Lecithin:cholesterol acyltransferase: role of N-linked glycosylation in enzyme function

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Lecithin:cholesterol acyltransferase (LCAT; phosphatidylcholine–sterol acyltransferase, EC 2.3.1.43) is a glycoprotein which is responsible for the formation of cholesteryl ester in plasma. The carbohydrate content has been estimated to be approx. 25% of the total LCAT mass, and four potential N-linked glycosylation sites have been predicted at residues 20, 84, 272 and 384 of the LCAT protein sequence. In the present study, we have examined which of these sites are utilized and how the *N*-glycosylation affects the secretion and function of the enzyme. Site-directed mutagenesis was performed to eliminate the glycosylation consensus sequence at each of the four potential sites, and the mutant proteins were expressed in COS cells. The amount of each mutant LCAT secreted was similar to that of the wild-type enzyme but the molecular mass was decreased by 3–4 kDa. The specific activity of each mutant LCAT was significantly different from the wild-type; however, the magnitude and direction of the change depended on the glycosylation site mutagenized. Loss of carbohydrate at position 20, 84 or 272 resulted in a decrease in the specific activity of the mutant enzymes by 18%, 82% and 62% respectively. In contrast, the mutant protein without glycosylation at position 384 displayed a 2-fold increase in enzyme activity. In addition, a quadruple mutant was constructed such that all four potential glycosylation sites were eliminated. The amount of the unglycosylated LCAT secreted into the culture medium was less than 10% of the wild-type level and the specific activity of this enzyme was decreased to 5% of that of the wild type. The results demonstrate that all four potential *N*-glycosylation sites in LCAT are used and the presence of carbohydrate at each site has diverse effects on the enzyme activity.

INTRODUCTION

Lecithin:cholesterol acyltransferase (LCAT; phosphatidylcholine–sterol acyltransferase, EC 2.3.1.43) is a glycoprotein enzyme responsible for the formation of cholesteryl ester in plasma via transfer of the *sn*-2 fatty acid from phosphatidylcholine to the 3-hydroxy group of cholesterol in plasma [1]. This enzyme is synthesized in hepatocytes and secreted into the blood where it primarily acts on high-density lipoprotein [2]. Full activity appears to require apolipoprotein A-I (apo A-I) which is also the major structural protein of high-density lipoprotein [3,4]. In plasma, LCAT protein has an apparent molecular mass of about 63–67 kDa [5,6]. However, analysis of the cDNA predicts that the mature LCAT protein contains 416 amino acids with a protein molecular mass of 46917 kDa and the carbohydrate content is estimated to be approx. 25% of total LCAT mass [5,7,8]. The structural and functional regions of the enzyme have been predicted from analysis of the human LCAT primary amino acid sequence and peptide alignment [8]. As a result of a search for homologies with some serine-type hydrolases, Fielding [6] has proposed that an Asp-His-Ser triad is involved in the deacylation of phospholipid substrate, formation of a Ser-*O*-acyl intermediate, and subsequent transfer of the acyl chain to cholesterol. Recently, Ser^{181} in the LCAT sequence has been identified as an essential residue for the catalytic function [9].

There are four potential glycosylation sites at Asn^{20}, Asn^{84}, Asn^{272} and Asn^{384} [5] each conforming to the consensus sequence (Asn-X-Ser/Thr) for N-linked glycosylation, where X can be any amino acid except proline [10]. Such N-linked glycosylation is a common post-translation modification in mammalian proteins but the role of the carbohydrate residues in LCAT function remains unclear. It has been postulated, however, that the two potential glycosylation sites in the N-terminal region (Asn^{20} and Asn^{84}) of the LCAT protein sequence are more likely to be occupied than Asn^{272} or Asn^{384} [11]. Recent studies have shown that blocking the glycosylation of recombinant LCAT secreted from Chinese hamster ovary (CHO) cells by tunicamycin does not affect LCAT protein secretion but inhibits the enzyme activity [11]. This suggests that the presence of carbohydrate is necessary for full enzyme activity. To date, however, it is unknown which of the potential sites are glycosylated and how the glycosylation at each site affects the secretion and function of the enzyme.

