Lecithin Retinol Acyltransferase Forms Functional Homodimers†

Wan Jin Jahng, Eric Cheung, and Robert R. Rando*

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

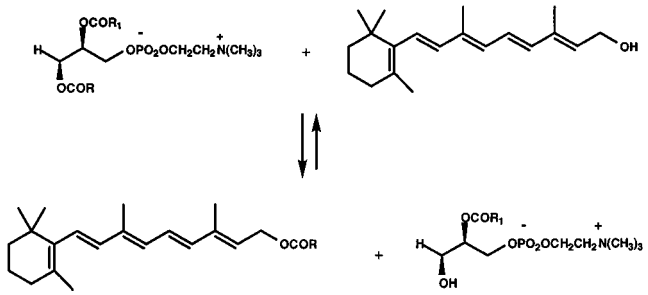
Received August 22, 2001

ABSTRACT: Membrane-bound lecithin retinol acyltransferase (LRAT), an essential enzyme in vitamin A processing, catalyzes the formation of retinyl esters from vitamin A and lecithin. Cloned and expressed LRAT has a molecular mass of 25.3 kDa. The enzyme is not homologous to known enzymes and is, therefore, of substantial interest mechanistically. Along these lines, the functional protomeric state of LRAT is of importance. Gel electrophoretic studies on LRAT in the presence of SDS and disulfide reducing agents show the expected 25 kDa monomer. However, gel electrophoresis in the absence of a reducing agent and/or strong denaturing conditions reveals substantial dimer formation. LRAT monomers can be efficiently and irreversibly cross-linked by thiol reactive bismaleimides in retinal pigment epithelial (RPE) membranes generating LRAT homodimers. Cross-linked LRAT homodimers are fully active catalytically. The experiments suggest that LRAT monomers interact in membranes and form functional homodimers through protein−protein interactions and disulfide bond formation.

Lecithin retinol acyltransferase (LRAT)[1] catalyzes the acylation of vitamin A with lecithin to generate retinyl esters and 1-acyl-lysophospholipids as products (Scheme 1) (*1*−*3*). The enzyme plays an essential role in general vitamin A metabolism and, in particular, is important in the processing of retinoids in the vertebrate visual cycle (*4*, *5*). The membrane-bound enzyme has been solubilized and partially purified (*2*, *6*, *7*). Its kinetic mechanism has been determined to be an ordered ping-pong bibi mechanism, with lecithin binding first and transferring an acyl group to an active-site nucleophile of LRAT (*8*). After the departure of the lysolecithin, the vitamin A binds to the enzyme and the acyl group is transferred to it.

Full purification of LRAT has been elusive, but partially purified LRAT has been specifically labeled with affinity labeling agents (*9*, *10*), enabling partial sequence information to be obtained (*11*). Expression cloning of the enzyme revealed a 25.3 kDa protein with predicted N- and C-terminal transmembrane helical segments (Scheme 2) (*11*). The active-site region of LRAT is presumably found in the cytoplasmic, soluble region of the protein (*4*). A study of the sequence in this region does not provide clues about the possible mechanistic class to which LRAT belongs, inasmuch as LRAT is not homologous with any known enzymes, including LCAT (*4*). This latter enzyme catalyzes a reaction very similar to that of LRAT involving the transfer of an *sn*-2 fatty acyl group of lecithin to cholesterol (*12*−*14*). In the case of LCAT, an active-site serine residue appears to be the essential active-site nucleophile (*12*−*14*). In fact, as expected, LCAT is homologous to serine proteases (*12*−*15*). Serine proteases carry out reactions similar to those of acyltransferases, and may only substantially differ from the latter by utilizing water as the attacking nucleophile, rather than an organic nucleophile. As mentioned above, LRAT is unlikely to be a serine-dependent enzyme and, moreover, shows none of the sequence motifs characteristic of this category of enzymes.

Scheme 1: Acylation of Vitamin A Catalyzed by LRAT



While the activity of LRAT is rather insensitive to serine-directed reagents, it is quite sensitive to organomercurial-based thiol reactive reagents, suggesting the presence of an essential cysteine residue (*6*, *7*). The enzyme possesses four cysteine residues (Scheme 2) (*4*, *11*). Biochemical studies

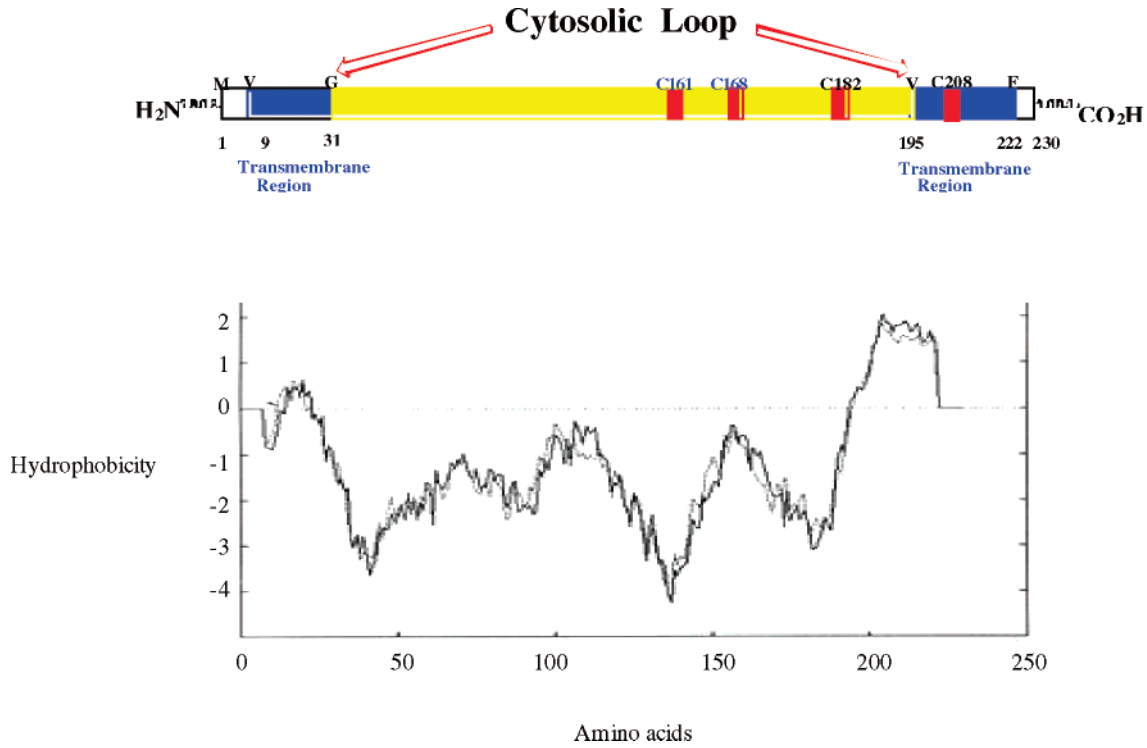
† The work described here was supported by U.S. Public Health Service NIH Grant EY-04096.

