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Two histidine residues are essential for catalysis by lecithin retinol acyl transferase

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Abstract Lecithin retinol acyl transferase (LRAT) is a novel membrane bound enzyme that catalyzes the formation of retinyl esters from vitamin A and lecithin. The enzyme is both essential for vision and for the general mobilization of vitamin A. The sequence of LRAT defines it as a novel enzyme unrelated to any other protein of known function. LRAT possesses a catalytically essential active site cysteine residue. The enzyme also contains six histidine residues. It is shown here that two of these residues (H57 and H163) are essential for catalysis. A mechanistic hypothesis is presented to account for these observations. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Lecithin retinol acyl transferase; Vitamin A; Acyl transferase; Enzyme catalysis; Enzyme mechanism; Site-specific mutagenesis

1. Introduction

Lecithin retinol acyl transferase (LRAT) is a novel membrane bound enzyme central to the processing of vitamin A in the visual system and in peripheral tissues [1–5]. The enzyme catalyzes a reversible transesterification reaction involving vitamin A and phosphatidylcholine (lecithin) by a ping-pong mechanism [6] (Scheme 1). LRAT is central for vision because the product of LRAT processing, all-*trans*-retinyl esters, are substrates along the pathway to 11-*cis*-retinol (al), the chromophore of rhodopsin [7,8]. LRAT is also generally important for the formation of retinyl esters in general vitamin A storage and mobilization processes [3,4].

While LRAT has never been fully purified, it can be specifically affinity labeled with electrophilic reagents, which led to its identification and partial sequencing [9]. The complete sequence of human LRAT has been determined by cloning based on the identified sequences [10]. The 25.3 kDa (calculated) protein is entirely novel, and showed only limited homology to a small group of proteins of unknown function [10]. The mechanism of action of LRAT is likely to be interesting, in as much as LRAT does not show any homology to enzymes that catalyze similar reactions, such as lecithin cholesterol acyl transferase [11–13]. This latter enzyme, which is typical of serine acyltransferases, catalyzes the reversible acylation of cholesterol with lecithin and is essential for cholesterol mobilization [11–13]. LRAT, of course, shows none of the motifs expected in a serine enzyme. In fact, LRAT is not inhibited by moderate concentrations of phenylmethylsulfonyl fluoride, a reagent known to inhibit serine-dependent enzymes [14]. LRAT, however, is readily inactivated by thiol-directed enzyme inhibiting reagents [9], and it has been shown to possess an active site cysteine (C161) essential for catalysis [15].

Typically, thiol-dependent enzymes in the protease/lipase category, as LRAT is, possess additional active site residues essential for catalysis. In the case of thiol proteases, the catalytic triad involves an essential histidine residue and an essential aspartate [16]. Sometimes an asparagine residue substitutes for the aspartate residue [17]. Consequently, it was of interest to probe the possible functional roles of the six histidine residues of LRAT. Histidine residues are typically involved in protonation–deprotonation reactions in biochemistry because histidine possesses a pK_A of close to 7. In the present studies, six Q/H mutations in LRAT were prepared and assessed for catalytic function. Of the six H residues, only H57 and H163 proved to be essential for catalysis. The remaining four Q/H mutants showed activities approximating or exceeding wild-type (WT) LRAT activity. A mechanism involving the two histidines in catalysis is presented.

2. Materials and methods

2.1. Materials

Frozen bovine eye cups were obtained from W.L. Lawson Co. (Lincoln, NE, USA). [11,12-^{3}H,^{3}H]All-*trans*-retinol (31.4 Ci/mmol) was obtained from NEN Life Sciences. L-α-Dipalmitoylphosphatidylcholine (DPPC), bovine serum albumin (BSA), dithiothreitol (DTT), all-*trans*-retinyl palmitate, and protein A were from Sigma. Triton X-100 was from Calbiochem. High performance liquid chromatography (HPLC) grade solvents were obtained from J.T. Baker. The enhanced chemiluminescence Western blotting kit was from Amersham. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Solubilization of WT and mutant LRAT activity in detergents. The WT and mutant LRAT proteins were solubilized in detergent containing Tris–HCl buffer. Briefly, the procedure involved incubation of 5 μl of cell suspension with 0.4% TX-100 in a 100 mM Tris–HCl buffer medium at pH 8.3. The cell suspensions containing detergent were gently agitated with the help of a continuous rotating device. All incubations were performed at 4°C for at least 2 h. Finally, the reaction mixture was centrifuged at 2000 rpm at 4°C for 2–5 min. The supernatant containing solubilized LRAT protein was collected and used for further kinetic analysis. The solubilized protein retains full activity for several days when stored at −70°C.