In order to determine which amino acid residues are glycosylated and the functional significance of N-linked glycosylation at each of the potential sites in LCAT, we now report on the construction of four mutants with a substitution of Gln for Asn at each of residues 20, 84, 272 and 384 of the LCAT protein. In addition, a quadruple mutant containing substitutions at all four sites was constructed. Our data show that all four glycosylation sites are used for N-linked carbohydrate addition in LCAT and the absence of carbohydrate at each site has profound effects on enzyme activity.

MATERIALS AND METHODS

Site-directed mutagenesis and transfection of COS cells

Oligonucleotides were designed so that each potential glycosylation site (Asn^{20}, Asn^{84}, Asn^{272} and Asn^{384}) had an alteration in the consensus sequence (Asn-X-Ser/Thr). Four mutagenic oligonucleotides were generated and each of them carried two mismatched bases creating a substitution of Gln for Asn in which

Abbreviations used: LCAT, lecithin:cholesterol acyltransferase (phosphatidylcholine–sterol acyltransferase; EC 2.3.1.43); apo A-I, apolipoprotein A-I; DMEM, Dulbecco's modified Eagle's medium.

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the original codon AAC was changed to mutant codon CAG: (1) L20, 5′-AGCTCAGTCAGCACACACG-3′; (2) L84, 5′-TTGTCTACCAGCGGAGCTC-3′; (3) L272, 5′-CCAGCTTCCAGTACACAGG-3′; (4) L384, 5′-TCTTCAGCCAGCTGACCCT-3′. The human plasma LCAT cDNA (a gift from Dr. J. McLean, Genentech) was transferred to the *Xho*I and *Bam*HI site of the pUC19 vector. Using a mutagenic oligonucleotide as primer, the mutation was introduced into the pUC19 LCAT cDNA by PCR as previously described [12]. DNA sequencing was performed to identify clones containing the desired mutations. The mutant cDNAs were excised from positive clones and then transferred to the mammalian expression vector pNUT [13,14]. A quadruple mutant was constructed by utilizing restriction enzymes to generate four different DNA fragments each of which contained a single mutation for each potential glycosylation site. Subsequently, these fragments were cloned into the pNUT expression vector. The final constructs containing a single or quadruple mutation were sequenced again to confirm the presence of the desired mutations. The expression vector pNUT containing either wild-type or mutant LCAT cDNA was transfected into COS-1 cells by DEAE-dextran transfection [12]. The transfected COS cells were incubated in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum for 12 h followed by incubation in serum-free medium (OptiMEM; GIBCO-BRL) for 48 h.

Radiolabelling and immunoprecipitation of recombinant LCAT

To identify cellular and secreted LCAT proteins, the transfected cells were radiolabelled after recovery from the transfection procedure. Briefly, the transfected COS cells were incubated for 30 min in methionine-free DMEM containing 100 μCi/ml [^{35}S]methionine (700 Ci/mmol; New England Nuclear) followed by a 4 h chase incubation in DMEM with 10% fetal bovine serum. Solid-phase immunoadsorption was performed to detect the presence of LCAT in the culture medium and cell lysate [12]. Polyclonal goat anti-(human LCAT) antibodies (kindly provided by Dr. A. Lacko, Texas College of Osteopathic Medicine, Fort Worth, TX, U.S.A.) were pre-adsorbed on to agarose-immobilized Protein G (GammaBind G Agarose; Pharmacia LKB) for 30 min at 4 °C. The LCAT antibodies were specific for LCAT protein in the culture medium as determined by Western-blot analysis of SDS/polyacrylamide gels. A portion of medium or cell lysate was added to the antibody–Protein G–agarose suspension and the mixture was rotated overnight at 4 °C. Agarose beads were pelleted by centrifugation and then washed twice with 1 ml of Tris-buffered saline containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1% sodium azide. The pellet was resuspended in the buffer containing 0.1 M Tris/HCl, pH 6.8, 2% (w/v) SDS and 40% (v/v) glycerol. The adsorbed materials were then eluted from the agarose beads by heating at 90 °C for 10 min. The agarose beads were removed by centrifugation. The supernatant was subjected to electrophoresis on SDS/10% (w/v) polyacrylamide gels; [^{14}C]methylated protein standards (Amersham Canada Ltd., Oakville, Ont., Canada) were used as molecular-mass markers. After electrophoresis, the gels were agitated in 25% methanol/5% acetic acid for 30 min, then in Amplify (Amersham Canada Ltd.) for 15 min. The gels were dried and autoradiography was performed with Kodak X-Omat AR film (Eastman-Kodak) at −70 °C for 24 h.