* To whom correspondence should be addressed.

[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; BMB, 1,4-bismaleimidobutane; BME, β-mercaptoethanol; BMH, 1,6-bismaleimidohexane; BMOE, bismaleimidoethane; BM[PEO]$_3$, 1,8-bismaleimidotriethylene glycol; BM[PEO]$_4$, 1,11-bismaleimidotetraethylene glycol; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; DPDPB, 1,4-bis[3′-(2′-pyridyldithio)propionamido]butane; DPPC, L-α-dipalmitoylphosphatidylcholine; DSP, dithiobis(succinimidyl propionate); DTME, dithiobismaleimidoethane; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGS, ethylene glycol bis(succinimidyl succinate); E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; HBVS, 1,6-hexane-bis(vinyl sulfone); HPLC, high-pressure liquid chromatography; LCAT, lecithin cholesterol acyltransferase; LRAT, lecithin retinol acyltransferase; PVDF, polyvinylidene fluoride; RPE, retinal pigment epithelium; SANPAH, *N*-succinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine; TMEA, tris(2-maleimidoethyl)amine.

10.1021/bi015710b CCC: $22.00
Published on Web 04/23/2002

Scheme 2: Cysteine Residues and Hydropathy Plot for LRAT



show that C161 is essential for catalysis, and may be an important involved active-site nucleophile (*16*). C182 and C208 are unimportant for catalysis (*16*).

Mechanistic studies on LRAT are ongoing because it may define a novel mechanistic category. How this might relate to the fact that LRAT is a membrane-bound enzyme is unclear, except bear in mind that most of what is known concerning enzyme mechanisms comes from studies on soluble proteins. It should also be noted that LRAT is homologous to a family of proteins of unknown function, some of which are thought to be tumor suppressors (*4*). Interestingly, all of these proteins have identical domains encompassing LRAT C161 (*4*).

An aspect of LRAT function of substantial interest to its mechanism of action is its protomeric state in membranes.

Since the primary sequence of LRAT in its monomeric form does not suggest a mechanistic class, it is possible that LRAT functions in a protomeric form. Interestingly, a study investigating the molecular size of microsomal LRAT by radiation inactivation analysis suggests an average target size of 52−56 kDa (*17*). This result would be consistent with LRAT being a dimer in microsomal membranes, rather than a monomer. SDS−PAGE of LRAT in the presence of disulfide reducing agents shows the expected 25 kDa molecular mass band, as detected by immunoblotting (*11*). However, as shown here, in the absence of strongly denaturing conditions or disulfide reducing conditions, distinct dimer formation is observed by PAGE analysis. Cross-linking studies show that LRAT monomers interact in RPE membranes. It is also demonstrated that the LRAT monomer is readily chemically cross-linked in RPE microsomal membranes to form catalytically active homodimers using bis-thiol reactive reagent, such as BMH (Scheme 3). These results require that LRAT monomers be normally associated with each other in membranes, and raise the possibility that LRAT functions catalytically as an active homodimer. No other proteins were found cross-linked to LRAT.