2.2.2. Steady-state kinetics. The kinetic experiments for LRAT

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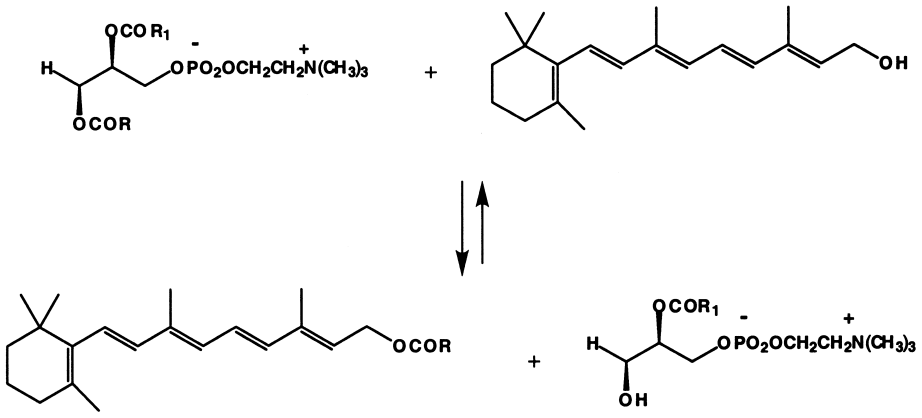


Scheme 1. LRAT catalyzed esterification of vitamin A.

activity were performed using solubilized enzyme by monitoring the formation of LRAT catalyzed retinyl esters from all-*trans*-retinol and DPPC. All procedures for the kinetic experiments were performed following the previously reported method [15] with slight modifications of reaction conditions. The incubation and reactions were carried out under dim red light at room temperature. All-*trans*-retinol [11,12-$^3H_2$] was dissolved in dimethyl sulfoxide (DMSO) in a range of concentrations to make various stock solutions. 1 μl of these stock solutions in DMSO was added to a 100 μl total buffer volume containing membrane suspension, DPPC, BSA, EDTA and DTT. The final concentrations of the components in the reaction mixtures are 100 mM Tris–HCl at pH 8.3, 220 μM DPPC, 0.6% BSA, 1 mM EDTA, 2 mM DTT, a varying concentration of all-*trans*-retinol and a desired amount of protein. The reactions were performed at room temperature for a certain length of time that depends on the specific activity of WT and mutant LRATs. The reactions were quenched by the addition of 500 μl of methanol, 100 μl of H_2O was added, and 500 μl hexane (containing butylated hydroxy toluene at 1 mg/ml) was used to extract the retinoids. The retinoids were analyzed on a PVA-Sil (250×4.0, YMC) HPLC column using 7% dioxane in hexane as eluant at a flow rate of 1.5 ml/min. Radioactivity was counted with an online Berthold LB 506-C HPLC radioactivity monitor interfaced with an IBM computer. All experiments were performed in duplicate. The average values from these measurements were used for analysis.

The initial rates of formation of retinyl ester products (catalyzed by WT and mutant proteins) were normalized with respect to the relative expression amounts of LRAT proteins as observed from the Western blotting analysis of solubilized WT and mutant samples. Therefore, these reaction rates provide a convenient method to compare the relative activity of WT and other mutant proteins. Protein concentrations were determined using Bradford method using a Bio-Rad protein assay kit.

