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Lecithin-cholesterol acyltransferase (LCAT) as a plasma glycoprotein: an overview

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This review is dedicated to Peter Dolphin, a good friend and valued colleague.

Abstract

This article reviews recent major efforts towards understanding the importance of carbohydrate chains for the physiological functioning of lecithin-cholesterol acyltransferase (LCAT), the plasma enzyme which esterifies cholesterol. The assembly of oligosaccharide chains in protein backbones is the most extensive of all the post-translational modifications, and can play a crucial role in protein folding, oligomer assembly and secretion, in regulating biological activity, as well as in clearance of glycoproteins from the bloodstream. Here, we describe modifications in LCAT-linked carbohydrate structures, arising from site-directed mutagenesis or from use of drugs and specific enzymes, which modify either the structure or the assembly of LCAT glycans, and evaluate how these help define their involvement in and importance to enzyme secretion, stability and activity.

Keywords: Cholesterol; Lipoproteins; N-glycosylation; Serum glycoprotein; Site-directed mutagenesis

1. Introduction

1.1. The LCAT reaction

Lecithin-cholesterol acyltransferase (EC 2.3.1.43; LCAT) is a plasma glycoprotein with a high content of carbohydrate residues, approx. one-quarter of its mass of 65,000 Da (Chong, Hara, Thompson, & Lacko, 1983; Chung, Abano, Fless, & Scanu, 1979; Lima, Harry, McIntyre, Owen, & Chaves, 1996). Like most plasma glycoproteins, LCAT is synthesized mainly in the liver, as demonstrated by: (a) its appearance in rat liver perfusates (Osuga & Portman, 1971; Soler-Argilaga, Russel, Goh, & Heimberg, 1977); its secretion by isolated rat hepatocytes (Nordby, Berg, Nilsson, & Norum, 1976); and its absence in isolated perfused rabbit liver treated with colchicine to disrupt the secretory process (De Parscau & Fielding, 1984); (b) its low activity in plasma of patients with liver disease (Calandra, Martin, & McIntyre, 1971); and (c) its secretion into the culture medium by the human hepatoblastoma cell line, Hep G2 (Chen, Forte, Cahoon, Thrift, & Albers, 1986; Erickson & Fielding, 1986; Lima, McIntyre, & Owen, 1988a; Lima, McIntyre, & Owen, 1988b). In humans, rabbits, rats and mice, LCAT gene expression is found predominantly in the liver (McLean et al., 1986; Murata, Maeda, Yoshino, & Kasuga, 1996; Tata et al., 1987). Noteworthy is the presence of LCAT mRNA, at appreciable levels, in brain and testis (Smith, Lawn, & Wilcox, 1990; Warden et al., 1989). LCAT measurement is

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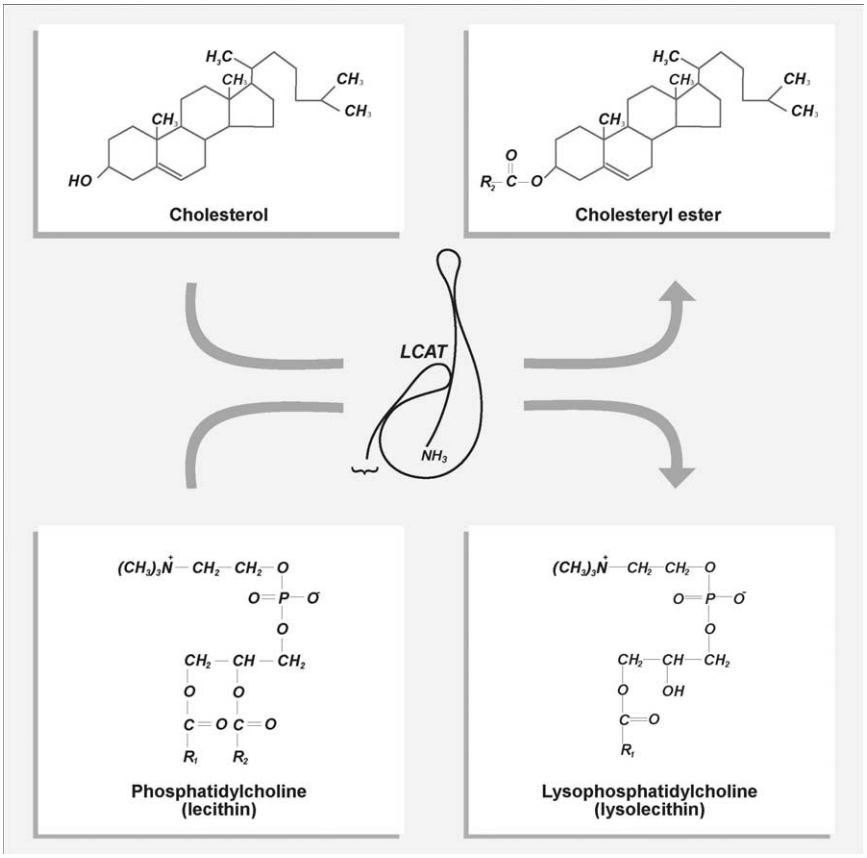

Fig. 1. Reaction catalyzed by lecithin:cholesterol acyltransferase. A schematic representation of the tertiary structure of LCAT, based on the model proposed by Yang et al. (1987), is placed between the arrows.

more sensitive than conventional liver function tests and is a good prognostic indicator in liver transplantation (Horton & Owen, 1990); its assay may also be useful in studies of hyperlipidemias.