MATERIALS AND METHODS

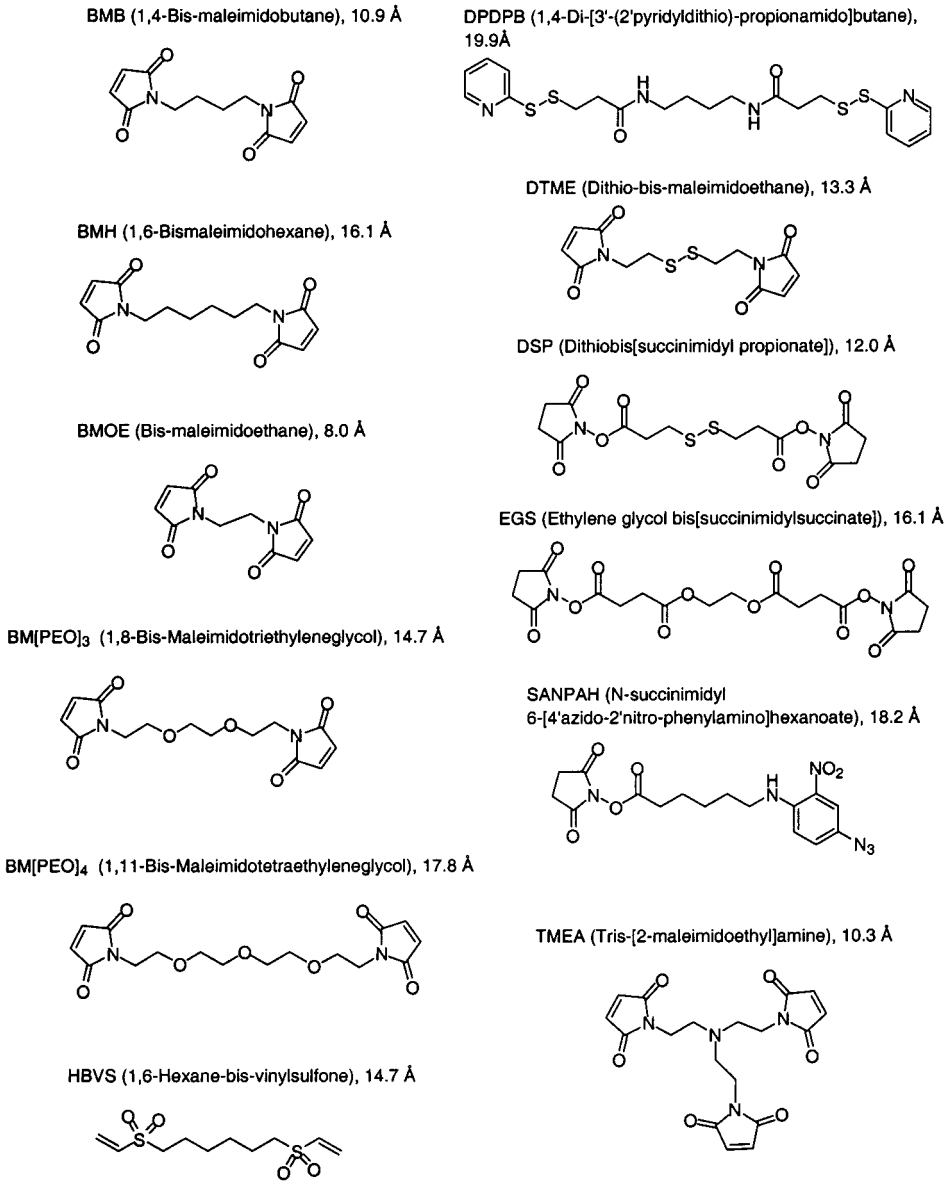
Materials

Frozen bovine eye cups were obtained from W. L. Lawson Co. (Lincoln, NE). [11,12-$^3H_2$]*all-trans*-Retinol (31.4 Ci/mmol) was obtained from NEN Life Sciences, Inc. L-α-Dipalmitoylphosphatidylcholine (DPPC), BSA, dithiothreitol (DTT), *all-trans*-retinyl palmitate, and protease inhibitor cocktail (AEBSF, aprotinin, bestatin, EDTA, E-64, leupeptin, and pepstatin A) were from Sigma. Triton X-100 was from Fluka. Centriprep was from Amicon. Cross-linking reagents, Western blot blocking buffer, and Gel-code blue were from Pierce. HPLC grade solvents were from J. T. Baker. Coomassie brilliant blue R-250 was from Bio-Rad. The silver staining kit, the polyvinylidene fluoride membrane, and the ECL-Western blotting kit were from Amersham Pharmacia Biotech. The precast gel (4 to 20%, 8 cm × 8 cm) for SDS−native-PAGE was from Invitrogen Life Technologies, and the preparative precast gel (4 to 20%, 16 cm × 18 cm) was from Jule Inc. (New Haven, CT). For molecular mass markers, Dalton VI markers for 14, 18, 24, 35, 45, and 66 kDa were from Sigma and Benchmark prestained markers for 9, 13, 19, 25, 36, 49, 61, 80, 111, and 173 kDa were from GibcoBRL Life Technologies. All other reagents were analytical grade.

Methods

Preparation of the Bovine Pigment Epithelium (RPE) Membranes. The procedure for preparation of bovine RPE membranes was described elsewhere (*6*−*11*). Prior to use, the membranes were irradiated with UV light (365 nm) on ice for 5 min to destroy endogenous retinoids. Solubilization

Scheme 3: Cross-Linking Reagents Used in Studies



of RPE in detergents was reported previously (*10*, *11*). Briefly, RPE membranes were solubilized in 10 mM Tris-HCl (pH 9.0), 2 mM DTT, 1 mM EDTA, and 1% Triton X-100. After thorough mixing for 1 h at 4 °C, the material was centrifuged at 10500*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 Triton X-100 (0.1%) after concentrating the sample with a Centriprep apparatus. The RPE stock solution contains 2−3 mg/mL proteins, as determined by the Bradford assay.

Gel Electrophoresis. Tris-glycine polyacrylamide gel (4 to 20%) electrophoresis was carried out using the Tris (25 mM), glycine (192 mM), and SDS (0.1%) buffer system described by Laemmli (*18*). Denaturing gels was performed by heating (97 °C, 2 min) in sample buffer (2×) 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 (0.1%) or silver staining. For the mini gel (8 cm × 8 cm), 20 μL of the protein solution (40 μg of total proteins, as determined by the Bradford assay) was loaded for each well, and for preparative gels (16 cm × 18 cm), 500 μL of sample (1−1.5 mg of proteins based on the Bradford assay) was loaded along with the molecular mass markers (20 μL).

Western Blot Analysis. Polyclonal anti-LRAT antibody formation was reported previously (*11*). After protein separation by SDS−PAGE, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the Tris-glycine buffer (25 mM) and ethanol (20%) via a semidry transfer apparatus. The membrane was blocked with 5% nonfat dried milk for 2 h at room temperature. Anti-LRAT antibody (1:4000 dilution), anti-rabbit Ig-linked horseradish peroxidase (1:8000 dilution) from donkey, and the enhanced chemiluminescence (ECL) system were used to detect the LRAT band.

LRAT Cross-Linking. Twelve different bifunctional or trifunctional cross-linking reagents were used to cross-link LRAT. For the 1,6-bismaleimidohexane (BMH) reaction with LRAT, crude RPE (90 μL) was incubated with BMH (10 μL, final concentration of 10−500 μM in DMF) at 4 °C for 1 h with gentle agitation. Other organic solvents such as DMSO, THF, and *p*-dioxane were also tested and showed

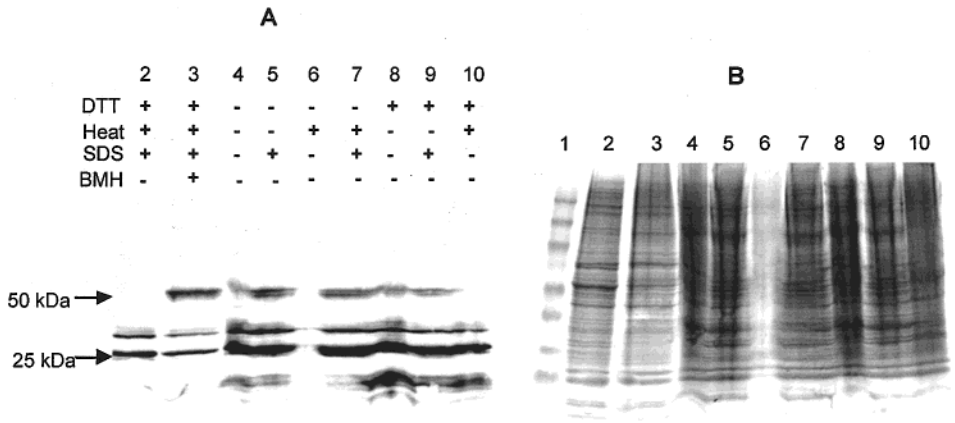


FIGURE 1: Effects of sample preparation on LRAT dimer formation. (A) Western blot of PAGE. The LRAT−LRAT dimer band was visualized with an antiLRAT antibody with ECL. Running buffer [25 mM Tris-HCl and 192 mM glycine (pH 8.3)] contained 0.1% SDS: lane 2, RPE, DTT (10 mM), and SDS (5%) with heating (97 °C for 2 min); lane 3, RPE and BMH (100 μM, incubation for 1 h at 4 °C); lane 4, RPE in native sample buffer without heating; lane 5, RPE and SDS (5%); lane 6, RPE with heating (97 °C for 2 min); lane 7, RPE and SDS (5%) with heating (97 °C for 2 min); lane 8, RPE and DTT (10 mM); lane 9, RPE, DTT (10 mM), and SDS (5%); and lane 10, RPE and DTT (10 mM) with heating (97 °C for 2 min). (B) Tris-glycine PAGE gel (4 to 20%). Forty micrograms of total proteins was loaded per each well. Proteins were visualized by Coomassie blue G-250 staining. Lane 1 contained molecular mass markers (173, 111, 80, 61, 49, 36, 25, 19, 13, and 9 kDa).

the same results. As control experiments, *N*-ethylmaleimide (NEM, 10 μL, final concentration of 100 μM) and iodoacetamide (10 μL, 100 μM) were reacted with RPE (90 μL). Other cross-linking reagents were incubated with LRAT under exactly the same conditions (10 μL, final concentration of 100 μM) as described above.

