health Service N. I. H. grant EY-015425

Abbreviations

BSA, bovine serum albumin
 DPPC, L- α -dipalmitoylphosphatidylcholine
 DP*PC, [1- ^{14} C]-L- α -dipalmitoyl diphosphatidylcholine
 LRAT, lecithin retinol acyltransferase
 tLRAT, truncated lecithin retinol acyltransferase
 tRE, all-*trans*-retinyl ester
 RPE, retinal pigment epithelium
 TFA, trifluoroacetic acid
 DTT, dithiothreitol
 EDTA, ethylenediaminetetraacetic acid disodium salt
 tRBA, all-*trans*-retinyl α -bromoacetate
 CHAPS, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
 CHAPSO, 3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate
 THF, tetrahydrofuran
 IEF, isoelectrofocusing
 DMSO, dimethyl sulfoxide
 SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

References

1. Sakmar TP, Menon ST, Marin EP, Awad ES. Rhodopsin: insights from recent structural studies. *Ann. Rev. Biophys. and Biomolec. Struct* 2002;31:443–484.

2. Wald G. The biochemistry of vision. *Annu. Rev. Biochem* 1953;22:497–526. [PubMed: 13139377]
3. Lamb TD, Pugh EN Jr. Dark adaptation and the retinoid cycle of vision. *Prog. Retin. Eye Res* 2004;23:307–380. [PubMed: 15177205]
4. Barry RJ, Cañada FJ, Rando RR. Solubilization and partial purification of retinyl ester synthetase and retinoid isomerase from bovine ocular pigment epithelium. *J. Biol. Chem* 1989;264:9231–9238. [PubMed: 2722827]
5. Rando RR. Membrane phospholipids and the dark side of vision. *J. Bioenerg. and Biomembr* 1991;23:133–146. [PubMed: 2010433]
6. Saari JC, Bredberg DL. Lecithin retinol acyltransferase in retinal pigment epithelial microsomes. *J. Biol. Chem* 1989;264:8636–8640. [PubMed: 2722792]
7. Gollapalli DR, Maiti P, Rando RR. RPE65 operates in the vertebrate visual cycle by stereospecifically binding all-trans-retinyl esters. *Biochemistry* 2003;42:11824–11830. [PubMed: 14529294]
8. Mata NL, Moghrabi WN, Lee JS, Bui TV, Radu RA, Horwitz J, Travis GH. Rpe65 is a retinyl ester binding protein that presents insoluble substrate to the isomerase in retinal pigment epithelial cells. *J. Biol. Chem* 2004;279:635–643. [PubMed: 14532273]
9. Redmond TM, Poliakov E, Shirley Y, Tsai J-Y, Lu Z, Gentleman S. Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:13658–13663. [PubMed: 16150724]
10. Moiseyev G, Chen Y, Takahashi Y, Wu BX, Ma J-X. RPE65 is the isomerohydrolase in the retinoid visual cycle. *Proc. Natl. Acad. Sci. U. S. A* 2005;102:12413–12418. [PubMed: 16116091]
11. Jin M, Li S, Moghrabi WN, Sun H, Travis GH. RPE65 is the retinoid isomerase in bovine retinal pigment epithelium. *Cell* 2005;122:449–459. [PubMed: 16096063]
12. Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma J-X, Crouch RK, Pfeifer K. Rpe65 is necessary for production of 11-*cis*-vitamin A in the retinal visual cycle. *Nat. Genet* 1998;20:344–351. [PubMed: 9843205]
13. Tsilou E, Hamel CP, Yu S, Redmond TM. RPE65, the major retinal pigment epithelium microsomal membrane protein, associates with phospholipid liposomes. *Arch Biochem Biophys* 1997;346:21–27. [PubMed: 9328280]
14. Jahng WJ, David C, Nesnas N, Nakanishi K, Rando RR. A cleavable affinity biotinylating agent reveals a retinoid binding role for RPE65. *Biochemistry* 2003;42:6159–6168. [PubMed: 12755618]
15. Xue L, Gollapalli DR, Maiti P, Jahng WJ, Rando RR. A palmitoylation switch mechanism in the regulation of the visual cycle. *Cell* 2004;117:761–771. [PubMed: 15186777]
16. Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, Shenmen CM, Schuler GD, Altschul SF, Zeeberg B, Buetow KH, Schaefer CF, Bhat NK, Hopkins RF, et al. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. U. S. A* 2002;99:16899–16903. [PubMed: 12477932]
17. Maiti P, Kong J, Kim SR, Sparrow JR, Allikmets R, Rando RR. Small molecule RPE65 antagonists limit the visual cycle and prevent lipofuscin formation. *Biochemistry* 2006;45:852–860. [PubMed: 16411761]
18. Lyubarsky AL, Savchenko AB, Morocco SB, Daniele LL, Redmond TM, Pugh EN Jr. Mole quantity of RPE65 and its productivity in the generation of 11-*cis*-retinal from retinal esters in the living mouse eye. *Biochemistry* 2005;44:9880–9888. [PubMed: 16026160]
19. Ruiz A, Winston A, Lim Y-H, Gilbert BA, Rando RR, Bok D. Molecular and biochemical characterization of lecithin retinol acyltransferase. *J. Biol. Chem* 1999;274:3834–3841.
20. Rothman DM, Vazquez ME, Vogel EM, Imperiali B. Caged phospho-amino acid building blocks for solid-phase peptide synthesis. *J. Org. Chem* 2003;68:6795–6798. [PubMed: 12919051]
21. Gollapalli DR, Rando RR. Molecular logic of 11-*cis*-retinoid biosynthesis in a cone-dominated species. *Biochemistry* 2003;42:14921–14929. [PubMed: 14674768]
22. Shi Y-Q, Hubacek I, Rando RR. Kinetic mechanism of lecithin retinol acyl transferase. *Biochemistry* 1993;9:1257–1263. [PubMed: 8448136]
23. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]