The LCAT glycoprotein catalyzes formation of almost all plasma cholesteryl ester in humans (Fig. 1) by transferring a long-chain fatty acyl residue from the *sn*-2 position of phosphatidylcholine (lecithin) to the 3-β-hydroxyl group of cholesterol, producing lysophosphatidylcholine (lysolecithin) and cholesteryl ester (Glomset, 1968; Struck et al., 1978). The reaction occurs predominantly on plasma high-density lipoprotein (HDL), which contains its principal activator, apolipoprotein A-I (apoA-I) (Fielding & Fielding, 1995; Francone, Gurakar, & Fielding, 1989). Current data suggest that LCAT dissociates from HDL after each catalytic cycle (Adimoolam, Jin, Grabbe, Shieh, & Jonas, 1998). Glycated apoA-I from plasma of diabetic subjects, however, is deficient in activating LCAT (Calvo, 1997), while natural and site-directed mutations indicate that the helical repeat formed by residues 143–165 of apoA-I is essential for full activation (Cho et al., 2001; Roosbeck et al., 2001). Although the preferred natural substrate for LCAT is HDL, the enzyme has demonstrable activity with low-density lipoproteins (LDL) (Barter, Hopkins, & Gorjatschko, 1984; Knipping et al., 1986). The presence of oxidized LDL or phospholipid hydroperoxides can reduce the enzyme activity (Bielicki & Forte, 1999).

LCAT activity was first reported in human plasma by Glomset (1962) and subsequently enzyme activity of this type was described in plasma of many other mammals (Chen & Albers, 1983, 1985; Lacko, Rutenberg, & Soloff, 1974; Lima, Sena, Stewart, Owen, & Dolphin, 1998), birds (Gillett, Lima-Filho, Lima-Filho, & Lima, 1984), fish (Gillett, 1978) and reptiles (Gillett, Lima, Costa, & Sibrian, 1979). In the absence of an appropriate sterol acceptor LCAT exhibits a phospholipase A_2 or esterase activity, liberating free fatty acids (Aron, Jones, & Fielding, 1978). Alternatively, LCAT may transfer fatty acyl groups to several alcohols, including a variety of sterols (Piran & Nishida, 1979; Zhou & Dolphin, 1995). On the other hand, when lysolecithin concentrations are elevated, LCAT catalyzes the reacylation of lysolecithin producing lecithin on LDL; this enzyme activity has been referred to as lysolecithin acyltransferase (LAT) (Dolphin, 1992; Fielding, Shore, & Fielding, 1972; Subbaiah & Bagdade, 1978). LCAT-catalyzed cholesteryl ester hydrolysis followed by re-esterification of a second cholesterol molecule has also been reported (Sorci-Thomas, Babiak, & Rudel, 1990) and was termed the cholesterol acyltransferase or CAT reaction by Jauhianen and Dolphin (1990).

1.2. LCAT structure

Although the complete tertiary structure of LCAT is unknown, the recent application by Peelman et al. (1998) of threading alignments to structural homology data for LCAT with human pancreatic lipase and *Candida antarctica* lipase, has demonstrated that LCAT belongs to the α/β hydrolase fold family. The central domain of LCAT was predicted to consist of seven conserved parallel beta-strands, which are connected by four α-helices and separated by loops containing the catalytic residues. By analogy with other serine-type esterases the putative active site of LCAT comprises the sequence Ile-Gly-His-Ser^{181}-Leu-Gly of LCAT primary structure, further studies have confirmed the importance of Ser^{181} for LCAT activity (Farooqui, Wohl, Kezdy, & Scanu, 1988; Francone, Evangelista, & Fielding, 1996; Francone & Fielding, 1991; Jauhiainen & Dolphin, 1986; Jauhiainen, Ridgway, & Dolphin, 1987; Yang et al., 1987). The existence of a putative Ser-His-Asp 'catalytic triad' in LCAT was first proposed by Jauhiainen and Dolphin (1990). Subsequent site-directed mutagenesis and modeling studies by Peelman et al. (1998) provided strong evidence that His^{377} and Asp^{345} formed such a triad with Ser^{181} which, based on previous studies (Jauhianen & Dolphin, 1986), would form a fatty-acyl-Serine acyl-enzyme intermediate, following lecithin cleavage and prior to donation of the fatty acid to the sterol acyl-acceptor. Consistent with other enzymes of this type, Peelman et al. (1998) identified Phe^{103} and Leu^{182} as the putative oxyanion hole residues with residues 50–74 potentially forming a 'lid' domain, which uncovered the catalytic residues following substrate binding. Unfortunately the putative lid region could not be modeled due to the lack of homologous templates. Subsequent studies using site-directed mutagenesis and enzymic assays, together with additional modeling and alignment of LCAT residues with *Candida rugosa* lipase sequences, confirmed the importance of this region, especially the Trp^{61} which is critical for full catalytic activity (Peelman et al., 1999).

1.3. LCAT and lipoprotein metabolism

LCAT is an enzyme that plays an important role in the complex process of normal intravascular lipoprotein metabolism (Owen & McIntyre, 1982), since it is closely involved in the maturation and transformation of HDL particles. Considering that HDL is an acceptor for cholesterol effluxed from peripheral cells for eventual hepatic removal, LCAT is intimately associated with the process of reverse cholesterol transport (Fielding & Fielding, 1995; Fruchart & Duriez, 1998; Nishida, Kato, & Nishida, 1990; Owen, 1984; Rothblat et al., 1999). Amongst the HDL subclasses the preferred substrate for the LCAT reaction is the HDL_3 fraction (Schmitz, Assmann, & Melnik, 1982). The initial step of the LCAT reaction involves the transfer of cellular membrane cholesterol to a small subfraction of HDL that exhibits pre-β electrophoretic modility (Fielding & Fielding, 1995). The cholesterol is then esterified by LCAT, which essentially traps it in the HDL particle, as cholesteryl esters will not freely exchange with cell membrane cholesterol (Owen, 1984). A major portion of the cholesteryl ester generated by the LCAT reaction in plasma is subsequently transferred to acceptor particles, preferentially very-low-density lipoprotein (VLDL) and the light LDL subclass, LDL_1 (Guérin, Dolphin, & Chapman, 1994), by the plasma cholesteryl ester transfer protein (CETP) in exchange for triglycerides, which are transferred from VLDL and LDL to HDL (Albers, Tollefson, Chen, & Steinmetz, 1984; Bagdade, Wagner, Rudel, & Clarkson, 1995; Fruchart & Duriez, 1998; Rye & Barter, 1992). Thus, cholesterol esterification by LCAT contributes to the formation of the hydrophobic lipid core of HDL particles and helps to maintain the gradient in cholesterol concentration between cell membranes and plasma that is important for cholesterol efflux from peripheral tissues (Rothblat, Mahlberg, Johnson, & Philips, 1992).

