G., Luo, M., Mosser, A. G., Rueckert, R. R., Sherry, B., & Vriend, G. (1985) *Nature (London) 317*, 145-153.
Roux, B., Yu, H.-A., & Karplus, M. (1990) *J. Phys. Chem. 94*, 4683-4688.
Ryckaert, J.-P., Ciccotti, G., & Berendsen, H. J. C. (1977) *J. Comput. Phys. 23*, 327-341.
Sheriff, S., Hendrickson, W. A., & Smith, J. L. (1987) *J. Mol. Biol.* 197, 273-296.
Shih, H. H.-L., Brady, J., & Karplus, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1697-1700.
Shortle, D., & Lin, B. (1985) *Genetics 110*, 539-555.
Shortle, D., & Meeker, A. K. (1986) *Proteins 1*, 81-89.
Singh, U. C., Brown, F. K., Bash, P. A., & Kollman, P. A. (1987) *J. Am. Chem. Soc. 109*, 1607-1614.
Tembe, B. L., & McCammon, J. A. (1984) *Comput. Chem. 8*, 281-283.
Thanki, N., Thornton, J. M., & Goodfellow, J. M. (1988) *J. Mol. Biol. 202*, 637-657.
Warshel, A., Sussman, F., & King, G. (1986) *Biochemistry 25*, 8368-8372.
Weaver, L. H., & Matthews, B. W. (1987) *J. Mol. Biol. 193*, 189-199.
Weaver, L. H., Gray, T. M., Grütter, M. G., Anderson, D. E., Wozniak, J. A., Dahlquist, F. W., & Matthews, B. W. (1989) *Biochemistry 28*, 3793-3797.
Wiley, D. C., & Skehel, J. J. (1987) *Annu. Rev. Biochem. 56*, 365-394.
Wolfenden, R., Anderson, L., Cullis, P. M., & Southgate, C. C. B. (1981) *Biochemistry 20*, 849-855.
Wong, C. F., & McCammon, J. A. (1986) *J. Am. Chem. Soc. 108*, 3830-3832.
Zwanzig, R. W. (1954) *J. Chem. Phys. 22*, 1420-1426.

Effects of Inhibitors of N-Linked Oligosaccharide Processing on the Secretion, Stability, and Activity of Lecithin:Cholesterol Acyltransferase†

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ABSTRACT: The structure and function of the carbohydrate moiety of human lecithin:cholesterol acyltransferase (LCAT) were determined by using several glycosidases in reaction with the isolated plasma protein or by using specific inhibitors of glycoprotein assembly with cultured cells secreting LCAT activity. Analysis of the plasma enzyme indicated that almost all of the large carbohydrate moiety of LCAT (approximately 25% w/w) was N-linked with part of the high-mannose and part of the complex type. This analysis was confirmed with metabolic inhibitors of carbohydrate processing by using CHO cells stably transfected with the human LCAT gene. Inhibitors of the subsequent processing of the N-linked high-mannose chains formed by glucosidase activity were without effect on either the secretion rate or the catalytic activity of LCAT. The inhibition of catalytic activity by glucosidase inhibitors applied to both the phospholipase and the acyltransferase activities of LCAT. The reduction of the LCAT catalytic rate by terminal glycosidase inhibitors was without effect on apparent K_m and did not affect enzyme stability. These data indicate an unusual specific role for high-mannose carbohydrates in the catalytic mechanism of LCAT.

Lecithin:cholesterol acyltransferase (LCAT), a glycoprotein of M_r 63 000 synthesized and secreted by the liver, is an integral part of several high-density lipoprotein (HDL) species (Fielding & Fielding, 1981; Cheung et al., 1986; Francone et al., 1989). The enzyme catalyzes the transfer of an acyl group from the 2-position of phosphatidylcholine to the 3-position hydroxyl group of cholesterol and as such is believed to be responsible for generating a major fraction of plasma cholesteryl esters (Glomset, 1983).

The primary structure of human LCAT has been determined by cDNA and amino acid sequencing (Mclean et al., 1986; Yang et al., 1987). This reveals several regions of clustered hydrophobic residues, which may be related to the lipid-binding functions of the enzyme. The LCAT sequence also includes four potential N-glycosylation sites (Asn-X-Ser/Thr) at positions 20, 84, 272, and 384 of the 416-residue protein moiety. Total carbohydrate content has been estimated at about 25% of the total LCAT mass (Chung et al., 1979; Chong et al., 1983). In vitro modification of the sialic acid content of LCAT was shown to increase catalytic activity about 1.5-fold (Doi & Nishida, 1983). Otherwise, there has been little research on the potential role of the LCAT carbohydrate moiety on the secretion, stability, or functions of the enzyme protein.

The initial transfer of dolichol-linked glucose-capped high-mannose assemblies to polypeptide asparagine residues, and finally the modification of these units into the oligosaccharide sequences of the mature protein, proceeds first in the endoplasmic reticulum (ER) and subsequently within the Golgi apparatus (Snider, 1984; Kornfeld & Kornfeld, 1985). Within the ER, sequential glucosidase activities generate

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uncapped high-mannose chains. Subsequent metabolic steps involve mannose trimming by specific glycosidases in the ER and Golgi, followed by the addition of hexose, hexosamine, and sialic acid residues in the Golgi compartment. Specific inhibitors have been identified for many of the reactions involved. In the present research, we have used both CHO cells stably transfected with the human LCAT gene (McLean et al., 1986) and LCAT-secreting human hepatoblastoma (HepG2) cells (Chen et al., 1986; Erickson & Fielding, 1986) to determine possible functions of the N-linked sugar moiety in the secretion, stability, and activity of this enzyme.

