Hepatic Lipase Acylates Dolichol in the Presence of a Plasma Cofactor *in Vitro*

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ABSTRACT: Phosphatidylethanolamine:dolichol acyltransferase (PEDAT), an enzyme previously partially purified and characterized in rat liver [Sindelar, P., Chojnacki, T., & Valtersson, C. (1992) *J. Biol. Chem.* *267*, 20594-20599], is here purified to homogeneity from a heparin perfusate of rat liver and is shown to be identical to hepatic lipase. However, in contrast to triglyceride hydrolysis by hepatic lipase, the PEDAT activity is strongly dependent on a heat-stable plasma cofactor. This cofactor stimulates the activity up to 15-fold and shifts the pH optimum for the reaction from 8.5 to 7.5. Upon gel filtration on Bio-Gel A-1.5, the factor is heterogeneously distributed, with a major peak at 220 kDa. The dolichol-acylating activity can also be detected in rat adrenals and ovaries, and evidence is presented that the PEDAT assay shows a higher degree of specificity for hepatic lipase than the standard assay with triolein-gum arabic emulsion in 1 M NaCl.

Dolichols are primary alcohols containing 14-23 isoprene units and occur in all mammalian tissues, either as free alcohols or as esters of fatty acids and phosphate (Hemming, 1983). The functions of the free alcohols and fatty acyl derivatives are still unknown, while glycosylated dolichyl phosphates are important intermediates in glycoprotein synthesis (Struck & Lennarz, 1980). In rat liver, dolichols and dolichyl esters are associated primarily with lysosomes, Golgi vesicles, and plasma membranes (Chojnacki & Dallner, 1988). Dolichols and their derivatives are synthesized both in the endoplasmatic reticulum (Wong & Lennarz, 1982) and in peroxisomes (Appelkvist & Dallner, 1987), and they are distributed to virtually all other organelles. However, redistribution of dolichol between organs via the blood, which occurs with cholesterol and retinol, does not seem to take place to any great extent, since blood contains very low levels of dolichol (Elmberger et al., 1988) and most organs are capable of synthesizing these lipids themselves (Elmberger et al., 1987).

Two enzymes responsible for the esterification of rat liver dolichol have been characterized. One of these is recovered in the microsomal fraction and utilizes acyl-CoA as substrate (acyl-CoA:dolichol acyltransferase) (Tollbom et al., 1988). The other is an extracellular enzyme, bound to the luminal surface of sinusoidal liver cells, and utilizes PE[1] as acyl donor (PEDAT), and liver perfusions with heparin were employed to partially purify and characterize this protein (Sindelar et al., 1992). PEDAT catalyzes transacylation of the acyl group in the *sn*-1 position of phospholipids, preferentially phosphatidylethanolamine, to dolichol. A fluid membrane structure containing both substrates is required for the reaction. The enzyme demonstrates a very high specifity toward dolichols and their α-unsaturated analogues, polyprenols, as acyl acceptors. In the purification published earlier, PEDAT activity copurified with hepatic lipase activity in three consecutive steps and was thereafter found to be part of a heterogeneous lipid-protein complex with an apparent size of 350 kDa. Further purification was hampered by the fact that the detergent Triton X-100 used to purify hepatic lipase (Kuusi et al., 1979a; Jensen & Bensadoun, 1981) irreversibly inhibited the PEDAT reaction. Various other detergents have since been tested in our laboratory, and Brij-35, employed in the isolation of hepatic lipase from human post-heparin plasma (Ikeda et al., 1989), turned out to be successful.

In the present study, we report the purification of PEDAT to homogeneity and proof that this enzyme is identical to hepatic lipase. We also present evidence for the existence of a cofactor in plasma that is required for maximal dolichol acylation by hepatic lipase.

MATERIALS AND METHODS

Chemicals. Dolichol-19 was isolated from autopsy specimens of human liver by reversed-phase chromatography (Mankowski et al., 1976) and was labeled with [^{3}H]sodium borohydride (57 Ci/mmol, Amersham Corp.) according to Keenan and Kruczek (1976). Upon thin-layer chromatography [^{3}H]dolichol-19 migrated as a single spot.