2. Microheterogeneity and LCAT carbohydrate content

Glycosylation of proteins represents one of the most important post-translational events because of the universality of the phenomenon. Carbohydrate moieties are covalently associated with the polypeptide backbone of most proteins secreted by the liver, including the enzyme LCAT. Studies using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that purified LCAT migrates as a single band with an apparent M_r of about 65,000–68,000 (Chung et al., 1979; Doi & Nishida, 1981; Lima et al., 1996). Treatment of native human plasma LCAT with various endoglycosidases converts this band to a single band corresponding to the molecular weight of about 47,000. Sequencing of the human LCAT cDNA showed the presence of 416 amino acid residues with a calculated M_r of 47,089 Da (McLean et al., 1986).

In addition to genetic variants expressed as modifications in the polypeptide chain of a protein, almost all glycoproteins reveal another form of polymorphism associated with their carbohydrate residues. A given glycan located at a given amino acid in a glycoprotein, often presents a structural heterogeneity, termed microheterogeneity, which is produced by partial substitution of sugar residues on a similar core structure (Montreuil et al., 1994). Despite only a single band being observed upon SDS-PAGE of plasma LCAT, microheterogeneity has been reported in the LCAT molecule, as for most other glycoproteins. At least six isoforms of this enzyme have been found by isoelectric focusing studies on polyacrylamide gels, and their apparent isoelectric points range from pH 3.9–4.8 (Doi & Nishida, 1983; Holmquist & Bjellqvist, 1988; Utermann, Menzel, Adler, Dieker, & Weber, 1980). Abnormal LCAT isoforms

are also reported in patients with hepatosplenic schistosomiasis mansoni (Lima, Cannizzaro, & Owen, 1997).

3. The uncertain role of carbohydrate chains in plasma LCAT

3.1. General considerations

The exact biological function of the oligosaccharide chain bound to the polypeptide backbone of LCAT is unknown. In some cases the carbohydrate portion may improve the solubility of the protein in water (Hickman, Kulczycki, Lynch, & Kornfeld, 1977). This could be important for LCAT and other lipid-binding proteins because of the predominantly hydrophobic nature of their polypeptide chains. For some glycoproteins the oligosaccharide chains may be essential for achieving proper conformation, whereas in other cases the carbohydrates may be only a minor determinant of the conformation. Thus, the function of the carbohydrate chains linked to proteins on glycoproteins seems to depend upon the particular glycoprotein in question. In vitro modification of LCAT by removing the sialic acid residues through the action of neuraminidase is associated with a 60% increase in the transacylase activity of the enzyme (Doi & Nishida, 1983).

The following considerations are important in deciding upon the role of LCAT-bound carbohydrates in the activity and secretion of this glycoprotein. A possible function of oligosaccharide chains is to provide a signal for the molecule to be transferred to the cell surface prior to the secretory process (Elbein, 1987; Gebhart & Huddon, 1986; Olden et al., 1985), although not all secretory proteins are glycosylated, for example, albumin, the major secretory product of the liver. The use of specific inhibitors of protein glycosylation has provided conflicting results (Elbein, 1987; Gebhart & Huddon, 1986; Olden et al., 1985). These inhibitors can impair the intracellular transport and secretion of some glycoproteins such as apoE (Mazzone, Papagiannes, & Magner, 1986), lipoprotein lipase (Buscá et al., 1995; Masuno et al., 1991; Olivecrona, Chernick, Bengtsson-Olivecrona, Garrison, & Scow, 1987), IgM (Hickman et al., 1977) and cruzipain (Labiola, Cazzulo, & Parodi, 1995) suggesting that carbohydrates are important in this process. In the case of other glycoproteins, for example, IgA (Williamson et al., 1980), VLDL and transferrin (Struck, Suita, Lane, & Lennarz, 1978), the inhibition of glycosylation does not alter their normal secretion. Adding to the confusion of the role of carbohydrates for glycoprotein secretion, the function of oligosaccharides in binding substrate and in maintaining the active site of glycoprotein enzymes is unclear. Thus, studies with inhibitors of glycosylation, or with either chemical or enzymatic agents that deglycosylate glycoproteins, have shown that enzymatic activity can be lost, as noted for lipoprotein lipase (Amri, Vannier, Etienne, & Ailhaud, 1986; Simsolo, Ong, & Kern, 1992), or retained as shown for invertase (Chu, Trimble, & Maley, 1978), or actually increased, as when sialic acid is removed from LCAT (Doi & Nishida, 1983). In order to reconcile these differences it was suggested that protein folding and conformation might be a critical factor for both secretion and maintenance of catalytic activity (Gibson, Kornfeld, & Schlesinger, 1980; Paulson, 1989).