Experimental Procedures

Cell Culture. CHO cells deficient in dihydrofolate reductase (DHFR) stably cotransfected with the LCAT and DHFR genes (LCAT–CHO cells) were kindly provided by Dr. Richard Lawn, Genentech Inc., South San Francisco, CA. These were cultured in a mixture of equal volumes of Dulbecco's modified Eagle's medium (DME) and Ham's F12 medium containing 10% fetal calf serum and 5 μg/mL gentamycin. The medium also contained 50 μM methotrexate, a potent inhibitor of DHFR function, to select for the coexpression of LCAT and DHFR genes (Kaufman & Sharp, 1982). Control (nontransfected) CHO cells (line CHO-KI) were grown under the same conditions. Both lines were seeded in 25-cm^2 flasks and cultured at 37 °C in 5% CO_2 for 5–6 days before use. Human hepatoma cells (HepG2) were cultured in DME with 10% (v/v) fetal calf serum (Erickson & Fielding, 1986). To determine LCAT secretion rates in the presence or absence of inhibitors, the cells were washed six times and then cultured in the same medium without fetal calf serum.

Immunoassay of Secreted LCAT. Immunoassays were carried out by using nitrocellulose membranes (0.45 μM Sartorius, West Coast Scientific, Oakland, CA) as the solid phase (Barkia et al., 1988). Anti-LCAT antibody raised in rabbits to the synthetic polypeptide corresponding to LCAT residues 165–183 (Francone et al., 1989) was affinity purified (Sydkowski & Fisher, 1985). Samples of cell culture medium and of pure standard were applied. The support was blocked with 5% bovine serum aluminin in 0.15 M NaCl and 0.05 M Tris-HCl (pH 7.4) for 30 min at 37 °C. Antibody was applied in 2% bovine serum albumin containing 0.2% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO), and incubation was carried out overnight at room temperature. The support was then washed three times (30 min) in albumin–0.2% Nonidet buffer and subsequently reacted (30 min) with goat anti-rabbit IgG labeled with ^{125}I (Castro & Fielding, 1988) and finally washed again three times. Bound radioactivity was determined by γ scintillation spectrometry. Radioactivity was proportional to LCAT mass up to 100 ng of protein.

LCAT Assay. LCAT activity was assayed from the medium of CHO or HepG2 cells by using lecithin–[^{3}H]cholesterol single-walled vesicles activated with apolipoprotein A-I (Fielding et al., 1972). [1,2-^{3}H]Cholesterol in benzene solution was obtained from New England Nuclear, Boston, MA. The label was repurified by thin-layer chromatography before use on silica gel layers (Merck) developed in cyclohexane–benzene (4/1 v/v). Liposomes containing egg lecithin (800 μg/mL) and unesterified cholesterol (100 μg/mL) [cholesterol specific activity (0.6–1.2) × 10^5 dpm/μg] were prepared by using the French pressure cell (Hamilton et al., 1980). Activation was carried out with human apolipoprotein A-I (final concentration 100 μg/mL) (Sigma Chemical Co., St. Louis, MO). Each assay contained 50 μL of dispersed lipid and an equal volume of recrystallized human albumin (20% w/v), 100 μL of 40 mM Tris-HCl buffer (pH 7.4) in 0.15 M NaCl, and up to 200 μL of cell culture medium in a total assay volume of 400 μL. Incubation was allowed to proceed for up to 60 min at 37 °C. The reaction was cooled in ice water and extracted with 800 μL of chloroform–methanol (1/1 v/v). Labeled cholesteryl ester generated by LCAT and extracted into the chloroform phase was separated by thin-layer chromatography on silica gel layers developed in hexane–diethyl ether–acetic acid (83/16/1 v/v), and radioactivity was determined by liquid scintillation spectrometry. Production of labeled cholesteryl ester was linear for at least 60 min.

To determine LCAT-dependent phospholipase activity, unesterified cholesterol was deleted from the assay medium, and lecithin was labeled with the incorporation of 2-[9,10-^{3}H]dipalmitoyllecithin (New England Nuclear) into the liposomes. Phospholipase activity was determined from the rate of production of labeled unesterified fatty acid (Aron et al., 1978).

Tunicamycin (TNM), which inhibits the synthesis of the dolichol-linked sugars initially attached to asparagine sites (Tkacz & Lampen, 1975), and the protonophore carbonyl cyanide *m*-chlorophenylhydrazone were both from Calbiochem-Behring, La Jolla, CA. Inhibitors of glycoprotein carbohydrate processing, swainsonine (SWSN), castanospermine (CSTP), (+)-1-deoxymannojirimycin (1-MWN), *N*-methyldeoxynojirimycin (NM-DNJ), and (+)-1-deoxynojirimycin (1-DNJ), were from Genzyme, Boston, MA. CSTP and MM-DNJ are specific for glucosidase I in the endoplasmic reticulum (Pan et al., 1983), while 1-DNJ inhibits both glucosidases I and II in the same compartment (Saunier et al., 1982). These three reagents therefore inhibit the removal of the glucose caps from high-mannose asparagine-linked oligosaccharides. 1-MMN and SWSN inhibit mannosidases I and II and therefore inhibit the trimming of uncapped high-mannose chains in the endoplasmic reticulum and Golgi compartments (Tulsiani et al., 1982; Fuhrman et al., 1984). Glycosidases, *N*-glycanase [peptide–N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase], endo-β-*N*-acetylglucosaminidase F (endoF), endo-β-*N*-acetylglucosaminidase H (endoH), and neuraminidase were from Genzyme, Boston, MA. [^{35}S]Methionine (600 Ci/mmol) was from New England Nuclear, Boston, MA. Incorporation of this label into total cell protein was determined as label present in the TCA-insoluble precipitate from extracts of control and inhibited cell cultures. Other reagents were as previously reported (Aron et al., 1978).