Isolation of Human Plasma. Blood obtained from male laboratory volunteers who had fasted overnight was collected in standard vacutainer tubes containing EDTA. Plasma was promptly separated at 4 °C by centrifugation at 2000*g* for 15 min. Rat plasma was isolated in a similar manner from male albino rats (Sprague-Dawley) fasted overnight.

Assay of Dolichol Acyltransferase Activity. Small unilamellar liposomes consisting of 2 mol % [^{3}H]dolichol-19 (330 mCi/mmol) and dioleoylphosphatidylcholine:dioleoylphosphatidylethanolamine (3:1) (Sigma) were prepared by sonication of 5 μmol of lipid/mL in 0.2 M Tris-HCl, pH 7.5. Esterification activity was measured in a total volume of 1.0 mL containing 0.2 M Tris-HCl, pH 7.5, 20 mM $CaCl_2$, 50 μL of human plasma, 0-300 μL of enzyme, and 300 μL of the substrate liposomes, unless otherwise indicated. Incubation was conducted at 37 °C for 50 min. The reaction was stopped by adding 15 mL of methanol and 10 mL of petroleum ether, followed by vigorous mixing on a Vortex mixer. After phase separation, the upper (petroleum ether) phase was removed and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 2 mL of hexane and applied to a

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[1] Abbreviations: DE, dolichyl ester; HDL, high-density lipoprotein; HL, hepatic lipase; IDL, intermediate density lipoprotein; PAGE, polyacrylamide gel electrophoresis; PE, phosphatidylethanolamine; PEDAT, phosphatidylethanolamine:dolichol acyltransferase; SDS, sodium dodecyl sulfate.

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column of Kieselgel 60 (Merck) packed in a Pasteur pipet with glass wool as filter. After passage, the gel was washed with an additional 15–20 bed volumes of hexane. The radiolabeled dolichyl esters formed were eluted with 10 bed volumes of 2% diethyl ether in hexane and collected in scintillation vials. The vials were supplemented with 10 mL of 2% 2,5-diphenyloxazole in toluene and thereafter maintained at room temperature for 30 min before counting. Enzyme activity was expressed as nanomoles of dolichyl esters formed during a 50-min incubation period at 37 °C.

Assay of Hepatic Lipase. Triglyceride hydrolysis was determined using a gum arabic-stabilized emulsion of [^{14}C]-triolein (120 mCi/mmol, New England Nuclear) in the presence of 1 M NaCl as described earlier (Ehnholm & Kuusi, 1986). Incubation was conducted at 32 °C for 30 min. The activity was expressed as micromoles of free fatty acids released per hour, by multiplying the activity obtained for the 30-min incubation by 2.

Purification of PEDAT. In order to remove all blood, livers were perfused at room temperature with 150 mL of 0.15 M NaCl at a flow rate of 20 mL/min (Sindelar et al., 1992). To release the enzyme into the perfusate, the perfusion medium was changed to 12 mL of 0.15 M NaCl containing 20 IU of heparin/mL and allowed to recirculate for 5 min. The heparin containing liver perfusates from 10 rats were pooled and centrifuged at 3000g for 20 min at 4 °C. The clear perfusate was mixed 1:1, by volume, with 40% glycerol, 20 mM Tris-HCl, 0.15 M NaCl, and 2 mM EDTA, pH 7.4, and applied at a flow rate of 0.2 mL/min to a 1 × 5 cm column of heparin–Sepharose (Pharmacia) preequilibrated with 0.15 M NaCl in buffer E (10 mM Tris-HCl, 1 mM EDTA, pH 7.4, containing 20% glycerol). After sample application, the column was washed with 100 mL of 0.4 M NaCl in buffer E, followed by elution of the PEDAT/HL activities with 0.8 M NaCl in buffer E. The pooled fractions were then supplemented with 10% Brij-35 to obtain a final concentration of 0.04% and subsequently applied to a 0.7 × 3 cm column of octyl-Sepharose (Pharmacia). After extensive washing with the equilibration buffer (0.04% Brij-35, 0.8 M NaCl in buffer E), the activities were eluted with 0.2% Brij-35 in buffer E. Fractions containing the PEDAT/HL activities were pooled and applied to a second column of heparin–Sepharose (0.5 × 3 cm) at a flow rate of 0.05 mL/min. The column was then extensively washed with 0.4 M NaCl in buffer E in order to remove detergent. Elution of the enzyme was carried out at 0.1 mL/min using a narrow linear gradient of 0.4–0.9 M NaCl in buffer E in a total volume of 20 mL.