LRAT Dimer Formation under Nonreducing Conditions. Nondenaturing sample buffer [without DTT and SDS, Tris-HCl (312.5 mM, pH 6.8) with 50% glycerol and 0.05% bromophenol blue] was used to reveal LRAT dimer formation compared to DTT (10 mM) and/or SDS (5%) control with or without heating. Running buffer contained Tris (25 mM), glycine (192 mM), and SDS (0.1%).

LRAT Kinetic Assays. The kinetic experiments for LRAT and LRAT dimer activity were performed by monitoring the formation of retinyl esters from *all-trans*-retinol and DPPC. Procedures for measuring LRAT kinetics were reported previously (*8*, *11*). The LRAT assay was performed by adding BMH/RPE mixture, Tris-HCl (pH 7.5, 100 mM), EDTA (1 mM), DPPC (1 mM in 2.5% BSA), and DTT (2 mM) into a dark Eppendorf tube followed by the addition of [11,12-$^3H_2$]*all-trans*-retinol (in DMSO, 3 μM). At 5, 10, 15, 20, 25, and 30 min, 100 μL of the mixture is transferred into another dark Eppendorf tube and quenched with 500 μL of methanol. Distilled water (100 μL) and hexane (500 μL) were added to the quenched mixture and vortexed for 5 min. The quenched mixture was then centrifuged at 14 000 rpm for 5 min. The top hexane layer (200 μL) was injected into the HPLC system (Berthold LB 506-C radioactivity monitor) for analysis.

Mass Spectrometry Analysis. Preparative SDS−PAGE (16 cm × 18 cm × 0.75 mm) was performed to generate a large amount of LRAT dimer. BMH (200 μM)-derived LRAT dimer band (50 kDa) was cut and dehydrated in MeCN for 10 min. Gel pieces were covered with DTT (10 mM) in NH_4HCO_3 (100 mM) to reduce proteins for 1 h at 56 °C. After the mixture had cooled to room temperature, the reducing buffer was removed, and iodoacetamide (55 mM) in NH_4HCO_3 (100 mM) was incubated for 45 min in the dark at room temperature. The gel washing−dehydration cycle was repeated three times with NH_4HCO_3/MeCN before trypsin (12.5 ng/μL, 5 μL/mm^2 gel, overnight) digestion. Mass spectrometric analysis of the trypsin-digested LRAT dimer was performed at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School. When peptides were analyzed by ion-trap mass spectrometry, the amino acid sequence was determined by tandem mass spectrometry (MS/MS) and a database search. LCQ DECA ion-trap mass spectrometers (ThermoFinnigan) were used. For microcapillary LC elution, a linear gradient from 100% buffer A [MeCN (5%), H_2O (95%), formic acid (0.1%), and heptafluorobutyric acid (0.005%)] to 100% buffer B [MeCN (95%), H_2O (5%), formic acid (0.1%), and heptafluorobutyric acid (0.005%)] was used. The capillary column was 75 μm (inside diameter) × 12 cm (bed length). The flow rate was split down from the pumps to 200 nL/min for the separation. The bovine database was extracted and downloaded by FTP from the NCBI (http://www.ncbi.nlm.nih.gov/).

RESULTS

SDS−PAGE of LRAT from RPE Membranes. When bovine RPE membrane-bound LRAT is solubilized in Triton X-100 and then subjected to SDS−PAGE under strongly denaturing conditions, a band is identified at approximately 25 kDa by a Western blot using antipeptide antibodies raised against an N-terminal LRAT peptide (*4*, *11*). This band contains bovine LRAT and is consistent with the molecular size of cloned LRAT as a monomer with a molecular mass of 25.3 kDa (*11*). In the current studies, gel electrophoresis is carried out under both strongly denaturing and weakly denaturing conditions and in the presence and absence of disulfide reducing agents (Figure 1A,B). The gel pattern under strongly denaturing conditions (boiling in SDS and DTT), stained using the antibody described above, appears exactly as published [Figure 1A (lane 2) (*11*)]. The faint band stained with a molecular mass slightly greater than that of LRAT is unrelated to LRAT, disappears upon further purification of the enzyme, and is identified as the nonmuscle myosin heavy chain (type b) by mass spectrometry (unpublished experiments). When gel electrophoresis was carried out in the

absence of DTT (Figure 1A, lanes 4−7), a clear LRAT band with a molecular mass of approximately 50 kDa can be observed, except in lane 6 where penetration into the gel does not reproducibly occur. This probably occurs because of the heat-induced aggregation of the integral membrane-bound proteins in the absence of detergent.

These data suggest that dimer formation is, at least partly, mediated by disulfide bond formation. Protein−protein interactions are also likely to be important as seen in lanes 8 and 9. In lane 9, DTT and SDS are included in the sample buffer, but boiling is eliminated. The apparent dimer is still found to be present. The same is true in lane 8 where both SDS and boiling are eliminated. These data suggest that dimer formation is driven by a combination of disulfide bond formation and protein−protein interactions. In no case is complete dimer formation observed. However, the running buffer in the gels still contains SDS (0.1%), which will have the effect of denaturing the protein. A way to circumvent this is to perform native gel electrophoresis in the absence of denaturing agents. However, in this instance, resolved native gels could not be obtained, even in the presence of 1% Triton X-100 (unpublished experiments). This is likely due to the fact that the proteins in question are membrane-bound.

Finally, in lane 3 of Figure 1A, data are shown for the covalent cross-linking of LRAT in RPE membranes using the bis-maleimide agent BMH (Scheme 3). As expected, this covalent cross-linked dimer is unaffected by DTT and boiling with SDS. The question of dimer formation was then further explored using thiol reactive cross-linking agents to covalently cross-link the monomers.

Covalent Cross-Linking of LRAT Monomers. Lane 3 of Figure 1A shows a clear covalent cross-linked LRAT product in the presence of BMH. LRAT contains four cysteine residues at positions 161, 168, 182, and 208 (Scheme 2), raising the possibility that interchain cross-linking of cysteine residues could produce dimer formation (*4*, *11*). To elaborate on this possibility, the series of cross-linking agents, shown in Scheme 3, were chosen for further study. This group of reagents includes thiol reactive maleimide and vinyl sulfone derivatives, along with amine reactive succinimides. Dimer formation by cross-linking reagents was dependent on the cross-linker structure (maleimide, reaction for sulfhydryl group vs succinimide, amino group reactive) and spacer arm distance (8−20 Å).