Purification of LCAT. LCAT was purified from normal human plasma by ultracentrifugation followed by sequential chromatography on phenylagarose, DEAE-cellulose, and hydroxyapatite (McLean et al., 1986). The purity of the isolated protein was confirmed by silver staining following SDS–polyacrylamide gel electrophoresis.

LCAT was also purified from the medium of CHO cells transfected with the human LCAT gene. Medium (40 mL) from T175 flasks of cells incubated for 12 h in the presence or absence of inhibitors of carbohydrate processing was passed through columns of phenylagarose (Pharmacia; 7.0 × 0.5 cm) that had been equilibrated in 3 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4). After being washed with 20 mL of Tris–EDTA, adsorbed LCAT was eluted with distilled water. The distilled water fraction (which contained all of the LCAT activity) were lyophilized, and the product was used in the assays of carbohydrate structure described below.

Enzymatic Deglycosylation of LCAT. To deglycosylate, all samples were boiled (3 min) in a final concentration of 0.5% SDS and 0.1 M β-mercaptoethanol, and then a mixed solution

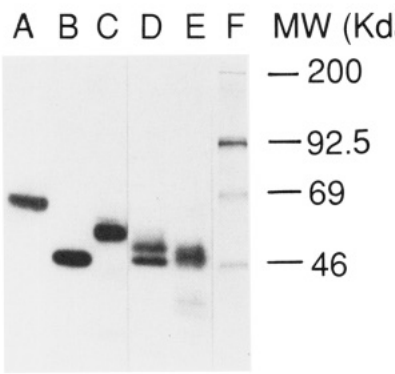

FIGURE 1: Effects of glycosidase treatment on plasma LCAT. The molecular weight of native human plasma LCAT or LCAT treated with the glycosidases indicated was determined by SDS–polyacrylamide gel electrophoresis, and LCAT protein was detected immunologically as described under Experimental Procedures. Lane A, native plasma LCAT; lane B, LCAT + *N*-glycanase; lane C, LCAT + neuraminidase; lane D, LCAT + endoglycosidase F; lane E, LCAT + neuraminidase + endoglycosidase F.

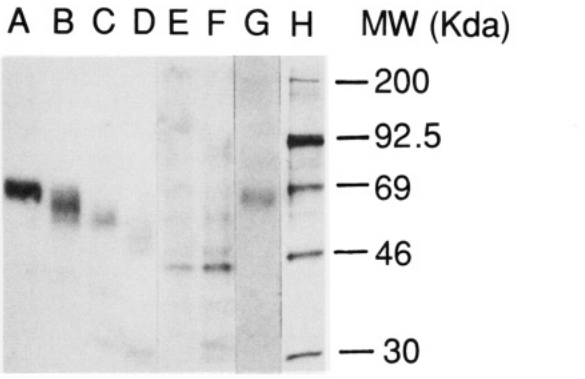

FIGURE 2: Effects of glycosidases and inhibitors of glycosylation on LCAT secreted from transfected CHO cells. Electrophoresis and visualization were as described in the legend to Figure 1. Lane A, native LCAT; lane B, LCAT + endoglycosidase F; lane C, LCAT from cells pretreated with 1-MMN (200 μg/mL); lane D, LCAT from cells pretreated with 1-MMN and digested with endoglycosidase F; lane E, LCAT from cells pretreated with TNM (5 μg/mL); lane F, LCAT from cells pretreated with TNM and digested with endoglycosidase F; lane G, native LCAT; lane H, molecular weight standards. Comparable results were obtained with endoH under the same protocol.

of protease inhibitors (Castro & Fielding, 1988) was added. Desialylation of purified plasma LCAT (100 ng, 10 μL) was carried out by addition of 7 μL of sodium acetate buffer (0.44 M), pH 6.5, and 5 μL of 7.5% Nonidet P-40. The sample was then incubated for 2 h at 37 °C with 5 μL of neuraminidase (1 unit/mL final concentration). *N*-Glycanase treatment to remove all N-linked oligosaccharide chains was carried in the presence of 11 μL of sodium phosphate (0.55 M), pH 8.7, 5 μL of Nonidet P-40 (7.5%), and 2 μL of *N*-glycanase (5 units/mL final concentration). The reaction mixture was incubated overnight at 37 °C. EndoF cleaves the glycosidic bonds of the chitobiose core structure of many high-mannose and biantennary complex N-linked sugars. Denatured samples were diluted with 7 μL of sodium acetate buffer (0.44 M), pH 6, 5 μL of Nonidet P-40 (7.5%), and 5 μL of endoF (10 milliunits/mL final concentration). When the sample was pretreated with neuraminidase, endoF was added directly and the solution was incubated overnight at 37 °C. EndoH catalyzes the hydrolysis of the chitobiose core of high-mannose and certain hybrid oligosaccharides. The samples were treated as indicated for endoF, except that the buffer was 0.44 M sodium citrate, pH 6. At the end of each enzymatic digestion, a 0.5 volume of buffer [0.2 M Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 0.08% bromophenol blue, and 40% glycerol] was added to the samples, and the solution was heated again at 100 °C for 3 min. Electrophoretic separation of samples was carried out on 10% SDS–polyacrylamide minigels (Novex, Encinitas, CA) at 125 V for 2 h at room temperature. Gels were calibrated with the following ^{14}C-methylated standards (Amersham): myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa). The gels were transferred overnight onto nitrocellulose (Castro & Fielding, 1988), and LCAT protein was detected with anti-LCAT antibody as described previously (Francone et al., 1989).