After this step, the HL activity and, in particular, the PEDAT activity were very unstable, even in the presence of 20% glycerol. The half-life for HL is 45 min at 4 °C (Twu et al., 1984; Ehnholm & Kuusi, 1986), and the half-life for PEDAT was estimated to be less than 30 min at this temperature. These activities could, however, be maintained by the addition of albumin to the collecting tubes. Therefore, each 15-min fraction was divided between two tubes, one of which contained 0.5 mg of albumin. The albumin-containing tubes were used for activity measurements, while the other tubes were used for monitoring purity by electrophoresis. The entire purification procedure took 3 working days.

Analytical Methods. SDS–polyacrylamide slab gel electrophoresis was performed using 10% gels as described previously (Laemmli, 1970), and silver staining of the gels was carried out by the method of Morrisey (1981). Proteins used as molecular weight standards were phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor. Nondenaturating polyacrylamide gel electrophoresis of the purified enzyme was carried out on a 3-mm slab gel consisting of 5% acrylamide and 15% glycerol at pH 9.5. Electrophoresis was performed at 4 °C for 3 h at 175 V. One lane was stained with Coomassie Brilliant Blue while the other lane was cut into 10 slices. The slices were homogenized in 2 mL of 250 mM Tris-HCl and 15% glycerol, pH 7.5, using a Turrax blender. After centrifugation at 2000g for 15 min the supernatants were used for determination of HL and PEDAT activities. Chromatofocusing of the purified protein was performed using a 0.5 × 7 cm column of PBE 94 polybuffer exchanger (Pharmacia) equilibrated with 25 mM imidazole hydrochloride and 15% glycerol, pH 7.4. About 0.25 mg of the purified protein was dialyzed overnight in this buffer and applied to the column at a flow rate of 4 mL/h. The pH gradient was generated with 40 mL of PBE 74 (Pharmacia) diluted 1:8 with 17% glycerol, pH 4.0. Fractions were collected and assayed for HL and PEDAT activities.

Gel Filtration. For determination of the molecular weight of the plasma cofactor, 3 mL of fresh human plasma was applied to a 1.6 × 80 cm column of Bio-Gel A-1.5 (Bio-rad) equilibrated in 0.15 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, and 0.02% azide, pH 7.4, at a flow rate of 0.11 mL/min at 4 °C. Fractions with a volume of 1.65 mL were collected and 300 μL of each fraction was tested for stimulation of PEDAT activity in the presence of 0.2 μg of partially purified enzyme (Sindelar et al., 1992). A standard gel filtration kit from Bio-Rad was used for column calibration.

Isolation of Human Lipoproteins. Lipoproteins from 12 mL of human plasma were isolated by ultracentrifugation using a discontinuous KBr gradient according to Terpstra et al. (1981). Fractions were collected by slicing the tubes and dialyzed for 48 h with three changes against 10 L of 0.15 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.5. The fractions were then tested for stimulation in the PEDAT assay as described above.