3.2. Use of drugs and enzymes

Apart from a few studies by site-directed mutagenesis on the four known glycosylation sites of LCAT very little data has been published on the potential role of the LCAT carbohydrate moiety on the secretion, stability, or function of the enzyme protein. One of the first attempts to evaluate the role of oligosaccharide chains on LCAT employed several glycosidases or specific inhibitors of glycoprotein assembly and was undertaken by Lima et al. (1988a,b) in Hep G2 cells, a culture line which expresses LCAT (Chen et al., 1986; Erickson & Fielding, 1986; Lima, Harry, Owen, & McIntyre, 1987), as well as all the major enzymes of intracellular cholesterol metabolism (Brissette & Falstrault, 1992; Cohen et al., 1984; Craig, 1993; Hayashi et al., 1993; Horie et al., 1993). This was followed by the study of Collet and Fielding (1991) in Chinese hamster ovary cells stably transfected with the human LCAT gene. In these studies, the importance of Asn-linked oligosaccharide in the secretion and activity of LCAT was probed by using some of the more common drugs and glycosidases. Examples of drugs used include: tunicamycin, an antibiotic which blocks the biosynthesis of $Glc_3Man_9GlcNAc_3$ pyrophosphoryl-dolichol (Tkacz & Lampen, 1975) in the dolichol-linkage oligosaccharide pathway; monensin, a Na^+ ionophore (Tartakoff, 1983; Uchida, Smilowitz, & Tanzer, 1979) which acts on the intracellular processing of both O- and N-linked oligosaccharides with both variable and non-specific effects; and swainsonine, which inhibits mannosidase I and II and thus prevents the trimming of uncapped high-mannose chains in the endoplasmic reticulum and Golgi compartments (Fuhrmann, Bause, Legler, & Ploegh, 1984; Tulsiani, Harris, & Touster, 1982). Examples of enzymes used to release saccharide units are endo H (endo-β-*N*-acetylglucosaminidase H), which preferentially hydrolyzes *N*-glycans of the high mannose type; endo F (endoglycosidase F), which cleaves the chitobiose core of high mannose and biantennary complex carbohydrate chains, respectively; and *N*-glycanase (glycopeptide-*N*-glycosidase), an enzyme that cleaves N-linked oligosaccharides between asparagine and the first *N*-acetylglucosamine moiety on the carbohydrate chain (Tarentino & Plummere, 1994). LCAT activity was found to be reduced in cells that were incubated with tunicamycin (Collet & Fielding, 1991; Lima et al., 1988a,b), or with monensin (Lima, 1989). However, tunicamycin, monensin, and swainsonine amongst others, had little effect on LCAT assembly and

secretion in either Chinese hamster ovary cells (Collet & Fielding, 1991) or in Hep G2 cells (Lima, 1989).

It has also been suggested that carbohydrates may increase the survival of a protein by reducing its susceptibility to the action of proteolytic enzymes (Montreuil et al., 1994; Olden, Pratt, & Yamada, 1978); this may be important for LCAT which has a plasma half-life of about 4 days (Glomset, Assmann, Gjone, & Norum, 1995; Stokke, Fjeld, Kluge, & Skrede, 1974). A report from Ridgway and Dolphin (1985) suggests that the high plasma LCAT activity in hypercholesterolemic rats results primarily from an impairment in LCAT clearance rather than an increase in the enzyme production. There is evidence that the carbohydrate moieties of LCAT contribute to its prolonged survival in the circulation. A rapid disappearance of abnormally glycosylated LCAT from within cell culture media exposed to Hep G2 cells was reported by Lima (1989), perhaps reflecting a rapid uptake of LCAT by the asialoglycoprotein receptor of these cells (Schwartz, Fridovitch, & Lodish, 1982); alternatively, this may reflect a rapid non-specific degradation of the protein. Thus, although the carbohydrate moieties of LCAT seem to have little influence on LCAT secretion, they appear important for its enzymatic activity and for prolonging its plasma survival, as seen for other glycoproteins (Amri et al., 1986; Bartalena & Robbins, 1984; Bauer, Parent, & Olden, 1985; Chajek-Shaul et al., 1985; Lodish, Kong, Snider, & Strous, 1983).

4. Glycosylation sites

The cDNA for human (McLean et al., 1986; Tata et al., 1987), mouse (Warden et al., 1989), rat (Meroni, Malgaretti, Magnaghi, & Taramelli, 1990), baboon (Hixson et al., 1993), chicken (Hengstschlager-Ottnad, Kuchler, & Schneider, 1995) and rabbit (Murata et al., 1996) LCAT have been cloned and sequenced. Mature human LCAT contains 416 amino acid residues, in a single polypeptide chain (McLean et al., 1986) with an apparent M_r of 47,090 Da, which is similar to the apparent M_r of 45,000 Da deduced by Chung et al. (1979). The rat, mouse and human LCAT sequences share an 80% homology (Hill, Wang, & Pritchard, 1993a), while in comparison to human LCAT the rabbit homology is higher than 90% (Murata et al., 1996). Chicken LCAT, in contrast has only 73% amino acid sequence homology with the human enzyme (Hengstschlager-Ottnad et al., 1995). The primary sequence of LCAT contains four potential sites for N-linked oligosaccharide chains (N···Asn-Xaa-Ser/Thr···C, where Xaa represents any of the common amino acids), which were identified at Asn positions 20, 84, 272 and 384 in the amino acid sequence of this glycoprotein (McLean et al., 1986; Yang et al., 1987). Initially, only one of these sites, Asn^{272}, was confirmed as being glycosylated (McLean et al., 1986), whereas a later study indicated that the two potential glycosylation sites in the N-terminal region, Asn^{20} and Asn^{84}, were more likely to be glycosylated than Asn^{272} and Asn^{384} (Collet & Fielding, 1991). On average, only about one-third of potential sites for N-linked sugars in mammalian proteins are utilized and occupied by carbohydrate (Struck & Lennarz, 1980).