Results and Discussion

Carbohydrate Structure of LCAT. As shown in Figure 1, SDS–polyacrylamide gel electrophoresis of plasma LCAT showed an apparent molecular weight for the protein of about 67 000, consistent with earlier determinations using the same procedure (Aron et al., 1978; Chung et al., 1979). When N-linked carbohydrate was digested with *N*-glycanase, LCAT was completely converted into a single species with an apparent molecular weight of about 46 000, which was comparable to the molecular weight (46 917) of the mature protein moiety (Fielding, 1990). This finding indicates that LCAT contained a large N-linked carbohydrate moiety, with little, if any, *O*-linked sugar. Reaction with neuraminidase confirmed previous data (Doi & Nishida, 1983) indicating a high sialic acid content in LCAT as part of the more complex-type carbohydrate chains. Treatment with endoF also reduced LCAT molecular weight, additionally indicating the presence of high-mannose chains. The presence of a molecular weight doublet under these conditions suggests heterogeneity within plasma LCAT. The same phenomenon has been reported for the secretion of Sindbis virus glycoproteins secreted from mammalian cells (Hsieh et al., 1983).

The molecular weight of LCAT secreted by stably transfected CHO cells (Figure 2) was indistinguishable from that of the plasma enzyme. As in the case of the plasma enzyme treated by *N*-glycanase, tunicamycin, which prevented the addition of N-linked carbohydrate chains, reduced the molecular weight of LCAT to a value comparable to that of the carbohydrate-free enzyme. This confirmed the preponderance of N-linked sugars in this enzyme. Inhibitors of high-mannose residue processing also decreased the molecular weight of LCAT, consistent with their expected activity in preventing the assembly of higher molecular weight complex chains. This conclusion is confirmed by the sensitivity of these chains to endoF. Similar results were obtained with endoH.

Inhibition of Intracellular Transport. The studies described above indicate that newly secreted LCAT, like the circulating plasma protein, contained a substantial N-linked carbohydrate moiety whose assembly was blocked by several metabolic inhibitors. Studies were then carried out to determine the effects of these inhibitors on the secretion and properties of LCAT secreted by CHO cells stably transfected with the LCAT cDNA and of that secreted by HepG2 cell lines. Under the conditions described above, the accumulation of LCAT activity in serum-free medium of LCAT–CHO cells was 132 ± 29 pmol of cholesterol esterified mL^{-1} h^{-1}. After 12 h, the mass of LCAT protein in the medium, measured by solid-phase immunoassay as described under Experimental Procedures, was 0.72 ± 0.18 μg/mL (n = 6). Control (nontransfected) CHO–KI cells cultured under the same conditions secreted no detectable LCAT protein. Part (35.5 ± 5.5%) of the total

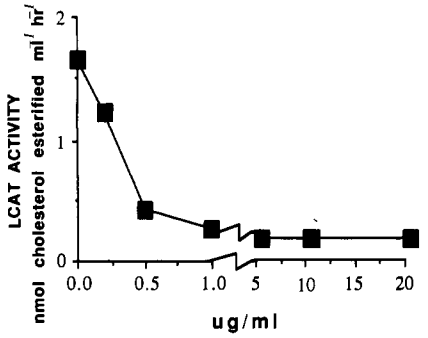

FIGURE 3: Inhibition of LCAT activity in the medium of transfected CHO cells in the presence of tunicamycin (TNM). Cells were cultured for 12 h in DME-Ham's medium without serum in the presence of the concentrations of TNM shown. LCAT activity was assayed with [^{3}H]cholesterol-lecithin liposomes as described under Experimental Procedures.

LCAT activity was recovered with the CHO-transfected cells after removal of the medium. After the cells were washed (six times) with DME-H16/F12 medium, this activity decreased to 16.4 ± 4.6% of the original LCAT activity in the medium. To further analyze this material, the washed cells were treated with 0.002% trypsin/0.01% EDTA (w/v) at 20 °C for 15 min. The cells were recovered by centrifugation at 1000*g* for 10 min at 4 °C and were then washed twice more. There was no further reduction in cell-associated LCAT protein.

The transit of cellular proteins from the endoplasmic reticulum (ER) to the Golgi complex is inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Tartakoff & Vassalli, 1979). The distribution of LCAT mass between medium and cells was determined, and the effect of CCCP was determined over a range of inhibitor concentrations. In the absence of inhibitor, 12% of LCAT was cell associated. In the presence of 5 μM inhibitor, when total activity was reduced to 5% of the original value, cell-associated LCAT was 20% of the total. In presence of 10 and 20 μM inhibitor, the activity in the medium was reduced to 0.6% and 0.5% of control values, respectively, and no detectable activity was found associated with the cells. This finding indicated that LCAT was actively secreted from the site of synthesis in the endoplasmic reticulum through the Golgi pathway.

Effects of Inhibitors of N-Linked Sugar Processing on LCAT Activity. TNM strongly inhibited the appearance of medium LCAT activity from CHO cells stably transfected with the human LCAT gene (Figure 3). The concentration required for half-maximal effect (about 1–5 μg/mL) is similar to that used to inhibit protein N-glycosylation in other systems (Struck et al., 1978). There was no effect on LCAT protein secretion (Table I) even at concentrations as high as 10 μg of TNM/mL. This is consistent with the effect of tunicamycin on other glycoproteins such as apolipoprotein E (Zannis et al., 1986) and apolipoprotein B and transferrin (Struck et al., 1978) secreted by rat hepatocytes and interferon secreted by leukocytes (Mizrahi et al., 1978). In others systems TNM reduced protein secretion, as in the case of immunoglobulins IgA and IgE (Hickman et al., 1977) and α_2-macroglobulin (Bauer et al., 1985). While the reasons for these differences are unknown, there is clearly little, if any, requirement for N-linked carbohydrate for the effective secretion of LCAT protein. The same data do indicate a requirement for one or more N-linked oligosaccharide chains for full LCAT activity.