RESULTS

Purification of Hepatic Lipase from Rat Liver. The purification of rat liver hepatic lipase was performed using the procedure of Ikeda et al. (1989), with the exception that heparinized rat liver perfusates were used as the enzyme source, instead of human post-heparin plasma. All preparations were assayed for both HL and PEDAT activities. In the first chromatographic step on heparin–Sepharose, both activities eluted with 0.8 M NaCl. In the next step, utilizing octyl-Sepharose, the activities coeluted in a broad peak when elution was performed stepwise with 0.2% Brij-35. However, in this case PEDAT activity was greatly reduced in comparison with HL activity. The pooled fractions were then applied to a second heparin–Sepharose column, and upon elution with a narrow linear gradient of NaCl (0.4–0.9 M), the HL and PEDAT activities coeluted in a symmetrical peak with approximately 0.6 M NaCl (Figure 1). After this step, the PEDAT/HL activity ratio was restored to its original value, indicating reversible inhibition of PEDAT by Brij-35. From 10 livers about 90 μg of purified protein was obtained with specific activities of 33.4 mmol of FFA/(h·mg) for HL activity and 40.0 μmol of DE/(50 min·mg) for PEDAT activity. The purity of the final enzyme preparation was assessed using 10% slab SDS–PAGE as shown in Figure 2. Electrophoresis of the peak fraction revealed a single protein band with an apparent molecular mass of 61 000 ± 1000 Da upon silver staining. This molecular mass is similar to that reported earlier for hepatic lipase (Kuusi et al., 1979a; Jensen & Bensadoun, 1981). Nondenaturating PAGE of the final product showed a single protein band (R_f = 0.8) when stained with Coomassie

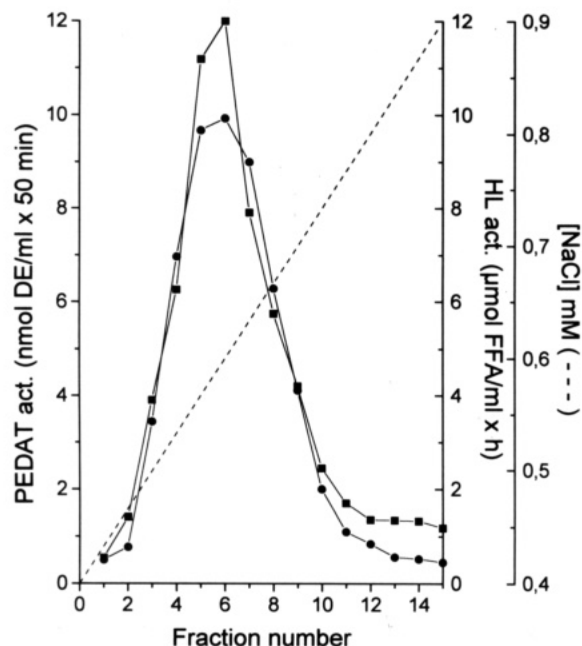

FIGURE 1: Elution profile of the final step in the enzyme purification. The pooled fractions from the octyl-Sepharose column were applied to a heparin–Sepharose column. Elution was performed as described in Materials and Methods. PEDAT (■) and HL (●) activities were measured using 0.2 mL of each fraction under standard assay conditions. The results shown are representative of six different experiments.

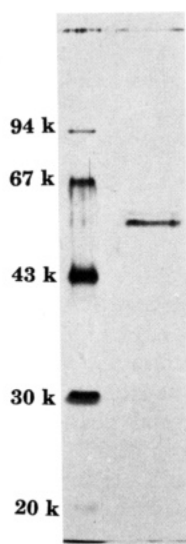

FIGURE 2: SDS–10% polyacrylamide gel electrophoretogram of fraction 6 from the final purification step. Fifty microliters of fraction 6 containing about 15 ng of protein was supplemented with SDS and β-mercaptoethanol to give final concentrations of 5% and 1%, respectively. The sample was heated at 100 °C for 3 min before application to the gel. Molecular weights of protein standards are indicated at the left.

Brilliant Blue. Assaying the gel slices for PEDAT and HL yielded only one peak for each activity, both corresponding to the single protein band (not shown). Chromatofocusing of the purified enzyme resulted in comigration of the two activities in a peak at pH 5.2 (data not shown). Since the two activities copurified throughout the entire procedure employed, demonstrated a single band upon SDS–PAGE and nondenaturating PAGE, and, in addition, coeluted on chromatofocusing, we conclude that hepatic lipase is responsible for catalyzing the transacylation from PE to dolichol.