2.2.3. Site-directed mutagenesis. Site-directed mutagenesis was performed by overlap extension using the polymerase chain reaction (PCR) procedure originally described by Ho et al. [18]. This methodology was previously used for the generation of several cysteine-LRAT mutants [15].

In order to mutate the six different histidines (H) in the LRAT polypeptide into glutamine (Q), different sets of forward (F) and reverse (R) primers were designed from the published human LRAT sequence [10] as shown in Table 1. A WT human LRAT construct described previously [10] was used as a positive control for the induction of LRAT activity in transfection experiments and as a template for the generation of mutants by PCR amplification. PCR experiments were performed according to the vendor's instructions provided in the GeneAmp PCR core reagents (Perkin-Elmer) and the amplifications were carried out in a Robocycler 40 apparatus (Stratagene, La Jolla, CA, USA).

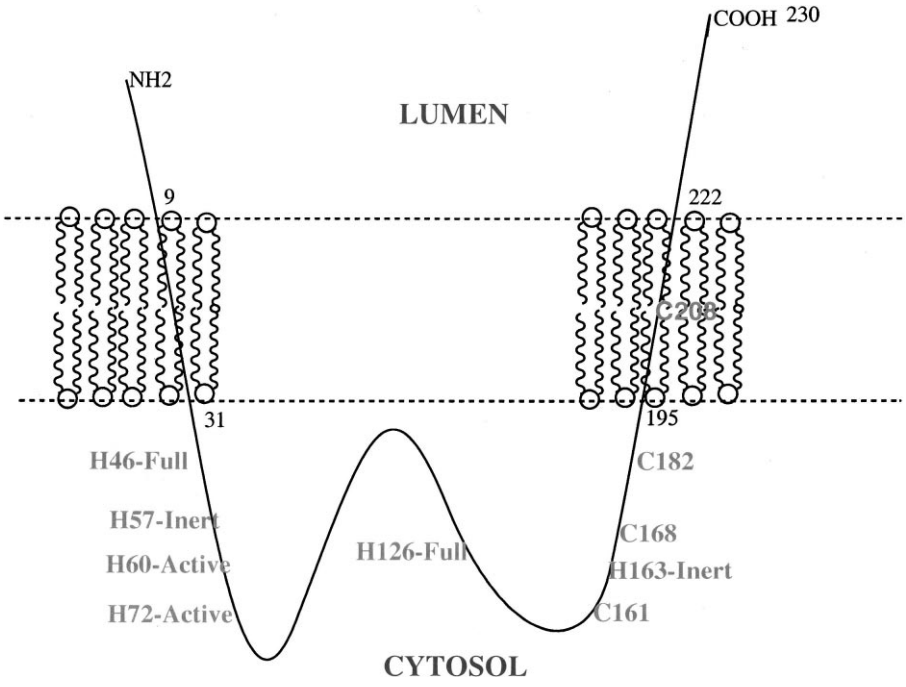
The final PCR product containing the single mutation was subcloned into the pCR II vector and subsequently inserted in the unique *Eco*RI site of the mammalian expression vector pcDNA3 (Invitrogen) where expression is driven by the human cytomegalovirus promoter. The presence of the insertion was confirmed by *Eco*RI digestion and its correct orientation determined by double-stranded DNA sequencing with the dideoxy chain termination method [19] using the Sequenase 2.0 sequencing system (US Biochemical Corp.).

2.2.4. Transfection of HEK-293T cells. A human embryonic kidney cell line (HEK-293T) was used to study the induction of LRAT activity after transfection with LRAT or LRAT mutant DNA. Maintenance and processing of the HEK-293T cells has been previously described in detail [20]. HEK-293T cells grown to around 80% confluency in 100 mm culture plates were transfected with 30 μg/plate of plasmid DNA using calcium phosphate as a vehicle, according to Kingston [21]. After 24 h of incubation transfected cells were collected

Table 1
Description of oligonucleotide primers utilized for the generation of LRAT mutants