In Figure 2A, data are shown for the cross-linking reactions on freshly harvested bovine RPE membranes. Lanes 2 and 12 show the migration of LRAT under reducing conditions (added BME). LRAT appears here as the 25 kDa protein. Lanes 3 and 13 show gel migration in the absence of BMH. Again, dimer formation is clearly evident. Of the cross-linking agents that were studied, the maleimide thiol acceptors were the most effective at producing dimeric LRAT. The DTME-treated LRAT dimer was cleaved with 5% BME in Laemmli buffer (lane 16). Finally, it should be noted that attempted cross-linking of detergent-solubilized LRAT does not lead to substantial cross-linking, as shown in Figure 2B.

The nature of the cross-linking process in RPE membranes was further investigated (Figure 3A,B). In Figure 3A are shown data demonstrating that BMH-cross-linked LRAT is unaffected by reducing agents DTT and TCEP, while the dimer generated using disulfide-containing DTME is readily

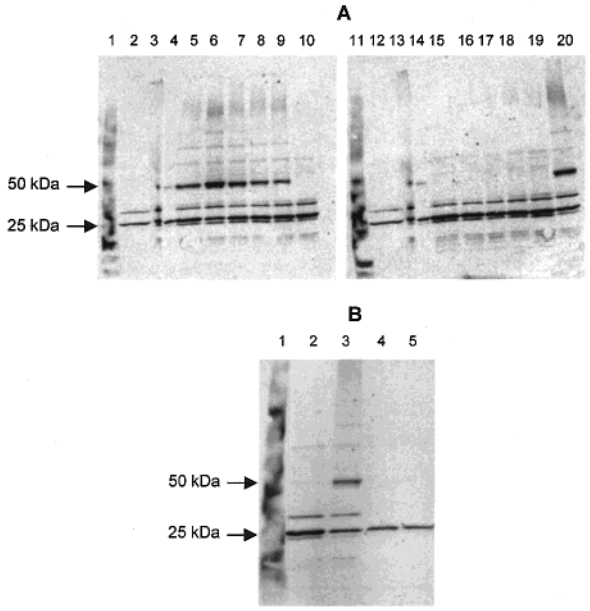


FIGURE 2: LRAT dimerization induced by various cross-linkers. (A) Western blot of the LRAT−LRAT dimer cross-linked by a series of cross-linkers. Sample buffer contained SDS (2%) and BME (5%): lane 1, molecular mass markers (173, 111, 80, 61, 49, 36, 25, 19, 13, and 9 kDa); lane 2, RPE; lane 3, RPE under nonreducing conditions without heating; lane 4, solubilized RPE under nonreducing conditions without heating; lane 5, RPE and BMB (100 mM); lane 6, RPE and BMH (100 mM); lane 7, RPE and BMOE (100 mM); lane 8, RPE and BM[PEO]$_3$ (100 mM); lane 9, RPE and BM[PEO]$_4$ (100 mM); lane 10, RPE and HBVS (100 mM); lane 11, molecular mass markers (173, 111, 80, 61, 49, 36, 25, 19, 13, and 9 kDa); lane 12, RPE; lane 13, RPE under nonreducing conditions without heating; lane 14, solubilized RPE under nonreducing conditions without heating; lane 15, RPE and DPDPB (100 mM); lane 16, RPE and DTME (100 mM); lane 17, RPE and DSP (100 mM); lane 18, RPE and EGS (100 mM); lane 19, RPE and SANPAH (100 mM); and lane 20, RPE and TMEA (100 mM). (B) Western blot of solubilized RPE with cross-linkers: lane 1, molecular mass markers (173, 111, 80, 61, 49, 36, 25, 19, 13, and 9 kDa); lane 2, RPE; lane 3, RPE and BMH (100 μM); lane 4, solubilized RPE; and lane 5, solubilized RPE and BMH (100 μM).

cleaved. The DTME-derived LRAT dimer was observed when none of the reducing reagents were used (Figure 3A, lane 6). That alkylation of cysteine residues by itself does not cause dimer formation is implied by the fact that disulfide cross-linking agents are ineffective under reducing conditions (Figure 3A). This was further investigated as shown in Figure 3B. In Figure 3B are shown data for the cross-linking of LRAT in RPE membranes using BMH and the lack of cross-linking evoked by *N*-ethylmaleimide (NEM) and iodoacetamide. Also, partial treatment with the latter two alkylating agents does not prevent cross-linking by BMH, as also shown in Figure 3. These experiments suggest that dimer formation can be understood to occur as a consequence of the cross-linking of two cysteine residues on adjacent monomeric proteins.

It is, of course, important to demonstrate that the dimer that is formed is a homodimer of LRAT monomers, rather than a heterodimer containing one LRAT monomer cross-linked to a different protein. As LRAT is not readily cross-linked when solubilized in detergent and partially purified, cross-linking reactions are performed in RPE membranes. To determine if the approximately 50 kDa protein is an LRAT homodimer, LRAT was cross-linked in RPE membranes using BMH as shown in Figure 4A,B. At high

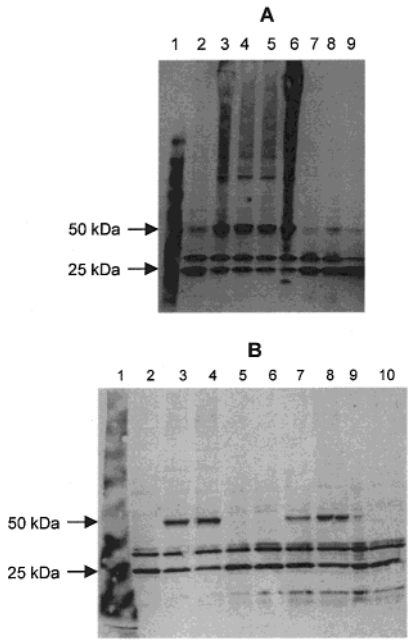


FIGURE 3: Studies on the LRAT cross-linking process. (A) Comparison of covalent vs reducible LRAT dimer formed by BMH and DTME. LRAT−LRAT dimer bands were visualized with an antiLRAT antibody and ECL: lane 1, molecular mass markers (173, 111, 80, 61, 49, 36, 25, 19, 13, and 9 kDa); lane 2, RPE; lane 3, RPE and BMH (100 μM); lane 4, RPE, BMH (100 μM), and DTT (5 mM); lane 5, RPE, BMH (100 μM), and TCEP (5 mM); lane 6, RPE and DTME (100 μM) without a reducing agent; lane 7, RPE, DTME (100 μM), and DTT (5 mM); lane 8, RPE, DTME (100 μM), and TCEP (5 mM); and lane 9, solubilized RPE. (B) NEM control. Western blot of SDS−PAGE gel: lane 1, molecular mass markers (173, 111, 80, 61, 49, 36, 25, 19, 13, and 9 kDa); lane 2, RPE; lane 3, RPE and BMH (50 μM); lane 4, RPE and BMH (100 μM); lane 5, RPE and NEM (100 μM); lane 6, RPE and iodoacetamide (100 μM); lane 7, RPE and NEM (100 μM), then BMH (100 μM); lane 8, RPE and iodoacetamide (100 μM), then BMH (100 μM); lane 9, RPE with nonreducing sample buffer without heating; and lane 10, RPE.

