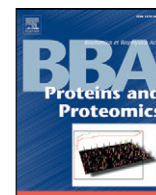
Biochimica et Biophysica Acta 1844 (2014) 1128–1136

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap



Enzymatic activity of Lecithin:retinol acyltransferase: A thermostable and highly active enzyme with a likely mode of interfacial activation

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ARTICLE INFO

Article history:
Received 29 January 2014
Received in revised form 21 February 2014
Accepted 25 February 2014
Available online 5 March 2014

Keywords:
Lecithin:retinol acyltransferase
Truncated LRAT
Visual cycle
Enzymatic activity
Retinol
Thermostability

ABSTRACT

Lecithin:retinol acyltransferase (LRAT) plays a major role in the vertebrate visual cycle. Indeed, it is responsible for the esterification of all-trans retinol into all-trans retinyl esters, which can then be stored in microsomes or further metabolized to produce the chromophore of rhodopsin. In the present study, a detailed characterization of the enzymatic properties of truncated LRAT (tLRAT) has been achieved using *in vitro* assay conditions. A much larger tLRAT activity has been obtained compared to previous reports and to an enzyme with a similar activity. In addition, tLRAT is able to hydrolyze phospholipids bearing different chain lengths with a preference for micellar aggregated substrates. It therefore presents an *interfacial activation* property, which is typical of classical phospholipases. Furthermore, given that stability is a very important quality of an enzyme, the influence of different parameters on the activity and stability of tLRAT has thus been studied in detail. For example, storage buffer has a strong effect on tLRAT activity and high enzyme stability has been observed at room temperature. The thermostability of tLRAT has also been investigated using circular dichroism and infrared spectroscopy. A decrease in the activity of tLRAT was observed beyond 70 °C, accompanied by a modification of its secondary structure, i.e. a decrease of its α-helical content and the appearance of unordered structures and aggregated β-sheets. Nevertheless, residual activity could still be observed after heating tLRAT up to 100 °C. The results of this study highly improved our understanding of this enzyme.

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1. Introduction

Lecithin:retinol acyltransferase (LRAT; EC 2.3.1.135) is a very important enzyme of the visual cycle. It catalyzes the esterification of retinol into retinyl esters in the retinal pigment epithelium (RPE) as well as in other tissues including testis, liver, and intestine [1–4]. The amino acid sequence of LRAT does not show any homology to enzymes that catalyze similar reactions, such as lecithin cholesterol acyltransferase (LCAT) [5,6], and is not related either to any protein of known function. LRAT was thus described as the founder member of a new class of Cys-His enzymes of unknown function [7–9] which includes class II tumor suppressors and a group of putative viral proteases [10–13]. The enzymatic reaction catalyzed by LRAT occurs in three steps: 1) it shows a phospholipase A1 activity leading to the hydrolysis of the *sn*-1 fatty acyl chain of phospholipids; 2) this fatty acyl chain is then used to self-acylate its Cys161; 3) this acyl group is finally transferred to all-*trans* retinol through an esterification reaction [1,2,4,14,15]. The formation of a thioester intermediate resulting from the addition of an acyl chain on Cys161 has clearly been demonstrated by mass spectrometry after incubation of tLRAT with phosphatidylcholine substrates [14]. This esterified form of retinol can thereby be accumulated in microsomes for storage, or hydrolyzed and isomerized by RPE65 to form 11-*cis*-retinol [16,17] which is then further metabolized to produce the chromophore of rhodopsin (for a review, see [7,18–22]).

The primary sequence of LRAT is made of 230 amino acids with a calculated mass of 25.3 kDa [8]. This sequence suggests the existence of N- and C-terminal hydrophobic segments at positions 9–31 and 195–222, respectively [8]. Only the C-terminal transmembrane domain has been suggested to be essential for membrane targeting [23]. However, these two individual hydrophobic segments were shown to bear the same

Abbreviations: BSA, bovine serum albumin; cmc, critical micellar concentration; DHPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; DMF, dimethylformamide; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; LRAT, lecithin:retinol acyltransferase; tLRAT, truncated form of LRAT; MES, 2-(*N*-morpholino)ethanesulfonic acid; OG, octyl β-D-glucopyranoside; RPE, retinal pigment epithelium; SDS, sodium dodecyl sulfate

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http://dx.doi.org/10.1016/j.bbapap.2014.02.022
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α-helical secondary structure and orientation in model membranes [24]. In every instance, full-length LRAT could not yet be overexpressed in *E. coli* [25] and expression in HEK (human embryonic kidney) cells has only led to a partially purified form of the protein [8], probably due to the very hydrophobic behavior of the enzyme termini [26]. A recombinant truncated form of LRAT (tLRAT) (amino acids 31–196), whose N- and C-terminal hydrophobic segments have been removed, has been produced [25]. The three essential residues forming the catalytic triad of LRAT (H60, C161 and Y154) are located within the sequence of this truncated enzyme [27]. tLRAT could thus be used to characterize the enzymatic properties of this enzyme. However, a detergent is required to achieve water-solubility of tLRAT [25] and enzymatic assays resulted in the measurement of a very low activity of this enzyme [9,25,27] compared to another enzyme having a similar activity (LCAT) [28]. Consequently, the enzymatic and biochemical properties of tLRAT must be thoroughly characterized using more appropriate experimental conditions. Moreover, thermal and time-dependent stability of tLRAT must be assayed because activity and stability are the most important qualities of enzymes [29,30], and also since the more general relationship between these properties and protein structure is still not well understood [29,30].