Endoglycosidase F digestion

Endoglycosidase F (peptide–*N*-endoglycosidase F; EC 3.2.2.8) (Boehringer-Mannheim, Laval, Que., Canada) digestion of wild-type recombinant LCAT was performed as recommended by the manufacturers. The assay mixture (containing 0.1 M sodium phosphate (pH 8.6), 1% Nonidet P40, 0.1 M EDTA, 0.5% 2-mercaptoethanol, 0.1% SDS and 100 ng of wild-type LCAT) was boiled for 10 min. The digestion was initiated by adding various amounts of endoglycosidase F and the reaction was carried out at 37 °C for the indicated time period. The digested products were analysed by Western immunoblotting. Briefly, a portion of the reaction mixture was subjected to electrophoresis on an SDS/7.5% (w/v) polyacrylamide gel. After the electrophoretic transfer of proteins from the gel to nitrocellulose membrane (pore size, 0.45 μm), the membrane was incubated with anti-(human LCAT) antibodies. Blots were developed with Protein G-conjugated horseradish peroxidase.

Determination of LCAT concentration

The amount of LCAT protein secreted into the culture medium was determined by solid-phase immunoassay as described previously [12]. The LCAT concentration measured was proportional to absorbance up to 80 ng of LCAT protein.

Determination of LCAT activity

The enzyme activities of wild-type and mutant LCAT gene products were determined using [^{3}H]cholesterol–phosphatidylcholine–apo A-I proteoliposome substrate (exogenous substrate) as previously described [12,15]. Apo A-I was purified from human plasma by chromatofocusing chromatography [16]. The enzyme activity of the recombinant LCAT was also determined by using [^{3}H]cholesterol-labelled plasma as a source of lipoprotein substrates (endogenous substrate) [12]. For this assay, blood was collected from normal volunteers after 12 h fasting and plasma was prepared by low-speed centrifugation (1200 ***g***, 20 min). The plasma was heat-inactivated at 56 °C for 30 min to eliminate endogenous LCAT activity [12,17,18]. The concentration of unesterified cholesterol in plasma was determined enzymically by a reagent kit (Boehringer-Mannheim). The endogenous lipoproteins present in the plasma were radiolabelled by equilibration with [^{3}H]cholesterol at 4 °C as described by Dobiasova and Schutzova [19]. The labelled plasma was incubated with recombinant LCAT and the rate of [^{3}H]cholesterol esterification in plasma was determined as previously described [12]. The results are expressed as nmol of free cholesterol esterified/h per μg of LCAT protein.

Treatment of transfected COS cells with tunicamycin

After transfection with wild-type LCAT cDNA, the COS cells were incubated in OptiMEM containing 0.5 μg/ml tunicamycin (Boehringer-Mannheim) for 2 h. Cells were then washed twice and incubated in OptiMEM containing 0.5 μg/ml tunicamycin for 48 h. The protein concentration and enzyme activity of unglycosylated LCAT secreted into culture medium were determined. The unglycosylated enzyme was also radiolabelled with [^{35}S]methionine and immunoprecipitated as described above except 0.5 μg/ml tunicamycin was included throughout the pulse–chase experiment.