Analysis by site-directed mutagenesis, in fact, indicates that all the four potential N-glycosylation sites in LCAT contain N-linked carbohydrate chains. Elimination of the glycosylation consensus sequence at each of the four potential sites results in a mutant LCAT with abnormal specific activity for each mutant enzyme (O, Hill, & Pritchard, 1995; O, Hill, Wang, McLeod, & Pritchard, 1993). New evidence for the presence of oligosaccharide chains at all the four potential sites of the enzyme emerged from a study using a combination of sequential glycosidase digestion of human plasma LCAT followed by reversed-phase high performance liquid chromatography coupled with electro-spray ionization mass spectrometry (Schindler, Settineri, Collet, Fielding, & Burlingame, 1995).

Site-directed mutagenesis at Asn^{20}, Asn^{84} or Asn^{272} decreased by 18, 82 and 62%, respectively, the specific activity of the mutant LCAT enzyme (O et al., 1993). Several other studies also indicate that mutation at Asn^{84} or Asn^{272} significantly reduces the LCAT activity (Francone, Evangelista, & Fielding, 1993; Kosman & Jonas, 2001; O et al., 1995; Qu, Fan, Vaca, & Pownall, 1993) whereas mutation of Asn^{384} causes either an increase in LCAT specific activity (Francone et al., 1993; O et al., 1995) or no change (Qu et al., 1993). Mutation of Asn^{20} produced an enzyme with a slight decrease in specific activity (Francone et al., 1993; O et al., 1995; Qu et al., 1993). Furthermore, Asn^{84} appears as a critical glycosylation site for full LCAT activity (Kosman & Jonas, 2001; O et al., 1995; Qu et al., 1993). On the other hand, removal of the glycosylation site at Asn^{272} inhibits the acyltransferase activity and converts this protein into a phospholipase A_2 (Francone et al., 1993). This change in the usual end product of the LCAT reaction seems to be mediated by the structural environment around Asn^{272} rather than the carbohydrate structures themselves (Schindler et al., 1995). Thus, much of our knowledge of the importance of glycosylation for LCAT activity is a result of the studies by site-directed mutagenesis on each of the four known N-linked glycosylation sites.

Despite the alterations in enzyme activity that have been found, the effect of site-directed mutagenesis on LCAT secretion is conflicting. Although O et al. (1993, 1995) reported that secretion of LCAT is unaltered by mutation at any of the four Asn sites, a different result has been reported by Qu et al. (1993) indicating that Asn^{272} and Asn^{84} are important for intracellular processing and secretion. In addition, mutation at all four glycosylation sites produced a recombinant enzyme the secretion of which is reduced to about 5% of that of the fully glycosylated protein (O et al., 1995). A recent study by site directed mutagenesis indicates that N-linked carbohydrates are important for stabilizing the tertiary structure of LCAT. Mutant

LCAT with a loss of an oligosaccharide chain at Asn^{20} or Asn^{384} exhibited increased thermolability, whereas mutation at Asn^{84} or Asn^{272} showed no change in enzyme thermostability (O et al., 1995).

5. LCAT-bound oligosaccharide chains

5.1. Plasma LCAT

To date, the precise structures of the carbohydrate chains linked to plasma LCAT are uncertain. Protein-bound oligosaccharides are classified according to the covalent linkage between amino acid and carbohydrate and the two common classes of glycoproteins are those containing N- or O-glycosidically linked oligosaccharide chains (Fig. 2) attached to Asn or Ser/Thr amino acid residues, respectively (Dwek, 1995; Fukuda, 1994; Lennarz, 1983). The synthesis of oligosaccharide chains occurs in the endoplasmic reticulum and Golgi apparatus (Schachter, 1994). Particular structures formed during O- and N-glycosylation are determined by the specificity of the glycosyltransferase and the order in which they act (Kornfeld, 1983; Paulson & Colley, 1989; Schachter, 1994). O-Linked oligosaccharides (Fig. 2) are mainly short, one to three sugar residues long (Montreuil et al., 1994). Asn-linked oligosaccharides are cotranslationally added to proteins, and the Asn residue acceptor must be located in the tripeptide sequence N···Asn···Xaa···Ser/Thr···C. The relationship between the translation of polypeptides destined to become glycoproteins and their N-glycosylation is shown schematically in Fig. 3. Generally, Asn-linked glycoproteins can be divided into two main types, high-mannose (Fig. 2) with eight D-mannose residues attached to the polypeptide chain, and complex (Fig. 2) oligosaccharide-containing glycoproteins with three D-mannose residues attached to at least two sialic acid, D-galactose and *N*-acetyl-D-glucosamine, moieties (Gebhart & Ruddon, 1986; Montreuil et al., 1994; Sharon, 1984). A third type is a hybrid (Fig. 2) mixture of the two (Dwek, 1995; Peters, Krzesicki, Perini, & Huddon, 1986). These different forms share a common 'core' pentasaccharide chain consisting of two *N*-acetyl-D-glucosamine and three D-mannose residues. Variation in the *N*-glycans arises from the number and positions to which *N*-acetylglucosamines are attached to D-mannose (Fig. 2). Diversity in complex type *N*-glycans is provided by a varying number of side (antennary) chains attached to the core D-mannose residues (Fukuda, 1994).