N-linked oligosaccharide requirements for effective LCAT activity were then further investigated by using specific inhibitors of ER and Golgi processing enzymes. Inhibitors of individual processing of N-linked sugars fall into two discrete

Table I: Effects of Inhibitors of Glycoprotein Processing on the Accumulation of LCAT Mass and Activities[a]

inhibitor	protein mass	acyltransferase	phospholipase
none	100	100	100
SWSN	91 ± 17	103 ± 21	82, 85
MMN	94 ± 13	94 ± 9	101, 93
CST	88 ± 17	20 ± 4	22, 34
DNJ	120 ± 27	10 ± 2	26, 23
NMDNJ	92 ± 16	14 ± 2	26, 22
TNM	103 ± 10	20 ± 6	13, 20

[a] Values are expressed relative to the mass of LCAT secreted over 12 h in the absence of inhibitor (0.72 ± 0.18 μg of LCAT protein mL^{-1}) and to the rate of LCAT and phospholipase activities under the same conditions (1.55 ± 0.35 nmol mL^{-1} h^{-1} and 1.48 nmol mL^{-1}1 h^{-1}1, respectively). The specific activity of LCAT secreted under these conditions was about 20% of that obtained with highly purified plasma LCAT (Aron et al., 1978). Mass and acyltransferase measurements are the mean ± SD for three to four experiments. Phospholipase activities are the values from two separate experiments. Concentrations of inhibitors were as follows: swainsonine (SWSN), 10 μg/mL; deoxymannojirimycin (MMN), 200 μg/mL; castanospermine (CST), 100 μg/mL; deoxynojirimycin (DNJ), 200 μg/mL; methyldeoxynojirimycin (NMDNJ), 200 μg/mL; tunicamycin (TNM), 5 μg/mL.

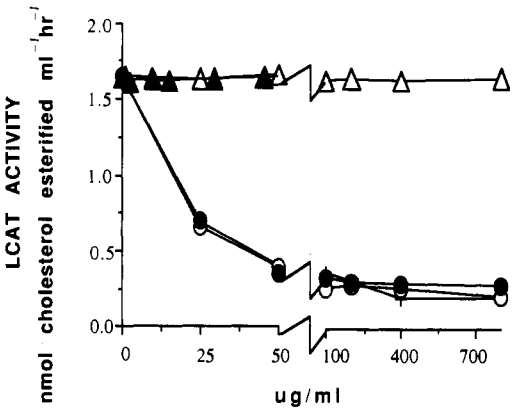

FIGURE 4: Inhibition of LCAT activity in the medium of transfected CHO cells in the presence of glucosidase and mannosidase inhibitors. Cells were incubated in DME-Ham's medium with the glucosidase inhibitors (●) castanospermine (CSTP), (○) methyldeoxynojirimycin (NM-DNJ), and (+) 1-deoxynojirimycin (1-DNJ), or the mannosidase inhibitors (△) deoxymannojirimycin (1-MMN) and (▲) swainsonine (SWSN). Incubation was for 12 h at 37 °C as described in the legend to Figure 3.

groups. Inhibitors of the hydrolysis in the endoplasmic reticulum of the glucose caps from the high-mannose chains (glucosidases I and II) were (like tunicamycin) very effective in suppressing LCAT activity (Figure 4). All three glucosidase inhibitors used in this study (CSTP, MM-DNJ, and 1-DNJ) strongly inhibited the accumulation of medium LCAT activity. None had any effect on the rate of accumulation of LCAT protein (Table I). Total protein synthesis in these cells (followed with [^{35}S]methionine) was slightly (16%) reduced under the same conditions. On the other hand, inhibitors of the microsomal and Golgi mannosidases, which catalyzed the subsequent trimming of these high-mannose chains (SWSN, 1-MMN), were without any effect on either LCAT activity or LCAT protein secretion, even at high concentrations (Figure 4). The specificity of these effects was maintained over the entire period, indicating the ongoing secretion of poorly active enzyme. Consistent secretion rates of LCAT activity in the presence and absence of inhibitors were obtained in different experiments (Table I).

Both control and partially inhibited LCAT activities from control cells and cells treated with glycosidase inhibitors were abolished (95%) by the addition of 1.5 mM dithiobis(2-

Table II: Inhibition of LCAT Activity in Human Hepatoblastoma (HepG2) Cell Medium

	LCAT activity[a] [pmol of cholesteryl ester h^{-1} (mL of medium)$^{-1}$]					
expt	control	SWN	MMN	NMDNJ	TNM	CST
1	29.9	27.3			8.9	8.3
2	25.3		30.6	5.7	6.0	8.6
3	26.0		27.2	2.7	5.2	4.4
mean ± SD	27.1 ± 2.5	27.3	28.9	4.2	6.7 ± 1.9	7.1 ± 2.3

[a] The identification and concentration of inhibitors were as given in the legend to Table I. LCAT activity was determined after 24 h of incubation with serum-free medium by using [^{3}H]cholesterol–lecithin liposomes.

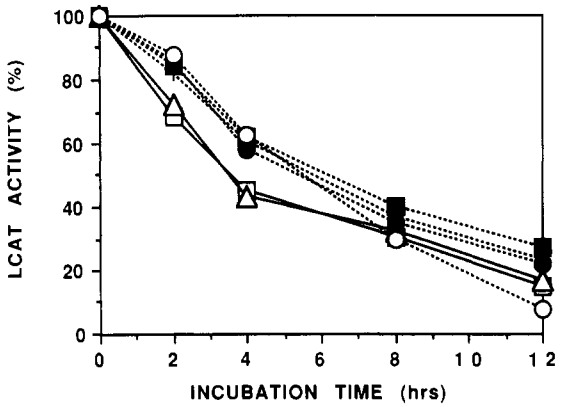

FIGURE 5: Stability of CHO cell derived LCAT activity. Medium from control cells or cells incubated with glucosidase or mannosidase inhibitors was incubated at 37 °C over the time course indicated. Interrupted lines indicate glucosidase inhibitors. Symbols for individual inhibitors are the same as in Figure 4. Continuous lines indicate (□) control cells or (△) cells in the presence of the mannosidase inhibitor 1-MMN.