RESULTS

Construction and expression of glycosylation mutants

Oligonucleotide-directed mutagenesis was used to introduce a substitution of Gln for Asn into the consensus sequence for the potential *N*-glycosylation sites in the human LCAT cDNA

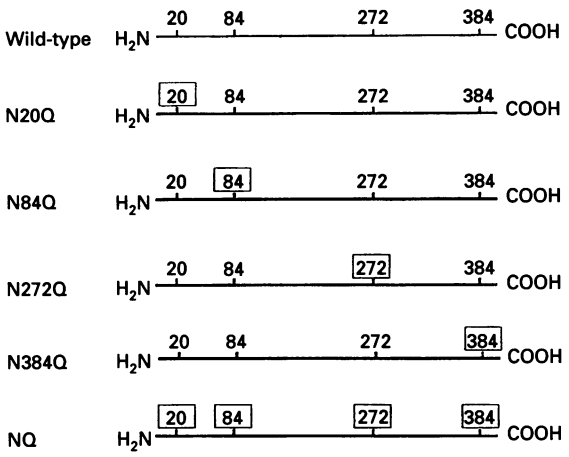

Figure 1 Site-directed mutagenesis of potential glycosylation sites in LCAT

The wild-type structure is shown at the top with four potential N-linked glycosylation sites at positions 20, 84, 272 and 384. Site-directed mutagenesis was performed to replace Asn with Gln at each or all of the above sites. The mutant LCAT proteins with a single substitution at positions 20, 84, 272 and 384 are designated N20Q, N84Q, N272Q and N384Q respectively, and the quadruple mutation is designated NQ (indicated by boxes).

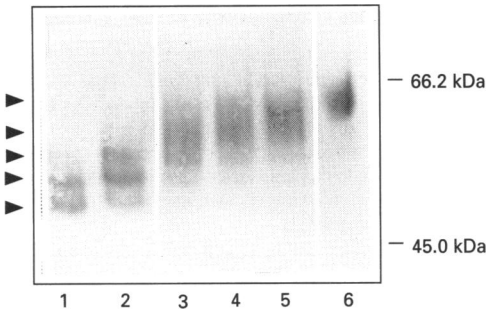

Figure 3 Determination of the number of N-linked glycans on recombinant LCAT

Wild-type recombinant LCAT (100 ng) was incubated with 250 munits (lane 1), 10 munits (lane 2), 5 munits (lane 3), 2 munits (lane 4), 1 munit (lane 5) and 0 (lane 6) of endoglycosidase F at 37 °C for 10 min. The treated samples were separated by SDS/PAGE (7.5% gels), followed by Western immunoblotting as described in the Materials and methods section. The arrowheads indicate the position of the five identifiable reaction products.

(Figure 1). This resulted in the loss of a single or multiple (quadruple) recognition site(s) for N-linked glycosylation. The mutant LCAT cDNA and the wild-type LCAT cDNA were independently inserted into the expression vector pNUT. After transfection of constructs into COS-1 cells, synthesis and secretion of mutant and wild-type LCAT were studied by a pulse–chase experiment with [^{35}S]methionine. As shown in Figure 2, COS cells transiently transfected with wild-type LCAT cDNA secreted an immunoprecipitable protein with an apparent molecular mass of about 66 kDa which was comparable with fully glycosylated plasma LCAT. This LCAT protein was not seen in the culture medium isolated from the cells transfected with vector DNA which did not contain the LCAT cDNA insert. The cells expressing mutant LCAT (N20Q, N84Q, N272Q or N384Q) secreted immunoprecipitable protein with a reduced size, which was consistent with the predicted loss of N-linked oligosaccharides (Figure 2). The apparent molecular mass was about 62–63 kDa for N20Q, N84Q, N272Q and N384Q. These results indicate that the replacement of Asn at Asn^{20}, Asn^{84}, Asn^{272} or Asn^{384} with Gln prevents the addition of N-linked carbohydrate to each of these positions, resulting in an apparent decrease in size for all mutant proteins.

Immunoprecipitation of the cell lysate of COS cells transfected with wild-type LCAT cDNA revealed a protein band with a lower molecular mass (57 kDa) than the mature LCAT secreted by COS cells (Figure 2). A specific band of immunoprecipitable protein was also observed within the cells expressing mutant proteins. As shown in Figure 2, a protein band with an apparent molecular mass of about 54 kDa was observed in the cells expressing N20Q and N84Q mutant LCAT, whereas a band with a molecular mass of about 52 kDa was seen in the cells expressing the N272Q and N384Q mutant proteins.