The present study was thus undertaken to perform a detailed characterization of the enzymatic and biochemical properties as well as of the thermal and time-dependent stability of tLRAT using in vitro assay conditions. The enzymatic properties of tLRAT and the effect of different parameters on its activity have been studied in detail, such as the usefulness of different detergents, the levels of the substrates, the pH and temperature of the enzymatic assay, the chain length selectivity of the phospholipid substrate, the content of BSA in the reaction mixture as well as the importance of the storage buffer and storage temperature and the influence of time on the activity of this enzyme. Furthermore, the thermal stability of tLRAT enzyme activity has been characterized and its related structural modifications were determined using circular dichroism and infrared spectroscopy.

2. Experimental procedures

2.1. Materials

The expression vector pET11a and the *E. coli* BL21(DE3) pLysS cells were from Novagen (Madison, WI, USA). 1,2-Diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) and all additional phospholipids used in this study were from Avanti Polar Lipids (Alabaster, AL, USA). N-lauroylsarcosine, sodium cholate, CHAPSO, dimethylformamide (DMF), dithiothreitol (DTT), globulin-free bovine serum albumin (BSA), MES and citrate buffers were from Sigma-Aldrich (St-Louis, MO, USA) whereas carbonate buffer and Triton X-100 were from Fisher Scientific (Montreal, Canada). Octyl β-D-glucopyranoside and n-dodecyl-beta-D-maltoside were from Calbiochem (San Diego, USA). Sodium dodecyl sulfate (SDS), hexane, methanol, ethyl acetate and Tris–HCl were from Laboratoire MAT (Quebec, Canada). Econo-Pac® 10DG and His-Trap columns were respectively from Bio-Rad (Hercules, CA, USA) and GE Healthcare (Piscataway, NJ, USA). All experiments were performed at least in triplicate with different enzyme preparations and the average value was used for the analysis of the data.

2.2. Cloning, expression and purification of tLRAT

tLRAT cDNA was cloned into the pET11a vector, overexpressed in *E. coli* Bl21(DE3) pLysS and purified using His-Trap columns as previously described [31]. In order to perform enzymatic assays in different experimental conditions, the elution buffer was changed to citrate (10 mM, pH 6, 0.05% SDS), MES (10 mM, pH 6, 0.05% SDS) phosphate (10 mM, pH 7.4, 0.05% SDS), Tris–HCl (10 mM, pH 8, 0.05% SDS) or carbonate (10 mM, pH 9.2, 0.05% SDS) buffers using an Econo-Pac® 10DG column.

2.3. Enzymatic assays of tLRAT

A reliable method was conceived to determine the enzyme activity of LRAT in order to characterize its biochemical properties and thermal stability in details. Activity assays have been reported with purified tLRAT produced in *E. coli* [9,14,23,25,27]. However, these experiments performed using water-insoluble substrates (phospholipid and all-*trans* retinol) resulted in values of maximum tLRAT activity which varied significantly from 3.4 [25] to 16.4 [9] and 42 [27] mmol of retinyl ester/min per mol of tLRAT and were much lower than that of LCAT (30,860 mmol of retinyl ester/min per mol of enzyme) (see Table 1 and Section 3.3). Therefore, a protocol has been devised to optimize enzymatic activity of tLRAT using a water-soluble phospholipid (DHPC) acting both as a substrate and as a detergent to solubilize the retinol substrate. The activity of tLRAT was then determined by monitoring the formation of all-trans retinyl heptanoate from all-trans retinol and DHPC. A stock solution of all-*trans* retinol was prepared in DMF. Then, 2.5 μL from this solution was added to a reaction mixture containing 1.25×10^{-2} μg tLRAT, 1% BSA, 6.25 mM DHPC and 1 mM DTT in a total volume of 250 μL to achieve a final concentration of 500 μM retinol. These particular concentrations are very important to obtain a maximum tLRAT activity. Indeed, after many trials and errors, the DHPC:retinol molar ratio was found to be very critical. It should be equal to at least 12.5:1 to allow proper solubilization of retinol by DHPC. In addition, a DHPC:tLRAT molar ratio of 4.2×10^6:1 should not be exceeded, otherwise a large decrease in tLRAT activity occurs. Unless otherwise stated, the tLRAT enzymatic reaction was measured at 20 °C under dim red light using dark Eppendorf tubes under stirring at 400 rpm using the Thermomixer R (Eppendorf). The reaction was then quenched after 15 min by mixing 100 μL of the reaction mixture with 200 μL methanol. The retinoids were extracted by adding 300 μL hexane to the latter mixture and vortexing vigorously for 30 s. Then, 50 μL of this retinoid extract was analyzed by HPLC (Hewlett-Packard 1100 series or a Shimadzu Prominence Modular) equipped with a diode array detector

Table 1
Comparison between the enzymatic properties of tLRAT and tLCAT reported in different studies.

Enzyme	V_{max} (mol of retinyl ester or cholesteryl ester/min per mol of enzyme)	Km (μM)	Catalytic efficiency (M^{-1} s^{-1})	Kcat (s^{-1})
tLRAT[a]	2426 ± 216	55 ± 16	7.3×10^5	40.4
tLRAT[b]	0.0034	0.24	235	5.6×10^{-5}
tLRAT[c]	0.0164	1.67	164	2.7×10^{-4}
tLRAT[d]	0.042	N.A.	N.A.	7×10^{-4}
tLCAT[e]	3.6	3.4	1.76×10^4	0.06
tLCAT[f]	30.86	2.3	2.57×10^5	0.514

[a] Present study.
[b] Bok et al. [25].
[c] Jahng et al. [9].
[d] Xue et al. [27].
[e] Adimoolam et al. [39].
[f] Chisholm et al. [28].

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