Comparison of HL and PEDAT Activites in Various Rat Organs. Hepatic lipase activity has been reported to be present

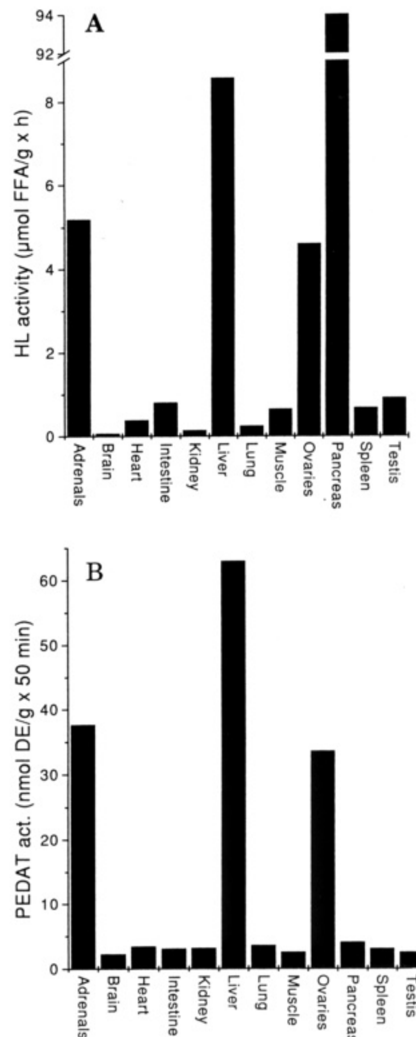

FIGURE 3: Distributions of hepatic lipase (A) and Dolichol acyltransferase (B) activities among various rat organs. The tissues were removed, weighed, and homogenized in 0.1 M Tris-HCl, pH 7.5, containing 25 IU of heparin/mL using a Turrax blender at 0 °C. Particle-free supernatants were obtained by ultracentrifugation of these homogenates at 45 000 rpm for 30 min in a 50 Ti rotor. HL and PEDAT activities in the supernatants were determined as described in Materials and Methods. The results shown are representative of four different experiments.

not only in the liver but also in the adrenal and ovary (Jansen & De Greef, 1981; Persoon et al., 1986). To determine whether this is also the case for its dolichol-acylating capacity, we measured PEDAT and HL activities in particle-free supernatant fractions from various rat organs originally homogenized in the presence of heparin. Figure 3A shows the distribution of HL activity in 10 different rat organs. As can be seen, the liver possesses the highest specific activity, whereas the corresponding values in adrenals and ovaries are about 30% lower. The extremely high rate of hydrolysis in the pancreas is most likely due to liberation of pancreatic lipase and colipase by the homogenization procedure employed. The other organs examined possessed no significant activity.

The dolichol acyltransferase activity showed a very similar pattern (Figure 3B) being detected in the liver, adrenal, and ovary in the same proportions as HL activity. The only difference was the absence of interference from pancreatic lipases in this case. These experiments strongly support the conclusion that the two different activities are catalyzed by the same enzyme. Furthermore, by assaying HL on the basis of its dolichol acyltransferase activity instead of using the standard assay procedure, interference by contaminating

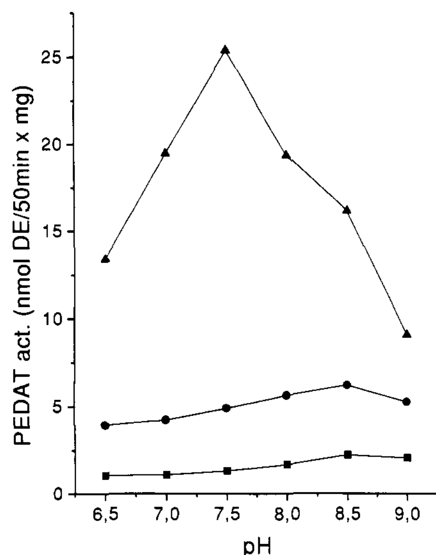

FIGURE 4: pH dependence of PEDAT activity in the presence of albumin and plasma. The assay was performed with 0.2 μg of partially purified enzyme (9) under standard assay conditions. ■, activity without additions; ●, activity in the presence of 50 μL of 6% albumin; ▲, activity in the presence of 50 μL of human plasma.

lipases can be eliminated. Thus, it appears that this assay procedure is more specific for HL than is the conventional one.