LRAT primers	Primer sequences	Position
5′ UTR	F5′tttaccttcctctctcctcagcggc3′	−59 to −35
mut H/Q 46	F5′agctctttc**cag**cgaggcgac3′	127–147
	R5′gtcgcctcg**ctg**gaaagagct3′	127–147
Mut H/Q 57	F5′ccccggacc**cag**ctgacccac3′	160–180
	R5′gtgggtcag**ctg**ggtccgggg3′	160–180
Mut H/Q 60	F5′cacctgacc**cag**tatggcatc3′	169–189
	R5′gatgccata**ctg**ggtcaggtg3′	169–189
Mut H/Q 72	F5′cgtgttgcc**cag**atgatgccc3′	205–225
	R5′gggcatcat**ctg**ggcaacacg3′	205–225
Mut H/Q 126	F5′ctggtcaat**cag**ctggacgag3′	367–387
	R5′ctcgtccag**ctg**attgaccag3′	367–387
Mut H/Q 163	F5′aactgcgag**cag**ttcgtgacc3′	478–498
	R5′ggtcacgaa**ctg**ctcgcagtt3′	478–498
3′ UTR	R5′tacagaatacacacactgacatgggg3′	702–727

Triplet nucleotide sets replacing the histidine cac with glutamine cag are shown in bold. The nucleotide position of primer sequences is numbered from the atg initiation site.

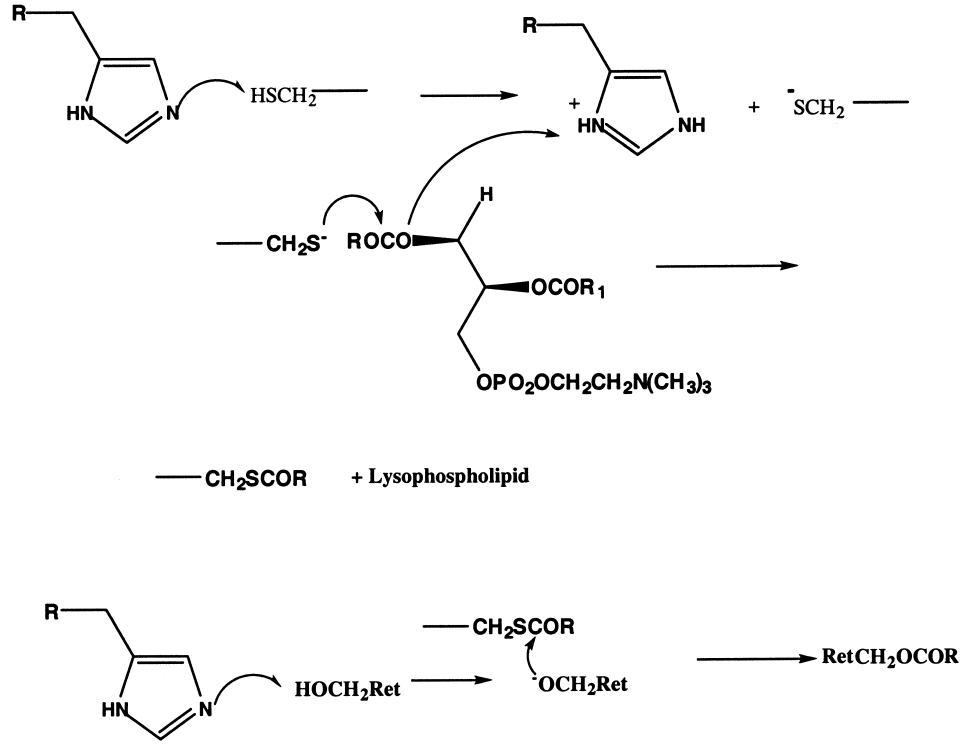


Scheme 2. Mutagenesis studies on LRAT.

in Hanks' balanced salt solution (Irvine Scientific, Irvine, CA, USA) and pelleted in 15 ml tubes at $1000 \times g$ for 5 min at 4°C. Cell pellets were frozen at −80°C until further analysis. A vector with no LRAT insert was used as a negative control. Western blot analysis and a peptide polyclonal antibody against LRAT described previously [10], were used for the evaluation of the expression of LRAT protein in transfected cells compared to extracts from RPE cells as a positive control.