Radiolabelling and immunoprecipitation were also performed on the COS cells expressing a quadruple mutant (results not shown). Only after a prolonged exposure of the gels to X-ray film (2 weeks) could a protein band with an apparent molecular mass of 47 kDa be detected. The size of this immunoprecipitable protein was comparable with that of unglycosylated LCAT [5]. Similarly, an immunoprecipitable protein band of equivalent molecular mass (47 kDa) was observed in COS cells transfected with wild-type LCAT cDNA treated with tunicamycin, an inhibitor of N-linked glycosylation (results not shown).

The secreted wild-type enzyme was also partially digested with endoglycosidase F, which cleaves all N-linked carbo-

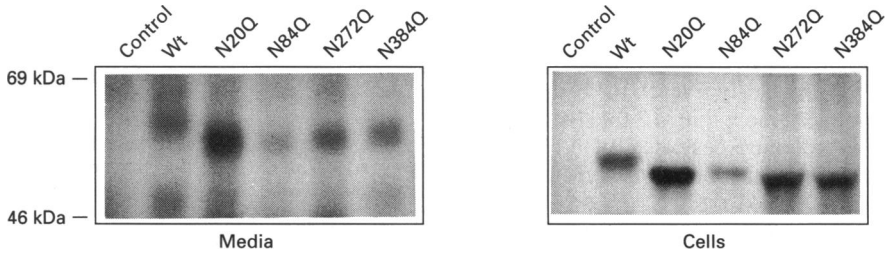

Figure 2 Immunoprecipitation of wild-type and mutant LCAT secreted into culture medium

The transfected COS-1 cells were pulse-labelled with [^{35}S]methionine (100 μCi/ml) for 30 min followed by a chase incubation with unlabelled methionine for 4 h. Subsequently, LCAT protein in the cell lysate and the culture medium were immunoprecipitated with polyclonal anti-(human LCAT) antibodies and electrophoresed in SDS/10% (w/v) polyacrylamide gels. The radiolabelled proteins of wild-type (Wt) and mutant LCAT were visualized by autoradiography. In the control experiments, the cells were transfected with vector DNA containing no insert. Migration position and size (in kDa) of protein standards are indicated.

Table 1 Protein mass and enzyme activity of LCAT secreted by COS cells

After a 48 h incubation of transfected COS-1 cells in serum-free medium, the culture medium was analysed for LCAT protein concentration and activity of wild-type and mutant LCAT as described in the Materials and methods section. The protein concentration and enzyme activity of wild-type LCAT were 0.18 ± 0.03 μg/ml ($n = 5$) and 12.25 ± 1.2 nmol/h per μg of LCAT protein ($n = 5$) respectively. The results are expressed as percentage of wild-type enzyme and depicted as means ± S.D. from five separate experiments. Control experiments in which the cells were transfected with vector containing no insert were also carried out. In all cases, no LCAT activity or mass could be detected.

Enzyme	LCAT concentration (% of wild-type)	LCAT activity (% of wild-type)
Wild-type	100	100
N20Q	90 ± 20	82 ± 15
N84Q	89 ± 7	18 ± 5
N272Q	87 ± 13	38 ± 7
N384Q	97 ± 12	215 ± 28
NQ	10 ± 2	5 ± 1

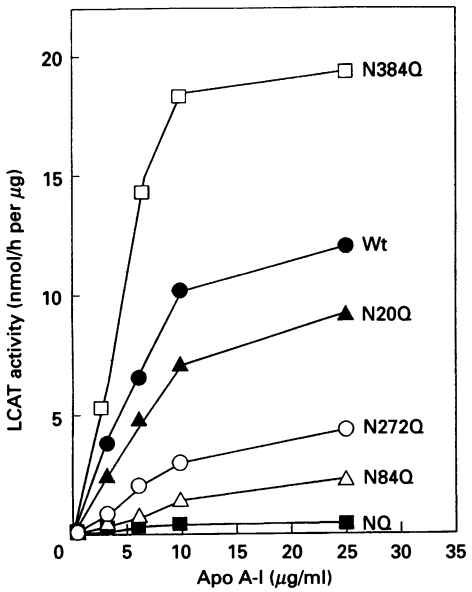

Figure 4 Activation of wild-type and mutant LCAT by apo A-I

The enzyme activities were determined with wild-type (Wt), mutant and unglycosylated LCAT. The substrate ([^{3}H]cholesterol–phosphatidylcholine) was preincubated with various amounts of purified plasma apo A-I at 37 °C for 30 min. Subsequently, the enzyme reaction was carried out as described in the Materials and methods section.