Dependence of PEDAT Activity on a Plasma Factor. In our previous report (Sindelar et al., 1992) we mentioned, but did not discuss the fact, that enzymatic acylation of dolichol requires a plasma cofactor for maximal activity. Figure 4 shows the effects of albumin and plasma on PEDAT activity at different pH's. When the enzyme is incubated without any additions, the rate of esterification is relatively low and the pH optimum is around 8.5. Addition of albumin causes nonspecific protein stimulation, activating the enzyme 2–3-fold without affecting the pH optimum.

When small volumes (50 μL) of plasma or serum are added to the assay mixture, two major effects are apparent. First, the pH optimum is shifted from 8.5 to 7.5. Second, the rate of esterification is increased 12–15-fold at this pH. A similar pattern can be observed with rat plasma although in this case the stimulation is only 6-fold (data not shown).

The low rate of acylation observed in the absence of plasma is not caused by enzyme instability, since activity could be maximally stimulated by addition of plasma following a 40-min preincubation without plasma (data not shown). Thus, we conclude that the enzyme is stabilized by the substrate liposomes and that the stimulation observed is solely due to the existence of a plasma cofactor activating the esterification of dolichol catalyzed by hepatic lipase.

Characteristics of the Plasma Cofactor. Heat treatment of plasma at 58 °C for 60 min did not reduce the stimulatory effect of plasma on the enzyme. Subfractionation of plasma on Bio-Gel A-1.5 revealed a heterogeneous distribution of the stimulating factor, with a major peak at approximately 220 kDa and shoulders on both sides (Figure 5). The high apparent molecular mass of the activating cofactor raised the possibility that it might be associated with HDL. Human plasma was therefore centrifuged on a discontinuous KBr gradient, and after slicing the tubes and dialysis, the fractions were assayed for stimulation. The cofactor was only detected in the lipoprotein-free ($d > 1.21$ g/cm^3) fraction (data not shown). We are currently working on the purification of this factor.

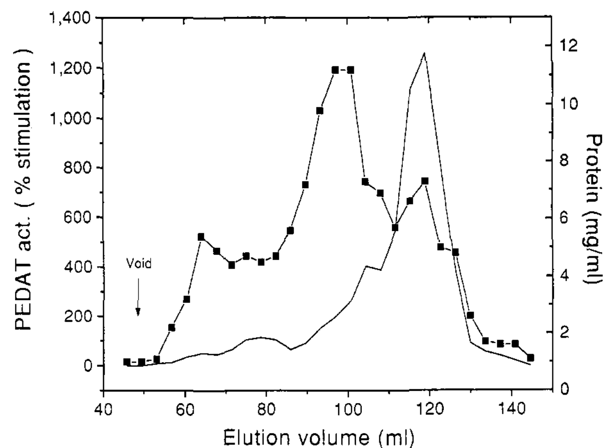

FIGURE 5: Distribution of the activating factor after gel filtration of human plasma on Bio-Gel A-1.5. Fractions were collected and tested for stimulation of PEDAT activity (■) as described in Materials and Methods and assayed for protein content (—) using the method of Lowry et al. (1951). The values are means of duplicate samples from a representative fractionation.

DISCUSSION

We have provided evidence in the present report that phosphatidylethanolamine:dolichol acyltransferase previously characterized by us (Sindelar et al., 1992) is identical to hepatic lipase and that this activity is strongly dependent on a plasma cofactor.

Purification of PEDAT requires delipidization, since the enzyme is complexed with lipids and other proteins when released by heparin (Sindelar et al., 1992). In our previous report we were unable to purify PEDAT to homogeneity, since Triton X-100 irreversibly inhibited this activity. Use of the uncharged detergent Brij-35 instead was found to be effective. Although the enzyme is also strongly inhibited in the presence of Brij-35, its activity is restored by removal of this detergent. Thus, by employing the purification procedure described earlier for hepatic lipase from human post-heparin plasma (Ikeda et al., 1989), we were able to maintain the PEDAT activity and purify the rat enzyme to homogeneity.