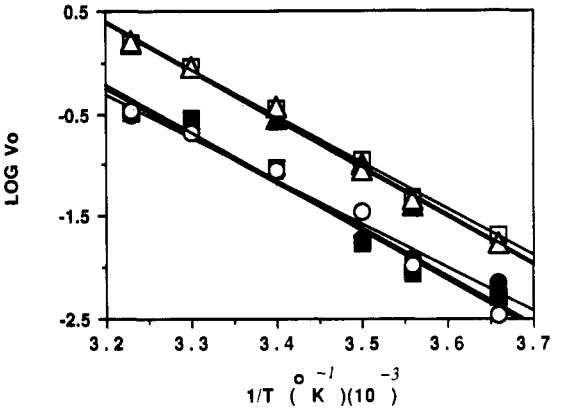

FIGURE 6: Arrhenius plot of LCAT activity from CHO cell medium over the temperature range 0–37 °C. The calculated values were fitted by least-squares analysis. The symbols for inhibitors are (□) control, (■) TNM, (△) 1-MMN, (▲) SWSN, (○) NM-DNJ, and (●) CSTP. The experimental points were fitted by least-squares analysis.

nitrobenzoic acid), which blocks LCAT activity by binding to an essential free sulfhydryl residue. This is comparable to the inhibition obtained with the plasma enzyme under the same conditions (Stoke & Norum, 1971).

These data suggest that LCAT activity (but not the synthesis or secretion of LCAT protein) is dependent upon the coupling of the N-linked sugar to the nascent LCAT polypeptide of at least one uncapped high-mannose chain.

As shown in Table II, an essentially identical pattern of inhibition was obtained for the much lower levels of LCAT [1.13 ± 0.1 pmol h^{-1} (mL of medium)$^{-1}$] secreted from the human hepatoblastoma line HepG2. This indicates a common requirement for N-linked sugar processing in the human hepatoma and transfected hamster cell lines.

Mechanism of Inhibition of LCAT Activity by N-Glycoprotein Glucosidase Inhibitors. Several functions have been suggested for the glycoprotein oligosaccharide moiety. In some systems the N-linked carbohydrate moiety may serve to promote the transport of glycoproteins from the endoplasmic reticulum to the Golgi compartment (Lodish, 1988). This is not the case with LCAT (like several other secreted glycoproteins), since none of the inhibitors reduced LCAT protein secretion rates. A second mechanism would involve the stabilization of protein three-dimensional structure (Rose & Domes, 1988; Hurtley & Helenius, 1989). In spite of the hydrophobic structure of the LCAT protein (McLean et al., 1986; Yang et al., 1987) and its large carbohydrate moiety (Chung et al., 1987; Chong et al., 1983; Doi & Nishida, 1983), a consistent stabilization (instead of the expected destabilization) of LCAT activity was obtained at 37 °C (Figure 5). This finding makes it unlikely that the N-linked sugar chains of LCAT have a stabilizing function in solution.

In view of the above, further experiments were directed toward investigating the effects of microsomal glucosidase inhibitors on the LCAT enzyme mechanism. In the absence of cholesterol, LCAT functions as a phospholipase in which water instead of cholesterol is the acceptor of the long-chain acyl group transferred from lecithin (Piran & Nishida, 1976; Aron et al., 1978). Glucosidase inhibitors reduced the phospholipase activity of LCAT to the same extent and with the same specificity as was obtained with the transferase activity of the same enzyme (Table I).

There is evidence from several laboratories that the rate-limiting step of the LCAT reaction is in the turnover of an acyl-enzyme intermediate that is the common precursor of the fatty acid or cholesteryl ester products of the phospholipase and transferase reactions, respectively (Aron et al., 1978; Jauhianen & Dolphin, 1986). Further analysis of substrate kinetics with a variety of soluble and interfacial esters indicated that the apparent Michaelis constant is probably proportional to the equilibrium constant of such an acyl-enzyme complex (Jonas et al., 1987). Further kinetic analysis of the mechanism of inhibition was undertaken by a study of the temperature and concentration dependence of the normal reactions and the reactions reduced by the activity of terminal glucosidase inhibitors. The concentration dependence of the activity was expressed in terms of its double-reciprocal plot. The apparent K_m of LCAT activity was 21.4 ± 3.0 mM cholesterol for native LCAT and 19.3 ± 3.3 mM for LCAT secreted from glucosidase-inhibited cells (n = 3 in both cases). Such values are similar to those previously reported (Matz & Jonas, 1982) and indicate that the apparent K_m was essentially unchanged by these inhibitors. On the other hand, the maximum reaction velocity is reduced about five-fold. The calculated V_{max} for the native enzyme was 0.64 ± 0.14 nmol mL^{-1} h^{-1} vs 0.15 ± 0.06 nmol mL^{-1} h^{-1} for the glycosidase-inhibited enzyme. The mannosidase inhibitors in Table I were without effect on either apparent K_m or V_{max}. The N-linked oligosaccharide therefore appears not to act to modify lipid binding, for example by

modulating the hydrophobicity of the substrate binding site, but to act more directly by affecting the rate of covalent catalysis at the active site.

Further studies indicated that the temperature dependence of normal and glucosidase-inhibited enzymes was unchanged by inhibition (Figure 6). The free energy (E) for the control and mannosidase-treated enzymes (21.3 ± 0.5 kcal/mol) was not significantly different from that of the glucosidase-treated inhibited enzyme preparations (20.9 ± 1.0 kcal/mol). These values are also similar to those previously reported for long-chain lecithins with LCAT purified from human plasma (Jonas et al., 1987).