hydrate regardless of complexity by hydrolysing the asparagine–oligosaccharide bond. As shown in Figure 3, on partial digestion, five protein bands were generated with molecular masses between 47 and 66 kDa. It is likely that these protein bands correspond to LCAT with four, three, two, one and no carbohydrate side chains.

To quantify LCAT in the culture medium, we determined the mass of the secreted LCAT protein using polyclonal antibodies specific for human LCAT. As shown in Table 1, after a 48 h incubation in serum-free medium, the transfected COS cells secreted an average 0.18 μg/ml LCAT for wild-type, 0.16 μg/ml for N20Q, 0.16 μg/ml for N84Q, 0.16 μg/ml for N272Q, 0.18 μg/ml for N384Q and 0.02 μg/ml for both quadruple mutant (NQ) and unglycosylated LCAT after tunicamycin treatment.

Functional analysis of secreted mutant LCAT

Our next objective was to assess the role of N-linked glycosylation at each individual *N*-glycosylation site in LCAT activity. The enzyme activities of the secreted mutant LCAT proteins were determined with a proteoliposome containing [^{3}H]cholesterol, egg phosphatidylcholine and apo A-I. Compared with the wild-type enzyme, a single mutation at position 20, 84 or 272 resulted in a decrease in specific enzyme activity (Table 1). Removal of a glycosylation site at position 20 had a mild inhibitory effect on LCAT activity (82% of wild-type activity), whereas the same substitution at position 272 caused a 62% reduction in enzyme activity. When the mutation occurred at position 84, the enzyme activity was only 18% of wild-type level. Interestingly, a mutation at position 384 produced more than a 2-fold increase in enzyme activity. The activity of unglycosylated LCAT in the culture medium was only 5% of that of the wild-type enzyme (Table 1).

Apo A-I has been shown to be the primary protein activator of LCAT [3,4]. In this study, we investigated the activation of mutant LCAT by this apolipoprotein. In the absence of apo A-I, no enzyme activity was detected with wild-type, unglycosylated or mutant LCAT proteins (Figure 4). When the activity was determined as a function of apo A-I concentration, the mutant enzymes were activated by apo A-I to different extents (Figure 4). Nevertheless, similar saturation curves were obtained with all

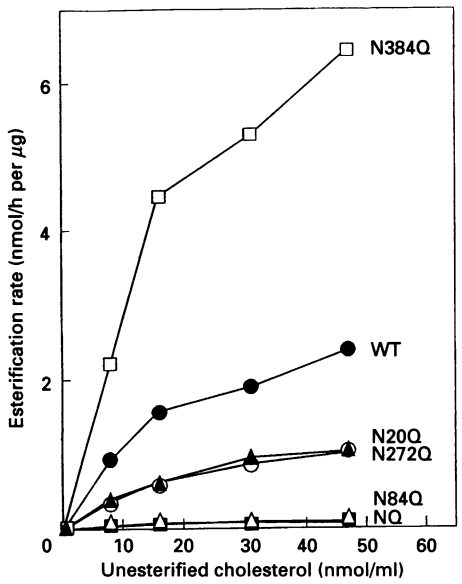

Figure 5 Cholesterol esterification in plasma catalysed by wild-type and mutant LCAT

A portion of cell culture medium containing wild-type (Wt) or mutant LCAT was incubated with various amounts of heat-inactivated [^{3}H]cholesterol-labelled plasma for 5 h at 37 °C. The cholesterol-esterification rate was determined and expressed as nmol of cholesteryl ester formed/h per μg of LCAT protein.

mutants carrying a single mutation as well as the wild-type LCAT.