The dolichol acyltransferase assay for HL is applicable not only to the liver enzyme, but also to the adrenal and ovarian HL. No other tissues examined contained significant levels of this activity, which indirectly points out hepatic lipase as the sole lipase capable of esterifying dolichol under these conditions. This assay can therefore be used for specific qualitative and quantitative determinations of hepatic lipase, being more specific than the standard assay with triolein–gum arabic emulsion in 1 M NaCl.

Hepatic lipase has an affinity for high-density lipoproteins (Bengtsson & Olivecrona, 1980), and one of its proposed biological functions is to convert HDL-2 to HDL-3 by phospholipid hydrolysis (Kuusi et al., 1980; Jansen et al., 1980; Shirai et al., 1981), thus promoting the uptake of cholesteryl esters (Kuusi et al., 1979b; Jansen & Hulsmann, 1980; Kadowaki et al., 1992). Regulation of hepatic lipase activity by apoproteins in the triolein–gum arabic emulsion has been extensively studied in the past without any convincing results for the existence of a cofactor (Kinnunen, 1984). Recently, Thuren et al. (1991a,b) have used phospholipid monolayers to show that Apo E activates the hydrolysis of phospholipids 3-fold at low surface pressure. The hydrolysis of triglycerides was only slightly activated by this apoprotein. In liposomes, the dolichol acyltransferase reaction of hepatic lipase is dependent on a plasma cofactor that coelutes with HDL on gel filtration on Bio-Gel A-1.5. The factor stimulates the

PEDAT activity more than 12-fold and shifts its pH optimum from an alkaline to a physiological one. The fact that human HDL isolated by ultracentrifugation on a KBr gradient was unable to stimulate the enzyme and that the cofactor was found exclusively in the lipoprotein-free fraction should be interpreted with caution. It is a well-known problem that isolation of lipoproteins by ultracentrifugation causes dissociation of several apoproteins from lipoproteins, such as Apo A-I, Apo A-IV, and Apo E, and that these can be recovered in the lipoprotein-free fraction (Lagrost et al., 1989; Fainaru et al., 1977).

Hepatic lipase possesses multiple activities, catalyzing the hydrolysis of phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, and tri-, di-, and monoglycerides (Jackson, 1983). The enzyme preferentially hydrolyzes the ester bond in the *sn*-1 position (Akesson et al., 1976). It also catalyzes the transacylation of acyl groups from these lipids, except triglycerides, to an acceptor alcohol. Acyl acceptors for transacylation include nonbulky compounds with either primary or secondary hydroxyl groups (Waite & Sisson, 1974), and the structure of dolichol fits well into this category. The ability to catalyze both hydrolase and transacylase reactions is not unique to hepatic lipase. For example, lecithin: cholesterol acyltransferase shows phospholipase activity in the absence of its acyl acceptor, cholesterol (Aron et al., 1978). However, the catalysis of a transacylation reaction by hepatic lipase *in vivo* remains to be demonstrated.

The finding that hepatic lipase esterifies dolichol raises once again questions concerning the physiological function(s) of this enzyme, a matter of ongoing controversy since its discovery some thirty years ago. In liver, hepatic lipase is bound to receptors on the plasma membrane of endothelial cells (Kuusi et al., 1979c). At this site, HL has been reported to participate in the catabolism of chylomicron remnants, IDL, or HDL (Kinnunen, 1984). Two pools of dolichol are available to hepatic lipase *in vivo*. One of these is in the blood, where dolichol is present exclusively in HDL at extremely low concentrations and mainly as dolichyl esters (Elmberger et al., 1988). HL readily acylates dolichol present in liposomes when these are injected intravenously in rats (Sindelar & Valtersson, 1992). The second pool is the dolichol present in the plasma membrane of endothelial cells.

We have succeeded in esterifying radiolabeled dolichols incorporated in isolated liver plasma membranes, and this activity was drastically reduced upon removal of HL with heparin (unpublished observations). These results imply that the enzyme, apart from its action on serum lipoproteins, is also capable of catalyzing reactions involving components of the plasma membrane *in situ*. The present findings of a new function for hepatic lipase and of a specific plasma activator of this function will hopefully stimulate further efforts to understand this multifunctional enzyme's biological role.

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