3. Results and discussion

LRAT contains six histidine residues (H46, H57, H60, H72, H126 and H163), and all of these histidine residues are predicted to be in cytoplasmic regions of the integral membrane protein [10]. The LRAT protein has already been expressed in HEK-293 cells, which are otherwise devoid of LRAT-like ac-



Scheme 3. Involvement of histidine residues in LRAT catalysis.

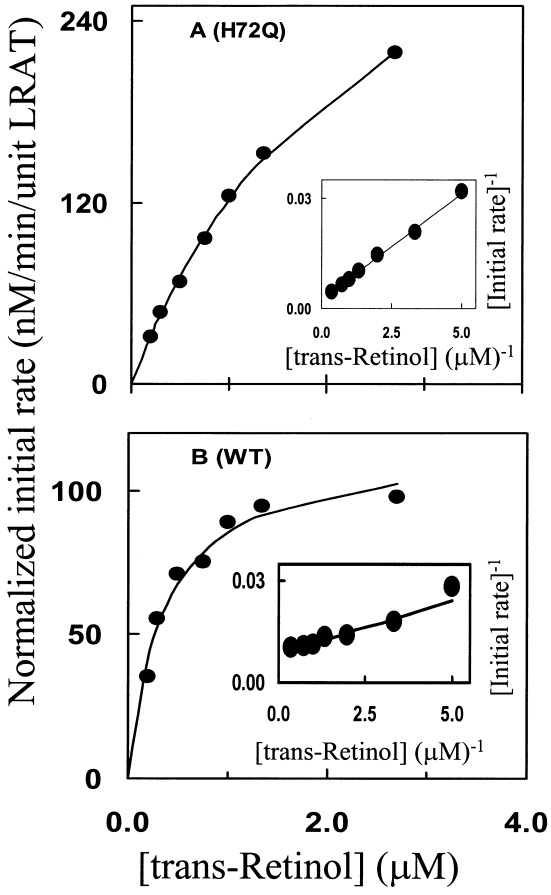


Fig. 1. Steady-state kinetic plots for the formation of retinyl ester products by H72Q (A) and WT (B) LRAT protein. The initial rates of the formation of ester products were measured at increasing concentrations of all-*trans*-retinol. The DPPC concentration was 220 μM. The symbols represent the mean value of duplicate determinations. The errors in the experimental data are in the range of 4–9%. The Lineweaver–Burk plots of these initial rate experiments are shown as insets to the figure.