concentrations of BMH (0.5 mM), virtually complete cross-linking is observed to occur. Protein samples eluted from one lane of a mini gel (Figure 4A, lane 5, 8 cm × 8 cm × 1 mm) failed to identify LRAT due to the low abundance of the protein. However, sufficient material could be harvested from preparative gels. When the 50 kDa protein band was removed from the preparative gel (16 cm × 18 cm × 0.75 mm) and subjected to MS/MS study. A control gel was run in the absence of BMH. The object of this experiment was to determine whether the approximately 25 kDa LRAT was observed in the 50 kDa mass region. Mass spectrometric study on the tryptic digest of the BMH gel showed, as expected, several approximately 50 kDa proteins, including vimentin (53 kDa) and the ubiquinol−cytochrome *c* reductase complex core protein 2 precursor (48 kDa) using the NCI bovine database. A table listing the observed peptides from the cross-linked sample (50 kDa band) is provided (Table 1). Apparent background such as trypsin, keratin, and crystalline which were also found in control band (50 kDa) was deleted from the list. Importantly, two peptides from LRAT were also found: THLTHYGIYLGDNR (residues 56−69) and VAHMMPDILLALTDDK (residues 70−85). These were the only peptides found derived from known approximately 25 kDa proteins uniquely found in the BMH-cross-linked sample. This experiment was performed twice

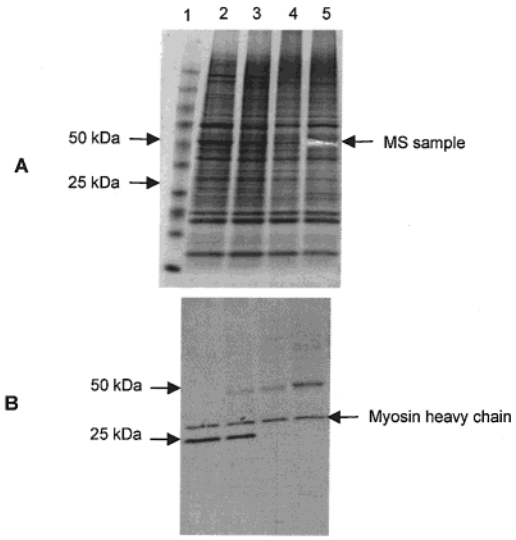


FIGURE 4: LRAT dimer sample preparation and dependence of dimer formation on BMH concentration. (A) SDS−PAGE Tris-glycine gel (4 to 20%). Protein from one lane of a mini gel was not sufficient to identify LRAT; 40−60 μg of total proteins was loaded per each well. Proteins were visualized by Coomassie blue G-250 staining: lane 1, molecular mass markers (173, 111, 80, 61, 49, 36, 25, 19, 13, and 9 kDa); lane 2, RPE; lane 3, RPE and BMH (10 mM); lane 4, RPE and BMH (50 mM); and lane 5, RPE and BMH (0.5 mM). (B) Western blot of SDS−PAGE gel. LRAT−LRAT dimer bands were detected with an antiLRAT antibody and ECL.

with the same results. The possibility of cross-linking LRAT with partial proteolysis products of other proteins cannot be ruled out, although the experiments were performed in the presence of a protease inhibitor cocktail. These data suggest that the 50 kDa dimer is indeed an LRAT homodimer, and not a heterodimer comprised of an LRAT monomer cross-linked to a second protein.

Kinetics of Covalenlty Cross-Linked LRAT Monomers. The observation that the 50 kDa protein is a homodimer of LRAT brings up the issue of its kinetic activity. To address this issue, LRAT was covalently dimerized using excess BMH to generate largely LRAT dimer (Figure 5B). The catalytic activity of this product was compared to that of an identical non-BMH-treated sample (Figure 5A,C). As can be seen, the two samples have virtually identical activities. The fact that the V_{max} values are identical means that the homodimer is fully active and that the results cannot be explained by the residual activity of a small amount of unreacted LRAT monomer in the sample. These data demonstrate that homodimeric LRAT is fully functional and leave open the possibility that RPE membrane-associated LRAT operates as a dimer.

DISCUSSION

SDS−PAGE in the presence of DTT shows LRAT as a 25.3 kDa protein (*4*, *11*). Expression cloning of this 25.3 kDa protein shows that this protein is necessary and sufficient for LRAT activity (*11*). Interestingly, radiation inactivation analysis studies suggest a molecular mass for LRAT of approximately 50 kDa (*17*). These conflicting observations are resolved by showing that LRAT is a dimer in RPE membranes. While native gel electrophoresis would have been useful for revealing the molecular size of LRAT,

Table 1: RPE Proteins of the 50 kDa (BMH-cross-linked) Band Identified by MS/MS[a]