The overall carbohydrate composition of LCAT by chemical analysis, as reported by Chung et al. (1979) and by Lima et al. (1996), respectively, are: 9.2 and 5.3% mannose, 6.0 and 6.4% galactose, 5 and 5.3% glucosamine, 0 and 3.0% galactosamine, 3.9 and 5% sialic acid. These carbohydrate compositions are consistent with varying chain types including N-linked and O-linked oligosaccharide

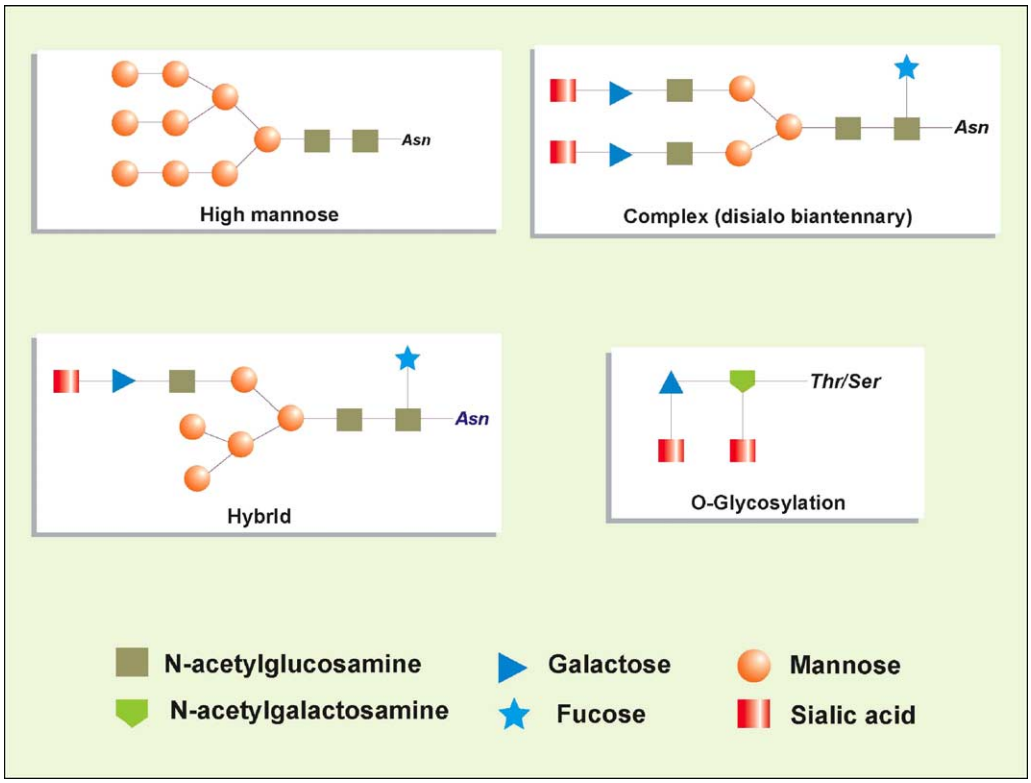

Fig. 2. Schematic representation of common oligosaccharide structures found in glycoproteins. Typical structures of high mannose, complex and hybrid N-linked oligosaccharides are shown, as well as of an O-linked carbohydrate chain.

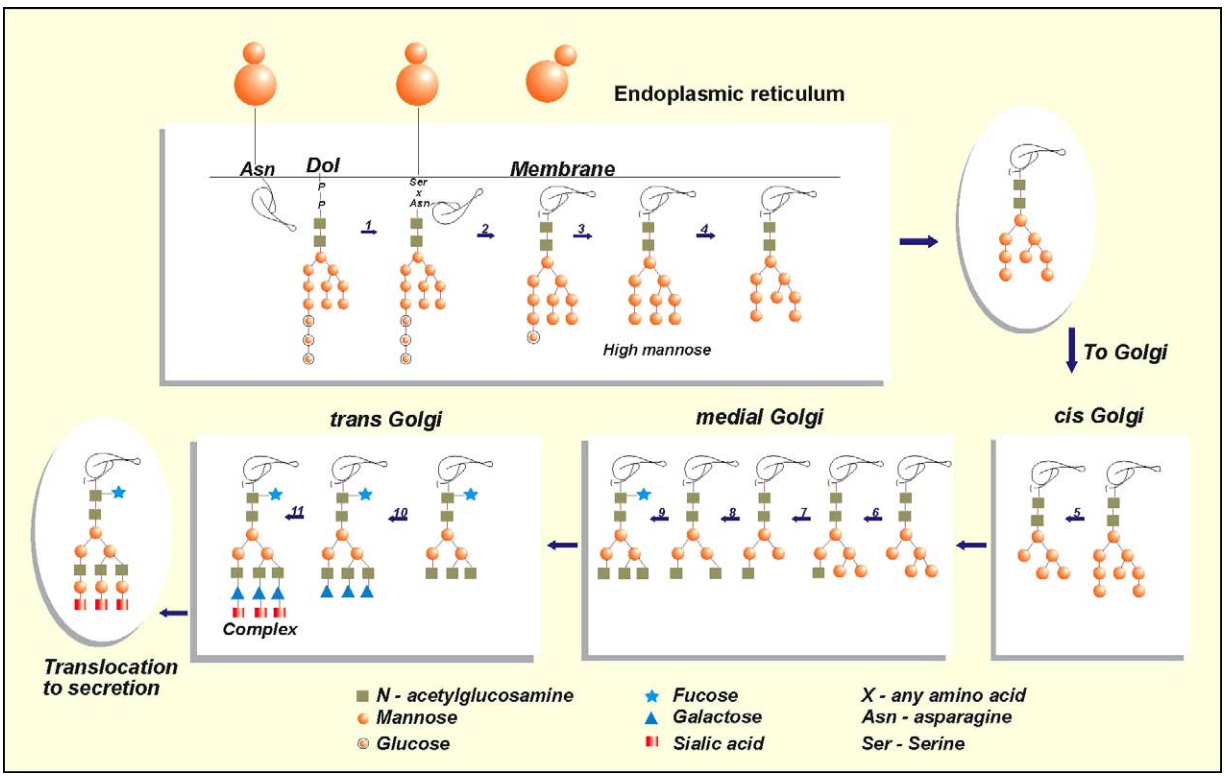

Fig. 3. Schematic representation of individual steps in the N-glycosylation pathway for secreted glycoproteins. The figure shows only one of many possible processing pathways. The reactions are catalyzed by: (1) dolichyldiphosphoryl-oligosaccharide: protein oligosaccharyltransferase, (2) D-glucosidase I, D-glucosidase II (3) D-glucosidase II, (4) Endoplasmic reticulum α-D-mannosidase I, (5) Golgi α-D-mannosidase I, (6) β-*N*-acetyl-D-glucosaminyltransferase I, (7) Golgi α-D-mannosidase II, (8) β-*N*-acetyl-D-glucosaminyltransferase II, (9) α-L-fucosyltransferase, β-*N*-acetyl-D-glucosaminyltransferase II, (10) β-D-galactosyltransferase, and (11) α-sialyltransferase (based on Fukuda (1994) and Schachter (1994)). The transfer of intermediates between the various subcellular compartments occurs via membranous vesicles.