The ability of mutant LCAT to esterify cholesterol in human plasma *in vitro* was also examined. Various amounts of radio-labelled heat-inactivated plasma were incubated with the

Table 2 Apparent kinetic parameters of wild-type and mutant LCAT

The values of K_m (μM of plasma unesterified cholesterol) and $V_{max.}$ (nmol of cholesteryl ester formed/h per μg of LCAT protein) were determined from linear regression analysis of two separate experiments, each performed in duplicate. Experimental differences were less than 10%. The apparent $V_{max.}/K_m$ is the reciprocal slope of the double-reciprocal plot of cholesteryl ester formation versus the amount of plasma cholesterol. ND, not determined. The low activities of mutants N84Q and NQ did not permit us to determine accurately the apparent K_m and $V_{max.}$.

Enzyme	K_m (μM)	$V_{max.}$ (nmol/h per μg)	$V_{max.}/K_m$ (nmol · h^{-1} · μg^{-1} · μM)
Wild-type	22	3.45	0.16
N20Q	31	1.83	0.059
N84Q	ND	ND	–
N272Q	20	1.43	0.072
N384Q	34	11.92	0.35
NQ	ND	ND	–

culture medium containing either wild-type or mutant LCAT for 5 h at 37 °C. We have previously shown that esterification of cholesterol occurs in a linear manner up to 6 h of incubation under the conditions described [12]. The activities of wild-type and mutant enzymes N20Q, N272Q and N384Q increased in a substrate-dependent manner (Figure 5). However, very low activity was detected in the reactions catalysed by either the N84Q mutant or the quadruple mutant (Figure 5). The mechanism by which the mutation at position 20, 272 and 384 altered enzyme activity was examined by kinetic analysis of the data obtained from Figure 5. The data obtained from the Lineweaver–Burk plot of cholesteryl ester formation versus plasma cholesterol are summarized in Table 2. Compared with the wild-type enzyme, the $V_{max.}/K_m$ was 37% and 45% for N20Q and N272Q mutants respectively. In contrast, the apparent $V_{max.}/K_m$ value of the enzyme carrying a mutation at position 384 was twice that of the wild-type. Kinetic parameters for N84Q and the unglycosylated LCAT were not obtainable from the present study because of their low reactivity with plasma substrates.

DISCUSSION

Carbohydrate accounts for approx. 25% of the molecular mass of human plasma LCAT [5,8]. It has been demonstrated that on *N*-Glycanase treatment, the molecular mass of plasma LCAT is reduced to 47 kDa, which suggests that LCAT contains a large amount of N-linked oligosaccharide moiety, with little, if any, O-linked carbohydrate [11]. On the basis of the consensus sequence (Asn-X-Ser/Thr), four potential sites for N-linked glycosylation have been predicted in the LCAT sequenced at Asn^{20}, Asn^{84}, Asn^{272} and Asn^{384} [8]. Although the sequence Asn-X-Ser/Thr has been shown to be necessary for *N*-glycosylation, it does not always result in glycosylation [20]. In order to obtain information on which of the four potential *N*-glycosylation sites in LCAT were utilized, we performed site-directed mutagenesis to eliminate each of the four sites. We found that LCAT proteins carrying a single mutation at Asn^{20}, Asn^{84}, Asn^{272} or Asn^{384} were secreted from transfected COS cells. All of the secreted mutant proteins were smaller than the wild-type LCAT. When all four sites were substituted, the mutant protein was the same size as the unglycosylated LCAT protein. The results obtained from digestion of wild-type enzyme with endoglycosidase F have also indicated that wild-type LCAT normally has four N-linked carbohydrate chains. The above results indicate that all four potential sites in LCAT are used for *N*-glycosylation of recombinant LCAT secreted by transfected COS cells. As the molecular mass of the recombinant LCAT studied here is similar to the LCAT protein purified from human plasma, we speculate that all potential glycosylation sites are also used during the synthesis and secretion of native LCAT by hepatocytes *in vivo*.

A band of immunoprecipitable LCAT was also observed in the cell lysates of the transfected COS cells. However, the molecular mass was lower by approx. 4–5 kDa when compared with the secreted form of LCAT. As only a single intracellular band was observed, it seems likely that this form of LCAT represents the substrate for a rate-limiting step in LCAT processing by COS cells. Moreover, as a similar increase in molecular mass was observed for all LCAT species studied, it appears that this slow step in LCAT transport and secretion was not affected by the absence of glycosylation at any of the four sites. However, the mechanisms associated with the accumulation of intracellular LCAT and its further processing before secretion cannot be addressed by the present study.