name	MH^+	X_{corr}	% sequence coverage[b]	MW (Da)	accession number	sequence[c]
ubiquinol−cytochrome *c* reductase complex core protein 2 precursor	2287.6 (−0.1)	5.1270	4.6	48149	SW:UCR2_BOVIN	K.AVALQNPQAHVIENLHAAAYR.N
elongation factor 1	2517.0 (−0.3)	5.0355	7.5	34379	GP:AF013213_1	R.VETGVLKPGMVVTFAPVNVTTEVK.S
vimentin	1534.8 (−0.6)	4.7409	3.0	53546	SW:VIME_BOVIN	R.KVESLQEEIAFLK.K
ubiquinol−cytochrome *c* reductase complex core protein 2 precursor	1435.8 (−0.5)	4.2117	3.1	48149	SW:UCR2_BOVIN	R.MALIGLGVSHPVLK.Q
glutamine synthetase	2151.3 (+0.6)	4.2088	18.7	12188	SW:GLNA_BOVIN	R.LTGFHETSNINDFSAGVANR.G
β-actin	2232.5 (+0.9)	4.1672	5.6	41737	SW:ACTB_HUMAN	+3K.DLYANTVLSGGTTM@YPGIADR.M
ubiquinol−cytochrome *c* reductase complex core protein 2 precursor	1886.2 (+0.6)	4.1230	4.0	48149	SW:UCR2_BOVIN	R.LPNGLVIASLENYAPASR.I
lecithin retinol acyltransferase	**1660.8 (−1.0)**	**4.0766**	**6.1**	**25701**	**GP:AF275344_1**	**R.THLTHYGIYLGDNR.V**
coatomer α subunit	1860.0 (+0.2)	3.8715	1.6	138359	SW:COPA_BOVIN	P.ELDIPPGAAGGAEDGFFVP.P
melanocortin-5 receptor	3186.8 (+1.0)	3.4814	8.0	36526	SW:MC5R_BOVIN	M.ISC#PHNLYCSCFMSHFNM@YLILIMCN.S
elastin-cBEL3	4096.8 (+0.7)	3.4412	7.4	56435	GP:M19372_1	+2V.GVPGLGVGVGVPGLGVGAGVPGFGAVPG-TLAAAKAAKFGPGGVG-ALGGV.G
lial fibrillary acidic protein, astrocyte	1216.3 (+0.8)	3.3823	2.6	49452	SW:GFAP_BOVIN	R.DNLAQDLGTLR.Q
isoform 1, bovine (fragment)	3318.8 (−1.3)	3.3095	28.3	10914	PIR2:PC4431	+2S.KPRVATPEVVSKIAQYKREC#PSIFAWEI.R
phosphatidylinositol glycan-specific phospholipase d precursor	1997.2 (+1.9)	3.2813	2.0	92602	SW:PHLD_BOVIN	E.KLNVEAANWM@VKGEEDF.A
lecithin retinol acyltransferase	**1784.1 (+0.0)**	**3.2161**	**7.0**	**25701**	**GP:AF275344_1**	**R.VAHMMPDILLALTDDK.G**
nitric oxide synthase, endothelial	4902.8 (−0.5)	3.1706	3.5	133156	SW:NOS3_BOVIN	+1E.WKWFRCPTLLEVLEQFPSVALPAPLLLTQ-LPLLQPRYYSVSS.A
ubiquinol−cytochrome *c* reductase complex core protein 2 precursor	1451.8 (+1.1)	3.1635	3.1	48149	SW:UCR2_BOVIN	R.M@ALIGLGVSHPVLK.Q
sodium/potassium transporting ATPase	1546.6 (−0.4)	3.0805	4.8	33401	SW:ATNC_BOVIN	+1R.INAANIATDDERDK.F
RPBH8M IgM	3882.5 (+1.0)	3.0535	22.4	17179	GP:U50557_1	W.TLLFVLSAPRGVLAQVQLRESGPSLVKPSQTLSLTC#.T
transcription factor 5A (STAT5A)	2225.7 (+0.4)	3.0523	19.8	11218	GP:AF079568_1	T.FLLRFXXSEIGGITIAWKF.D
anti-GST IgVL3	3426.7 (−1.3)	3.0382	29.7	11432	GP:AJ012801_1	T.LGQRVSITC#SGSSSNVGLGDYVGWFQQVPGSAP.R
acetylcholinesterase	1843.0 (−0.4)	3.0290	3.1	64239	SW:ACES_BOVIN	+1G.GAGGNDTELVAC#LRARPA.Q
tyrosyl-tRNA synthetase	3649.2 (−1.6)	3.0268	6.1	59149	GP:AF087021_1	K.RIHLM@NPM@VPGLTGSKM@SSSEEESKIDLLDRK.E

[a] Data for LRAT peptides are in bold. [b] By amino acid count. [c] M@ is oxidized methionine, and C# is carboxymethylated cysteine.

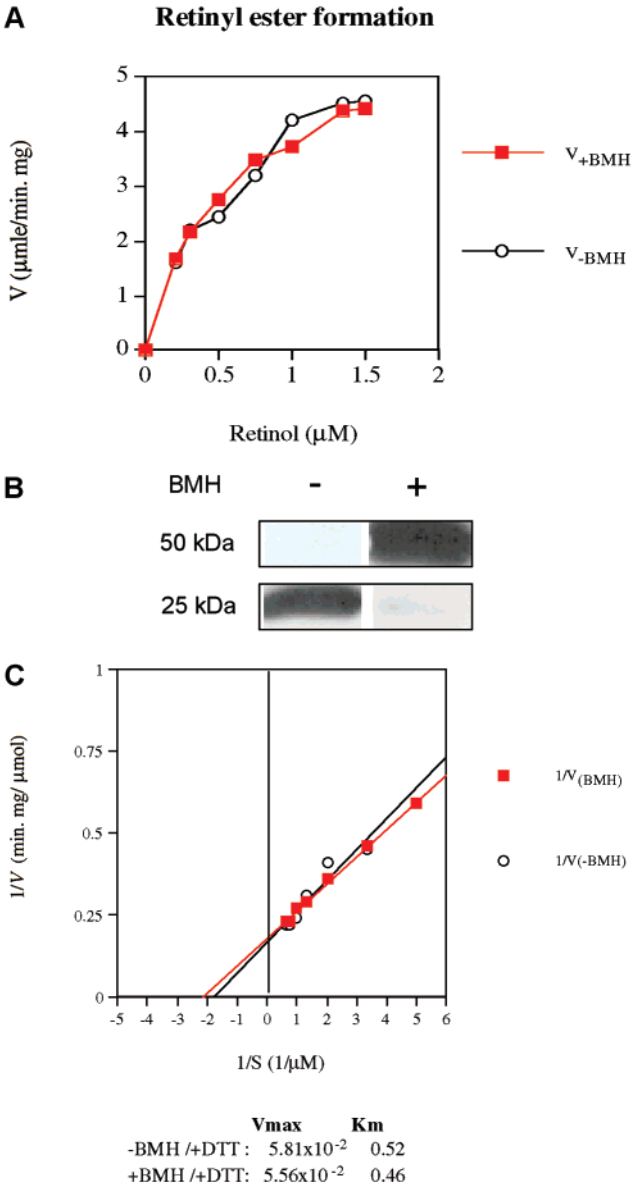


FIGURE 5: LRAT dimer kinetics. (A) Initial rates of retinyl ester formation of un-cross-linked LRAT (without BMH) and LRAT dimer (with BMH). (B) Western blots of LRAT and LRAT dimer showing virtually complete dimer formation under the conditions of the cross-linking process. (C) Lineweaver−Burk plot of LRAT dimer kinetics showing almost the same K_m and V_{max}. Data for the LRAT dimer are shown with a red line with squares, whereas data for the LRAT monomer are shown with a black line with circles. All points are averages of triplicates, and the determinations were within 5% of each other.