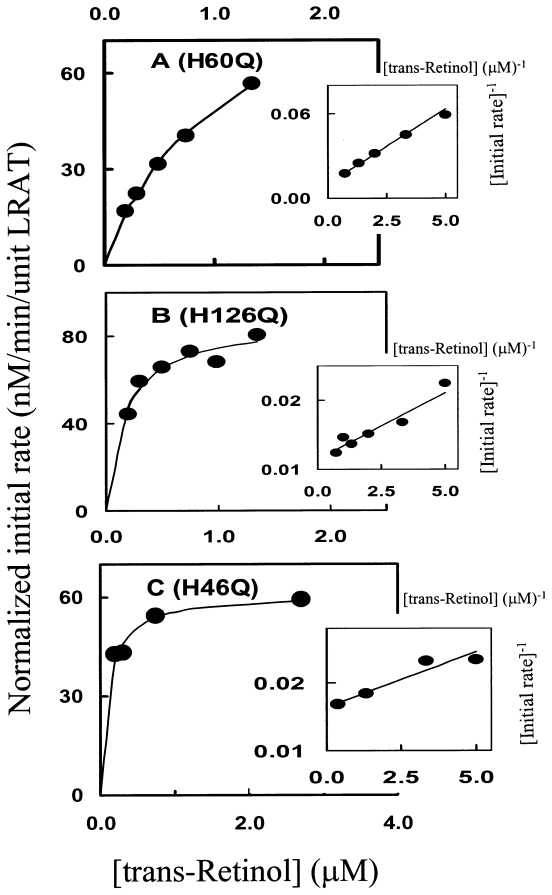


Fig. 2. Steady-state kinetic plots for the formation of retinyl ester products by H60Q (A), H126Q (B) and H46Q (C) mutant LRAT proteins. The initial rates of the formation of ester products were measured at increasing concentrations of all-*trans*-retinol. The DPPC concentration was 220 μM. The symbols represent the mean value of duplicate determinations. The errors in the experimental data are in the range of 4–9%. The Lineweaver–Burk plots of these initial rate experiments are shown as insets to the figure.

tivity [10]. Previously, a series of mutations at the cysteine residues of LRAT were prepared and studied [15]. In the case of the histidine residues under consideration here, Q for H substitutions for each of the six histidine residues were prepared and expressed in HEK-293T cells. Yields of the expressed proteins appear to be greater in HEK-293T cells as compared to the HEK-293 variant. An illustration of LRAT and the positions of the six histidine residues as well as the catalytically essential C161 residue is shown in Scheme 2. The series of the six mutants studied are H72Q, H60Q, H126Q, H46Q, H57Q and H163Q. In Fig. 1 are shown data for WT LRAT and the H72Q mutant.

Table 2
The kinetic parameters for WT and mutant LRAT proteins at room temperature

Protein	K_M (μM)	Normalized V_{max} (nM/min/unit LRAT)	V_{max} (μM/min/mg of protein)
WT	0.36 ± 0.06	116 ± 6	23.2 ± 1.2
H46Q	0.1 ± 0.02	61 ± 5	8.9 ± 0.7
H60Q	1.08 ± 0.11	101 ± 6	5.0 ± 0.3
H72Q	2.4 ± 0.2	416 ± 17	31.3 ± 1.3
H126Q	0.167 ± 0.04	87 ± 5	11.4 ± 0.6

The experiments are performed as described in Section 2.

In Fig. 2 are shown data for mutants H60Q, H126Q and H46Q. The data are summarized for these active mutants in Table 2. Clearly, all of these mutants are active and, in fact, H72Q is substantially more active than WT LRAT. Therefore, H72, H60, H126 and H46 can be removed from consideration as being either catalytically or structurally important for LRAT action. The same is not true for H57 and H163, because both H57Q and H163Q proved to be catalytically inert. The LRAT activities of these two mutants were indistinguishable from that of H293 cells transfected with empty vector and are thus inert.

The experiments described here rule out the possibilities that H72Q, H60Q, H126Q and H46Q are catalytically important. H57 and H163, on the other hand, are important for catalytic function. It is interesting to note that only these two histidine residues are found in regions of LRAT predicted to be α-helical. Moreover, C161 is the catalytically active thiol group of LRAT. This cysteine residue is then quite close spatially to H163. Since no structural information is available on LRAT, the relative spatial orientation of H57 and CC161 are unknown at present but, of course, they could be nearby in

space. A possible mechanism involving the two histidine residues is provided in Scheme 3. The ping-pong kinetic mechanism for LRAT indicates that a *sn*-1 acyl moiety of lecithin is first transferred to an active site nucleophile of LRAT, probably C161 [15]. In ensuing steps, the lysolecithin leaves and vitamin A is bound. Finally, the acyl moiety of the acyl–enzyme intermediate is transferred to the vitamin A, generating the retinyl ester [6]. Base catalysis is required in this kind of mechanism, and the putative involvement of the two histidine residues in base catalysis is shown in Scheme 3. One of the histidine residues could act as a base to generate the thiolate anion in the first step, which then reacts as the nucleophile attacking lecithin and generating the thioacyl intermediate. In step two, the second histidine residue could act as a general base to help abstract the HO proton of vitamin A, thus increasing its nucleophilicity and enhancing its ability to attack the acyl enzyme intermediate (Scheme 3). The validity of this hypothesis is currently being tested experimentally.

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