These data suggest that the effects of glucosidase inhibitors of N-linked oligosaccharide processing in LCAT are specific and most directly affect the catalytic rate without a significant effect on substrate properties or the synthesis or secretion of the enzyme protein. However, it cannot be ruled out that the change in glycosylation pattern associated with the inhibited reaction velocity exerts its effect indirectly, for example by influencing another posttranslational modification. As inhibitors of later processing were without effect on the catalytic rate, it is likely that the oligosaccharide involved is retained as a high-mannose chain.

On average, only about one-third of potential sites for N-linked sugars in mammalian proteins are substituted (Struck & Lennartz, 1980). There are some data suggesting that sites in the anterior part of the polypeptide chain are more likely to be modified (Pollack & Atkinson, 1983). Two of four potential sites in LCAT (those at residues 20 and 84) are in the anterior half of the molecule where available data places other functional groups also (Fielding, 1990). There is some local similarity between the sequence at position 84 (-YN_{84}RSS-) and the site of the sole high-mannose chain in lipoprotein lipase (LPL) (-FN_{43}HSS-) (Wion et al., 1987; Semenkovich et al., 1990). In LPL this chain is required for both secretion and full activity. In the presence of tunicamycin, adipocytes accumulated catalytically inactive LPL intracellularly (Olivecrona et al., 1987). This is in contrast to the situation with LCAT described here, where the poorly active product of tunicamycin inhibition is secreted normally. The balance of data in the case of LCAT appears to favor a mechanism such that a high-mannose chain, possibly at position 84 in LCAT, interacts sterically with other active site components (Jauhianen & Dolphin, 1986; Park et al., 1987; Farooqui et al., 1988). These data, with other information on the location of active site residues, should help in understanding the mechanism of this key enzyme of plasma cholesterol metabolism.

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REFERENCES

Aron, L., Jones, S., & Fielding, C. J. (1978) *J. Biol. Chem. 253*, 7220–7226.

Barkia, A., Martin, C., Puchois, P., Guesquiere, J. C., Cachera, C., Tartar, A., & Fruchart, J. C. (1988) *J. Lipid Res. 29*, 77–84.

Bauer, H. C., Parent, J. B., & Olden, K. (1985) *Biochem. Biophys. Res. Commun. 128*, 368–375.

Castro, G. R., & Fielding, C. J. (1988) *Biochemistry 27*, 25–29.

Chen, C. H., Forte, T. H., Cahoon, B. E., Thrift, R. N., & Albers, J. J. (1986) *Biochim. Biophys. Acta 877*, 433–439.

Cheung, M. C., Wolf, A. C., Lum, K. D., Tollefson, J. H., & Albers, J. J. (1986) *J. Lipid Res. 27*, 1135–1144.

Chong, K. S., Jahani, M., Hara, S., & Lacko, A. G. (1983) *Can. J. Biochem. Cell Biol. 61*, 875–881.

Chung, J., Abano, D. A., Fless, G. M., & Scanu, A. M. (1979) *J. Biol. Chem. 254*, 7456–7464.

Doi, Y., & Nishida, T. (1983) *J. Biol. Chem. 258*, 5840–5846.

Erickson, S. K., & Fielding, P. E. (1986) *J. Lipid Res. 27*, 875–883.

Farooqui, J. Z., Wohl, R. C., Kezdy, F. J., & Scanu, A. M. (1988) *Arch. Biochem. Biophys. 261*, 330–335.

Fielding, C. J. (1990) in *Advances in Cholesterol Research* (Esfahani, M., & Swaney, J., Eds.) pp 270–314, Telford Press, Caldwell, NJ.

Fielding, C. J., & Fielding, P. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3911–3914.

Fielding, C. J., Shore, V. G., & Fielding, P. E. (1972) *Biochem. Biophys. Res. Commun. 46*, 1493–1498.

Francone, O. L., Gurakar, A., & Fielding, C. J. (1989) *J. Biol. Chem. 264*, 7066–7072.

Fuhrmann, U., Bause, E., Legler, G., & Ploegh, H. (1984) *Nature 307*, 755–758.

Glomset, J. A. (1983) in *Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., & Frederickson, D. S., Eds.) pp 643–654, McGraw-Hill, New York.

Hamilton, R. L., Goerke, J., Guo, L. S. S., Williams, M. C., & Havel, R. J. (1980) *J. Lipid Res. 21*, 981–992.

Hickman, S., Kulczycki, A., Lynch, R. G., & Kornfeld, S. (1977) *J. Biol. Chem. 252*, 4402–4408.

Hsieh, P., Rosner, M. R., & Robbins, P. W. (1983) *J. Biol. Chem. 258*, 2548–2554.

Hurtley, S. M., & Helenius, A. (1989) *Annu. Rev. Cell Biol. 5*, 277–307.

Jauhiainen, M., & Dolphin, P. J. (1986) *J. Biol. Chem. 261*, 7032–7043.

Jonas, A., Zorich, N. L., Kezdy, K. E., & Trick, W. E. (1987) *J. Biol. Chem.* 262, 3969–3974.

Kaufman, R. J., & Sharp, P. A. (1982) *J. Mol. Biol. 159*, 601–621.

Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.

Lodish, H. F. (1988) *J. Biol. Chem. 263*, 2107–2110.

Matz, C. E., & Jonas, A. (1982) *J. Biol. Chem. 257*, 4541–4546.

McLean, J., Fielding, C., Drayna, D., Dieplinger, H., Baer, B., Kohr, W., Henzel, W., & Lawn, R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2335–2339.

Mizrahi, A., O'Malley, J. A., Carter, W. A., Takatsuki, A., Tamura, G., & Sulkowski, E. (1978) *J. Biol. Chem. 253*, 7612–7615.