structures. Apparently, most LCAT molecules contain high mannose and complex type oligosaccharide chains covalently linked to its polypeptide structure, and no or very little O-linked sugar.

Collet and Fielding (1991) demonstrated that digestion of purified plasma LCAT by *N*-glycanase F produced a protein with an apparent M_r of 46,000 Da by SDS-PAGE, which was similar to the size of the protein backbone with no carbohydrate suggesting the absence of O-linked carbohydrate. Similar results were obtained using tunicamycin. This is typical of N-linked sugar chains. However, the more recent study of Schindler et al. (1995) using mass spectrometric analysis, suggested that there are two O-linked carbohydrate residues. These chains were identified in the proline-rich C-terminal region at Thr^{407} and Ser^{409} and contained sialylated β-D-galactosyl-(1 → 3)-*N*-acetyl-D-galactosamine structures.

LCAT carbohydrate modifications resulting from the use of glycosidases and specific inhibitors of glycosylation indicate that LCAT is heterogeneously glycosylated. Collet and Fielding (1991) showed that treatment of plasma LCAT with endo-β-*N*-acetylglucosaminidase H or endoglycosidase F yielded two species of LCAT on SDS-PAGE, while other hand, the use of neuraminidase also reduced the molecular weight of LCAT, indicating the presence of LCAT having high mannose chains and complex type oligosaccharides, respectively. By contrast, Miller, Wang, Sorci-Thomas, Anderson, and Parks (1996) found LCAT from plasma and from Chinese hamster ovary cells was insensitive to endoglycosidase F, although there was sensitivity to *N*-glycanase and neuraminidase. From all studies with neuraminidase digests of LCAT, this glycoprotein appears to have a high sialic acid content (Collet & Fielding, 1991; Doi & Nishida, 1983; Hill et al., 1993b; Miller et al., 1996). Therefore, microheterogeneity due to glycosylation differences is evident in LCAT isolated from different sources. The carbohydrate structures of Hep G2-derived LCAT appear distinct from those of plasma as, unlike plasma LCAT, it resists *N*-glycanase digestion (Miller et al., 1996). Recombinant LCAT from transfected baby hamster kidney cells has greater glycosylation heterogeneity compared to plasma LCAT. The recombinant protein migrates as a broader band than that from plasma, and changes to a sharp band, with no apparent difference in mobility, when removal of the N-linked carbohydrates is performed with *N*-glycanase (Hill et al., 1993b).

Recently, the heterogeneously glycosylated N-linked carbohydrate chains of LCAT were shown to contain

sialylated triantennary and/or biantennary complex structures. Schindler et al. (1995) established that Asn at position 20, 84, 272 and 384 are glycosylated with complex type oligosaccharide chain. The latter, Asn^{384}, contained only mono- and disialo biantennary structures. The other sites, Asn^{20} Asn^{84} and Asn^{272}, contained as a major carbohydrate structure trisialo triantennary, and as a minor component fucosylated trisialo triantennary, disialo triantennary, and disialo biantennary oligosaccharide chains. Although they found no evidence of high mannose oligosaccharides, probably due to the source for endo H (Trimble & Tarentino, 1991) used in their work, there is strong evidence for LCAT containing N-linked high mannose type chains as described above. Other evidence for the presence of N-linked oligosaccharide of the high mannose type on LCAT arises from the use of lectins which are glycoproteins consisting of a variable number of subunits that possess at least two sugar-binding sites (Kennedy et al., 1995). LCAT can be isolated by lectin-affinity column chromatography. Most of the plasma LCAT purified by Ridgway and Dolphin (1985) was bound to Concanavalin A-Sepharose (Con A). On the other hand, Lima et al. (1997) found only a small portion of plasma LCAT bound to mannose/glucose-binding lectins such as Con A and *Cratylia mollis*.

5.2. Recombinant LCAT

The LCAT source is very important because it influences the oligosaccharide structures of the protein. Several mammalian cell lines used to express LCAT, such as baby hamster kidney (BHK) (Hill et al., 1993b), Chinese hamster ovary (CHO) (Collet & Fielding, 1991; Francone et al., 1993; Miller at al., 1996) and COS (O et al., 1993; Qu et al., 1993), all produce recombinant LCAT with an apparent molecular weight indistinguishable from that of plasma LCAT (~68,000 Da). Moreover, a detailed analysis of the BHK-derived LCAT by fast atom bombardment mass spectrometry (Lacko et al., 1998) revealed that, like plasma LCAT, it contained mostly tri- and tetraantennary oligosaccharides. By contrast, baculoviral-expressed LCAT appears to be underglycosylated. The apparent molecular weight of baculoviral-expressed LCAT (M_r 53,000) is smaller than the plasma glycoprotein (Chawla & Owen, 1995; Miller at al., 1996). LCAT from Sf21 cells (M_r 50,000) infected with a recombinant baculovirus (Miller et al., 1996) appears to contain little or no terminal sialic acid residues since it was resistant to digest with *N*-glycanase, endo F and neuraminidase. This finding is consistent with that of Chawla and Owen (1995) whose LCAT from *Trichoplusia ni* was sensitive to endo H digestion, interacted with Con A, and appeared to have only high mannose, or possible bi- or tri-antennary hybrid glycans. Differences in carbohydrate structures may thus arise by choosing one recombinant host cell line and cell culture conditions over another (Jenkins, Parekh, & James, 1996). However, caution is needed as the rat hepatoma cell line, Mc-7777, produced recombinant LCAT with biantennary structures rather than the predicted 'complex-type' found in plasma LCAT (Ayyobi et al., 2000).