Our primary objective in this study was to examine the role of *N*-glycosylation in the biological function of LCAT. There was no significant change in the secretion rate of the mutant proteins carrying a single mutation from transiently transfected COS cells. However, the absence of *N*-glycosylation at each site had diverse effects on the activity of LCAT for proteoliposome substrates. For example, the loss of the N-linked glycosylation site at position 84 produced a mutant LCAT with substantially decreased enzyme activity (18% of the wild-type enzyme). The LCAT proteins containing a mutation at position 20 or 272 also displayed lower enzyme activity. This result indicates that glycosylation at Asn^{84} may have an important role in the catalytic activity of LCAT or the interaction of the enzyme with substrates. Interestingly, the absence of glycosylation at position 384 resulted in a 2-fold increase in the enzyme activity. This result suggests that the carbohydrate group at position 384 has an inhibitory effect on LCAT activity possibly by sterically hindering access of the substrate to the active site. In contrast, the secretion and catalytic activity of the quadruple mutant was reduced by approx. 90%. Such a decrease might reflect the requirement of the correct conformation of LCAT protein for efficient secretion by COS cells as well as enzyme activity. Alternatively, loss of N-linked glycosylation might change the secondary structure of the LCAT protein so that the improperly folded unglycosylated enzyme might be degraded more rapidly within the cells. As caution should be exercised when interpreting results obtained from site-directed mutagenesis, we also examined the secretion and activity of unglycosylated LCAT synthesized by transfected COS cells in the presence of tunicamycin, an inhibitor of N-linked glycosylation [21]. The unglycosylated protein exhibited similar properties to those of the quadruple mutant.

As apo A-I is a cofactor for the LCAT reaction, the mechanism by which apo A-I activates this enzyme has been extensively studied [3,4,22]. In the present study, we examined the effect of changes in *N*-glycosylation of LCAT on its activation by apo A-I. In agreement with the results obtained with the plasma enzyme, the wild-type recombinant LCAT was activated by apo A-I in a dose-dependent manner. The activation by apo A-I was also observed in the reaction catalysed by the mutant enzymes carrying a mutation at one of the four *N*-glycosylation sites. These results suggest that the removal of some of the N-linked carbohydrate groups does not affect LCAT activation by apo A-I.

We have also examined the ability of mutant LCAT to esterify cholesterol in human plasma. We found that partial or complete loss of *N*-glycosylation had a profound effect on LCAT activity towards plasma substrates. Changes in the consensus sequence at

all four *N*-glycosylation sites completely abolished the ability of the enzyme to utilize substrates in the plasma. The apparent $V_{max.}/K_m$ ratios have been used by a number of investigators to study the kinetics of the LCAT reaction [23–25]. In the present study, this parameter was decreased for the mutants carrying a substitution (Asn to Gln) at position 20 or 272. These results suggest that the efficiency with which these two mutant enzymes utilized substrates in the plasma was reduced. Kinetic analysis also revealed that the increased activity associated with the N384Q mutant enzyme was not a result of an enhanced affinity of the enzyme for the substrate, but was due to more efficient utilization of the substrate, as the ratio $V_{max.}/K_m$ was about twice as high as that of the wild-type enzyme. This suggests that the oligosaccharide at position 384 has an inhibitory effect on LCAT activity, but the exact mechanism remains unclear. The N84Q mutant exhibited lower reactivity with plasma substrates, and the quadruple mutant completely lost the ability to utilize substrates in the plasma.

In summary, the present study has demonstrated that all four *N*-glycosylation sites of LCAT are utilized: two are in the N-terminal region (Asn^{20} and Asn^{84}) and two are in the C-terminal region (Asn^{272} and Asn^{384}). Glycosylation of all four sites is required to generate the full-size mature LCAT protein. Although complete N-linked glycosylation does not appear to be required for LCAT secretion, the pattern of glycosylation has a profound effect on the catalytic activity of the enzyme.

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