experimental difficulties precluded the use of non-SDS-based gel electrophoresis, a nonunique occurrence with intrinsic membrane proteins. However, SDS−PAGE could be performed under conditions that are suitable for revealing dimer formation. SDS−PAGE performed in the absence of DTT and/or in the absence of strongly denaturing conditions shows the presence of substantial dimer formation (Figure 1). The data suggest that dimer formation is dependent both on disulfide bond formation and on protein−protein interactions. However, complete dimer formation is not observed, even in the absence of a reducing agent. There are several possible reasons for this. The denaturant SDS is still maintained in the running buffer during electrophoresis and may interfere with protein−protein interactions. SDS should not affect disulfide bond formation, however. It is possible that endogenous reducing agents cleave some of the dimer in RPE membranes, resulting in the substantially less than quantitative recovery of dimer observed on gels. Finally, it is also possible that there is some denatured or oxidized LRAT in RPE membranes which is not dimerized. While these experiments indicate that LRAT forms a dimer in RPE membranes, they are not informative with respect to the functional activity of the dimer. Since native LRAT has resisted purification, it is not possible to purify the homodimer and determine its activity directly. In addition, since LRAT is a very minor membrane protein (<1%), it was not possible to obtain sufficient quantities of the LRAT dimer for mass spectrometric studies. To approach the issue of functional activity and mass spectrometric analysis, a method for irreversibly cross-linking monomers was sought.

The results described here suggest that LRAT monomers in RPE membranes can be readily cross-linked by a series of bis-maleimides to form functional homodimers. Maleimides are known to act as Michael acceptors in reactions with thiols (*19*). These experiments minimally demonstrate that LRAT monomers are adjacent to one another in native membranes. Other cross-linked products were not found, although, of course, the possibility that they may be present at undetectable levels cannot be ruled out. Most importantly, the cross-linked dimer is fully active kinetically when compared to untreated LRAT in membranes. Of course, it is not possible to determine whether membrane-associated LRAT functions catalytically as a dimer, a monomer, or both. The possibility that LRAT can be a functional homodimer in membranes, though, reconciles the known molecular mass with its apparent molecular size in membranes as determined by radiation inactivation analysis (*17*). Along these lines, it is interesting to note that a truncated version of LRAT (tLRAT) has been prepared [LRAT(31−195), 18.8 kDa], purified to homogeneity (unpublished experiments, D. Bok and A. Ruiz), and shown to form homodimers on SDS−PAGE. The extent of dimer formation is enhanced in the absence of reducing agents (W. J. Jahng and R. R. Rando). Therefore, native LRAT forms dimers in RPE membranes and can be cross-linked to form functional dimers in RPE membranes, and purified tLRAT can form dimers in solution.

It is tempting to speculate that the putative disulfide bond between LRAT monomers occurs between the same pair of cysteine residues involved in the bis-maleimide-mediated cross-linking step. This remains to be determined. Of the four cysteine residues of LRAT (C161, C168, C182, and C208), C161 and C168 are essential for catalytic activity, and are thus highly unlikely to be involved in any cross-linking process (*16*). Neither C182 nor C208 is essential for catalytic activity, and hence, either cysteine residue could be involved in disulfide bond formation (*16*). In this regard, C208 is likely to be part of a transmembrane segment at the C-terminus of LRAT (Scheme 2), and is considered a prime candidate for the cysteine residue involved in cross-link formation. It is also interesting to note that solubilized LRAT cannot be readily cross-linked by the bis-maleimides. Ongoing studies on LRAT mutants will determine which cysteine residue is involved in monomer cross-linking.

An important aspect of this is that LRAT is apparently not mechanistically related to other enzymes, even enzymes such as LCAT, which catalyze similar chemical reactions

(*12*−*14*). This can also be concluded from the fact that the sequence of LRAT is not homologous to that of any other known enzymes (*4*, *11*). The idea that LRAT might function catalytically as a dimer clearly has an impact on mechanistic and structural hypotheses about the enzyme. Of central concern here is whether the LRAT monomer is catalyltically active and the associated question of whether the LRAT dimer has two functional active sites or one.

ACKNOWLEDGMENT

The technical assistance of Dr. Dong-Won Choo and Svetlana Shulga-Morskaya are greatly acknowledged. The anti-LRAT polyclonal antibody was a generous gift from Dr. Dean Bok of the University of California (Los Angeles, CA).

REFERENCES

1. MacDonald, P. N., and Ong, D. E. (1988) *J. Biol. Chem. 263*, 12478.
2. Barry, R. J., Cañada, F. J., and Rando, R. R. (1989) *J. Biol. Chem. 264*, 9231.
3. Saari, J. C., and Bredberg, D. L. (1989) *J. Biol. Chem. 25*, 6473.
4. Rando, R. R. (2001) *Chem. Rev. 101*, 1881.
5. Saari, J. C. (2000) *Invest. Ophthalmol. Visual Sci. 41*, 337.
6. Rando, R. R., Bernstein, P. S., and Barry, R. J. (1991) *Prog. Retinal Res. 10*, 161.
7. Bernstein, P. S., Law, W. C., and Rando, R. R. (1987) *J. Biol. Chem. 262*, 16848.
8. Shi, Y.-Q., Hubacek, I., and Rando, R. R. (1993) *Biochemistry 32*, 1257.
9. Trehan, A., Cañada, F. J., and Rando, R. R. (1990) *Biochemistry 29*, 309.
10. Shi, Y.-Q., Furuyoshi, S., Hubacek, T., and Rando, R. R. (1993) *Biochemistry 32*, 3077.
11. Ruiz, A., Winston, A., Lim, Y.-H., Gilbert, B. A., Rando, R. R., and Bok, D. J. (1999) *J. Biol. Chem. 274*, 3834.
12. Francone, O. L., and Fielding, C. J. (1991) *Biochemistry 30*, 10074.
13. Francone, O. L., and Fielding, C. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1716.
14. Jauhiainen, M., Yuan, W., Gelb, M. H., and Dolphin, P. J. (1989) *J. Biol. Chem. 264*, 1963.
15. Yang, C.-Y., Manoogian, D., Pao, Q., Lee, F.-S., Knapp, R. D., Gotto, A. M., Jr., and Pownall, H. J. (1987) *J. Biol. Chem. 262*, 3086.
16. Mondal, M. S., Ruiz, R., Bok, D., and Rando, R. R. (2000) *Biochemistry 39*, 5215.
17. Ross, A. C., and Kempner, E. S. (1993) *J. Lipid Res. 34*, 1201.
18. Laemmli, U. K. (1970) *Nature 227*, 680.
19. Brown, R. D., and Matthews, K. S. (1979) *J. Biol. Chem. 254*, 5128.

BI015710B