5.3. LCAT deficiency

A review by Turner (1992) emphasized that different types of liver disease are associated with different patterns in the glycan branching of several serum glycoproteins. These changes may provide the basis for more sensitive and better discriminative clinical tests, which can be done by using simple techniques for glycoprotein analysis including lectin-based methods. The high content of carbohydrate molecules in LCAT is of future interest in studies to investigate the nature of the oligosaccharide branches in patients with secondary (acquired) and primary (familial) LCAT deficiency. Forty unique mutations in the human LCAT gene which result in significant impairment in plasma LCAT activity have been reported worldwide to date. Phenotypically, LCAT deficiency is segregated into classical or familial LCAT deficiency (FLD) and a smaller subset termed Fish Eye Disease (FED) due to the pronounced corneal opacity associated with this variant of LCAT deficiency (Carlson, 1982). LCAT gene mutations producing the FED phenotype result in an LCAT protein that is inactive against HDL but will esterify cholesterol present in VLDL and LDL (Carlson & Holmquist, 1985). Mutations causing the FLD phenotype are associated with variable plasma enzyme mass which has either no or very low activity (Guérin et al., 1997). The general clinical and biochemical features of LCAT deficiency have been previously reviewed (Assmann, Eckardstein, & Funke, 1991; Glomset et al., 1995; McIntyre, 1988). These include corneal opacity, anemia, proteinurea, which typically results in renal failure in the fourth to fifth decade, HDL deficiency, lipid changes in both VLDL and LDL, and very low levels of plasma cholesteryl esters in FLD, though not in the FED phenotype. The lack of plasma LCAT activity may result from a reduced secretion of a fully or partially active enzyme, or from the normal secretion of an enzyme with reduced activity, the secretion of an unstable or rapidly catabolized form, or from a complete absence of protein secretion. For example, in plasma from patients presenting with homozygous FLD the enzyme activity is undetectable or represents about 2% of the control values, in spite of its protein mass being from 10% (Guerin et al., 1997) to 50% of the normal plasma circulating LCAT (5.5 ± 0.90 μg/ml) reported by Albers, Adolphson, and Chen (1981).

Most of the molecular mutations in the human LCAT gene have recently been reviewed by Kuivenhoven et al. (1997) and in vitro expression in eukaryotic cells of the molecular defects demonstrate at least 60% of normal secretion of an inactive or partially active enzyme (Funke et al., 1993; Gotoda et al., 1991; Humphries et al., 1988; Maeda et al., 1991; Miettinen, Gylling, Ulmanen, Miettinen, & Kontula, 1995; Moriyama et al., 1995; Owen et al., 1996).

It is important to focus again on the study by Qu et al. (1993), using site directed mutagenesis of the LCAT N-linked glycosylation sites, which demonstrated that the oligosaccharide chain linked to Asn^{272} is absolutely required for the secretion of an active LCAT enzyme. Moreover, the deletion of a G nucleotide at position 4571 of the LCAT gene results in the loss of N-glycosylation sites Asn^{272} and Asn^{384} and the complete absence of active LCAT protein in the plasma (Moriyama et al., 1995). Therefore, the glycosylation of Asn^{272} should be considered as one of the several structural signals that facilitate the normal secretion of LCAT as a fully active enzyme. Of the ten unique mutations that result in the FED phenotype, four involve changes in either threonine or asparagine residues (Guérin et al., 1997). However, none of these residues represent known glycosylation sites and thus carbohydrate chain deletion is probably not the basis for the altered substrate specificity of LCAT noted in the FED phenotype.

6. Conclusions

Although we are aware of the general classes of carbohydrate that associate LCAT, and have some information concerning their sequence and types of branching, it is evident that we are far from understanding the precise function of the carbohydrate chains. Clearly, the terminal sialic acid residues are not essential for enzyme activity yet may preserve the plasma half-life. Equally if the retention of enzymatic activity is to be the criterion of conformational integrity then a limited amount of heterogeneity within the carbohydrate moiety of LCAT is permissible as evidenced by the variably glycosylated, yet functional, LCAT molecules synthesized and secreted by different cell lines and the liver. Similarly, there is evidence to suggest that not all of the N-linked carbohydrate chains are essential for activity (e.g. Asn^{384}) as some sites can be deleted without effect while the addition of carbohydrate to others is required. One of the most likely functions of the carbohydrate moiety of LCAT is the provision of an interface between the relatively hydrophobic polypeptide backbone of the enzyme (Yang et al., 1987) and the hydrophilic environment of the plasma. Until very recently we had little knowledge of how the LCAT polypeptide chain may be folded and the only structural model was that proposed by Yang et al. (1987) based upon their own studies and those of Jauhianen and Dolphin (1986) and shown in Figs. 1 and 3. The application of threading techniques by Peelman et al. (1998) has provided a sophisticated model for how LCAT may be folded and would certainly explain why many of the LCAT gene mutations would result in a dysfunctional protein. The ultimate test of the validity of this model, however, will require direct structural determinations by either X-ray diffraction or NMR. To that end recombinant, underglycosylated yet functional forms of LCAT may be of use in that they may be more easily crystallized and provide more interpretable data than the fully glycosylated form. We look forward to future studies upon the structure of this important enzyme of plasma lipoprotein metabolism.

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