Olivecrona, T., Chernick, S. S., Bengtsson-Olivecrona, G., Garrisson, M., & Scow, R. O. (1987) *J. Biol. Chem. 262*, 10748–10759.

Pan, Y. T., Hori, H., Saul, R., Sanford, B. A., Molyneux, R. J., & Elbein, A. D. (1983) *Biochemistry 22*, 3975–3984.

Park, Y. B., Yuksel, K. V., Gracy, R. W., & Lacko, A. G. (1987) *Biochem. Biophys. Res. Commun. 143*, 360–363.

Piran, U., & Nishida, T. (1976) *J. Biochem. (Tokyo) 80*, 887–889.

Pollack, L., & Atkinson, P. H. (1983) *J. Cell Biol. 97*, 293–300.

Rose, J. K., & Domes, R. W. (1988) *Annu. Rev. Cell Biol. 4*, 257–288.

Saunier, B., Kilker, R. D., Tkacz, J. S., Quaroni, A., & Herscovics, A. (1982) *J. Biol. Chem. 257*, 14155-14161.
Semenkovich, C. F., Luo, C.-C., Nakanishi, M. K., Chen, S.-H., Smith, L. C., & Chan, L. (1990) *J. Biol. Chem. 265*, 5429-5433.
Snider, M. D. (1984) in *Biology of Carbohydrates* (Ginsburg, V., & Robbins, P. W., Eds.) Vol. 2, pp 163-198, Wiley-Interscience, New York.
Stokke, K. T., & Norum, K. R. (1971) *Scand. J. Clin. Lab. Invest. 27*, 21-27.
Struck, D. K., & Lennartz, W. J. (1980) in *Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W., Ed.) pp 35-83, Plenum Publishing, New York.
Struck, D. K., Siuta, P. B., Lane, M. D., & Lennartz, W. J. (1978) *J. Biol. Chem. 253*, 5332-5337.
Sydkowski, A. J., & Fisher, J. W. (1985) *J. Biol. Chem. 260*, 14727-14731.
Tartakoff, A. M., & Vassalli, P. (1979) *J. Cell Biol. 83*, 284-299.
Tkacz, J. S., & Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun. 65*, 248-257.
Tulsiani, D. R. P., Harris, T. M., & Touster, O. (1982) *J. Biol. Chem. 257*, 7936-7939.
Wion, K. L., Kirchgessner, T. D., Lusis, A. J., Schotz, M. C., & Lawn, R. M. (1987) *Science 235*, 1638-1641.
Yang, C.-Y., Manoogian, D., Pao, Q., Lee, F.-S., Knapp, R. D., Gotto, A. M., & Pownall, H. J. (1987) *J. Biol. Chem. 262*, 3086-3091.
Zannis, V. I., van der Speck, J., & Silverman, D. (1986) *J. Biol. Chem. 261*, 13415-13421.

The Galactose-Binding Sites of the Cytotoxic Lectin Ricin Can Be Chemically Blocked in High Yield with Reactive Ligands Prepared by Chemical Modification of Glycopeptides Containing Triantennary N-Linked Oligosaccharides†

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ABSTRACT: A glycopeptide containing a triantennary N-linked oligosaccharide from fetuin was modified by a series of chemical and enzymic reactions to afford a reagent that contained a terminal residue of 6-(*N*-methylamino)-6-deoxy-D-galactose on one branch of the triantennary structure and terminal galactose residues on the other two branches. Binding assays and gel filtration experiments showed that this modified glycopeptide could bind to the sugar-binding sites of ricin. The ligand was activated at the 6-(*N*-methylamino)-6-deoxy-D-galactose residue by reaction with cyanuric chloride. The resulting dichlorotriazine derivative of the ligand reacts with ricin, forming a stable covalent linkage. The reaction was confined to the B-chain and was inhibited by lactose. Bovine serum albumin and ovalbumin were not modified by the activated ligand under similar conditions, and we conclude, therefore, that the reaction of the ligand with ricin B-chain was dependent upon specific binding to sugar-binding sites. Ricin that had its galactose-binding sites blocked by the covalent reaction with the activated ligand was purified by affinity chromatography. The major species in this fraction was found to contain 2 covalently linked ligands per ricin B-chain, while a minor species contained 3 ligands per B-chain. The cytotoxicity of blocked ricin was at least 1000-fold less than that of native ricin for cultured cells in vitro, even though the activity of the A-chain in a cell-free system was equal to that from native ricin. Modified ricin that contained only 1 covalently linked ligand was also purified. This fraction retained an ability to bind to galactose affinity columns, although with a lower affinity than ricin, and was only 5- to 20-fold less cytotoxic than native ricin.

Ricin, a toxic lectin isolated from castor beans (*Ricinus communis* beans), is a glycoprotein that consists of two nonidentical subunits, the A-chain (M_r 30500) and the B-chain (M_r 32000), that are linked by a single disulfide bond (Yoshitake et al., 1978; Funatsu et al., 1979; Olsnes & Pihl, 1982). The A-chain is a specific *N*-glycosidase that inactivates the 60S subunit of eukaryotic ribosomes by hydrolyzing the adenine–ribose bond of residue 4324 of the 23S rRNA (Endo et al., 1987; Endo & Tsurugi, 1987). The B-chain has lectin activity and binds to galactose and galactose-terminated oligosaccharides (Olsnes & Pihl, 1973). Following binding to cell surfaces, ricin is internalized (Sandvig & Olsnes, 1982; Youle & Colombatti, 1987) and ultimately the A-chain (or the entire toxin) is transported across a membrane into the cytosol, where free A-chain inactivates ribosomes, ultimately causing cell death. The details of the events that occur between binding of ricin to the cell surface via the B-chain and the inactivation of ribosomes by the A-chain are not known.

The B-chain of ricin has long been known to have two binding sites for galactose[1] or a galactoside (Villafranca &

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