24. Jahng WJ, Xue L, Rando RR. Lecithin retinol acyltransferase is a founder member of a novel family of enzymes. *Biochemistry* 2003;42:12805–12812. [PubMed: 14596594]
25. Mondal MS, Ruiz A, Bok D, Rando RR. Lecithin retinol acyltransferase contains cysteine residues essential for catalysis. *Biochemistry* 2000;39:5215–5220. [PubMed: 10819989]
26. Xue L, Rando RR. Roles of cysteine 161 and tyrosine 154 in the lecithin-retinol acyltransferase mechanism. *Biochemistry* 2004;43:6120–6126. [PubMed: 15147196]
27. Shi Y-Q, Furuyoshi S, Hubacek I, Rando RR. Affinity labeling of lecithin retinol acyltransferase. *Biochemistry* 1993;32:3077–3080. [PubMed: 8457568]
28. Huang K, Yanai A, Kang R, Arstikaitis P, Singaraja RR, Metzler M, Mullard A, Haigh B, Gauthier-Campbell C, Gutekunst C-A, Hayden MR, El-Husseini A. Huntingtin-interacting protein HIP14 is a palmitoyl transferase involved in palmitoylation and trafficking of multiple neuronal proteins. *Neuron* 2004;44:977–986. [PubMed: 15603740]
29. Fukata M, Fukata Y, Adesnik H, Nicoll RA, Brecht DS. Identification of PSD-95 palmitoylating enzymes. *Neuron* 2004;44:987–996. [PubMed: 15603741]

Scheme 1.
The Mammalian Visual Cycle

Scheme 2.
Palmitoylated Analogs of Cysteine and Serine

Scheme 3.
Exchange of Palmitoyl Moieties by LRAT

Figure 1. Steady-state kinetic analysis of tLRAT mediated esterification of all-*trans*-retinol by using **FSP** (A) and **FS2P-OH** (B) as acyl donors. The values of V_{MAX} and K_M derived from Hill-Plots are listed in Table 1. Experiment details are described in Methods section.

Figure 2. Steady-state kinetic analysis of tLRAT mediated esterification of all-*trans*-retinol by using **FCP** (A) and **FC2P-OH** (B) as acyl donors. The values of V_{MAX} and K_M derived from Hill-Plot were listed in Table 1. Experiment details were described in Methods section.

Figure 3.

RPE labeling by DPPC. **3A:** Crude RPE membranes were incubated with $[1-^{14}\text{C}]$ -DPPC. Labeled proteins were analyzed by 1D SDS-PAGE, and Western blot. Lane 1 represents the labeled RPE membranes and lane 2 is control RPE. Western blot for RPE65 of RPE alone is shown on the right side of SDS-PAGE. **3B:** Radioactive RPE65 band was excised, trypsin digested and analyzed by scintillation counting (Lane 1: RPE with DP*PC, Lane 2: control RPE). **3C:** 2D SDS-PAGE analysis of RPE incubated with DPPC. Crude RPE membranes were incubated with DP*PC. Labeled proteins were analyzed by 2D SDS-PAGE and Western blot (data no shown). **3D:** Radioactivity of RPE65 from the 2D gel spot was analyzed by trypsin digestion and scintillation counting (Spot 1: RPE65 pI=6, spot 2: RPE65 pI=9, spot 3: blank gel control). Experiment details were described in Methods section.

Figure 4.

Inhibition of DP*PC labeling of RPE by tRBA. Crude membranes were incubated with tRBA (5 μ M) for 1 hr, followed by DP*PC labeling for 2 hrs. Gel band corresponding to RPE65 was excised and digested by trypsin. Radioactivity of the labeled protein was measured by scintillation counting. Condition 1: RPE + DP*PC, condition 2: RPE + RBA + DPPC, condition 3: control RPE.

Figure 5.

Inhibition of tLRAT-mediated DP*PC labeling of RPE by RBA. Purified mRPE65 was incubated with DP*PC and tLRAT or RBA + tLRAT + DP*PC. Condition 1: mRPE65 + DP*PC + tLRAT, condition 2: mRPE65 + DP*PC + tLRAT + RBA, condition 3: mRPE65 + DP*PC, condition 4: control mRPE65.

Table 1

Kinetic parameters of different acyl donors for tLRAT mediated esterification of V_A. The experimental details were described in Methods Section.

Acyl Donor	V _{MAX} (mmol/min/mol)	K _M (μM)
DPPC/BSA *	16.41 ± 0.38	1.67 ± 0.24
FMOC-Ser-Palm FSP	4.39 ± 0.16	198.64 ± 7.26
FMOC-Ser-Ser-Dipalm	7.26 ± 1.04	183.65 ± 7.25
FS2P-OH		
FMOC-Cys-Palm FCP	19.34 ± 3.05	164.49 ± 13.54
FMOC-Cys-Cys-Dipalm	149.25 ± 23.94	135.28 ± 22.54
FC2P-OH		
mRPE65 **	11.00 ± 0.45	0.030 ± 0.002

* Previously published in Reference (24)

** Previously published in Reference (15)