Interaction of Human Plasma Lecithin:Cholesterol Acyltransferase and Venom Phospholipase A₂ with Apolipoprotein A-I Recombinants Containing Nonhydrolyzable Diether Phosphatidylcholines*

John B. Massey†, Quein Pao, W. Barry Van Winkle, and Henry J. Pownall

From the Department of Internal Medicine, Baylor College of Medicine, and The Methodist Hospital, MS A601, Houston, Texas 77030

(Received for publication, April 18, 1985)

Partially reassembled high density lipoproteins (R-HDL) composed of apolipoprotein A-I and nonhydrolyzable analogues of phosphatidylcholine have been prepared, and their physical properties and reactivities as substrates for lecithin:cholesterol acyltransferase and three phospholipases were tested. The stereochmical pairs L-DMPC-ether (1,2-O-ditetradecyl-sn-glycero-3-phosphorylcholine) and D-DMPC-ether (2,3-O-ditetradecyl-sn-glycero-1-phosphorylcholine) or L-DMPC (1,2-dimyristoyl-sn-glycero-3-phosphorylcholine) and d-DMPC (2,3-dimyristoyl-sn-glycero-1-phosphorylcholine) have similar thermal properties. R-HDL composed of these four lipids also have similar thermal properties as well as lipid/protein ratios, molecular weights, and protein conformations. Vmax and apparent Km values for lecithin:cholesterol acyltransferase on R-HDL consisting of linear combinations of L-DMPC and d-DMPC, L-DMPC-ether, or D-DMPC-ether plus 6 mol % cholesterol were measured. For the ether lecithins, there was a linear increase in Vmax with percentage of the acyl donor, L-DMPC, in R-HDL; over the same range, there was no change in Km. A comparison with bee venom and Naja melanoleuca phospholipase A₂ demonstrated that the venom enzymes have turnover numbers almost 3 orders of magnitude greater than has lecithin:cholesterol acyltransferase; the activity of the phospholipases was profoundly affected by the physical state of the lipid, whereas lecithin:cholesterol acyltransferase activity was not. The differences between these two types of enzymes, which cleave the same bonds of a phosphatidylcholine, are assigned to different catalytic mechanisms. These studies show that R-HDL containing sn-glycero-3-phosphorylcholines and sn-glycero-3-phosphorylcholine ethers have similar structure, properties, and affinities for phospholipolytic enzymes.

The plasma lipoproteins transport lipids from their sites of secretion to various extravascular compartments where they are utilized or stored. The HDL, low density lipoproteins, very low density lipoproteins, and chylomicrons represent important lipoprotein classes that are modified by two plasma enzymes (1). The free cholesterol of HDL is esterified by lecithin:cholesterol acyltransferase, an enzyme that also has phospholipase A₂ activity; both of these processes are activated by apo-A-I (1). Lipoprotein lipase hydrolyzes the triglycerides of VLDL and chylomicrons via a reaction that is stimulated by apo-C-II, a component of HDL, VLDL, and chylomicrons. This enzyme also has an apo-C-II-dependent phospholipase A₁ activity. The specificities and molecular description of the activities of both enzymes are key components of a thorough understanding of lipid metabolism.

Although several mechanistic models for lipolysis have been proposed (2-4), it has not been possible to validate these because binding and catalysis could not be measured independently. To distinguish the effects, nonhydrolyzable substrate analogues consisting of the D-lecithins, which are the opposite stereoisomers of the natural substrate, or ether-linked phospholipids have been utilized (5-7). D-Lecithins are competitive inhibitors of porcine pancreatic phospholipase A₂ in which the inhibition is in the catalytic step and not due to changes in binding specificity (7). Reassembled HDL composed of a single pure protein and single well-defined molecular species of PCs are well-suited for the study of lipolytic enzymes including those that have protein cofactors and various other perturbants or substrates, including cholesterol, which can be added and their effects studied in a systematic way. Unlike single bilayer vesicles, reassembled HDL have only one surface so that there is no ambiguity about the number of lipid molecules that are exposed to the surrounding aqueous phase that contains the enzyme. In addition, reassembled HDL composed of DMPC and apolipoproteins have been extensively studied by a variety of physicochemical methods that includes low angle x-ray (8, 9) and neutron scattering (10, 11), hydrodynamics (12, 13), differential scanning calorimetry (14, 15), fluorescence (16, 17), Raman (18), electron paramagnetic (19), and nuclear magnetic resonance (20) spectroscopies. R-HDL formed by assembling apo-A-I, PC, and cholesterol contain the minimal number of components required for a lecithin:cholesterol acyltransferase substrate (21, 22). We have synthesized 1,2-O-ditetradecyl-sn-glycero-3-

choline; rac-DMPC, 1,2-dimyristoyl-rac-glycero-3-phosphorylcholine; d-DMPC-ether, 2,3-O-ditetradecyl-sn-glycero-1-phosphorylcholine; DSC, differential scanning calorimetry; MPNPC, 1-myrystoyl-2-(9-(1-pyrenyl)nonanoyl)-sn-glycero-3-phosphorylcholine; M/M, molar ratio of phospholipid to protein; T_m, gel to liquid crystalline phase transition temperature; ΔH, enthalpy of the gel to liquid crystalline phase transition; Δωₓ, width of phase transition at half-maximal change; LCAT, lecithin:cholesterol acyltransferase; PLA₂, phospholipase A₂.

* This research was supported by National Institutes of Health Grant HL27341 SCOR in Atherosclerosis (H. J. P.), Grant HL30914 (H. J. P.), and Grant HL27341 SCOR in Atherosclerosis (H. J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

The abbreviations used are: HDL, high density lipoproteins; R-HDL, partially reassembled high density lipoproteins; PC, sn-glycero-3-phosphorylcholine; L-DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine; D-DMPC, 2,3-dimyristoyl-sn-glycerol-1-phosphorylcholine; sn-glycero-3-phosphorylcholine; d-DMPC-ether, 2,3-O-ditetradecyl-sn-glycero-1-phosphorylcholine; DSC, differential scanning calorimetry; MPNPC, 1-myrystoyl-2-(9-(1-pyrenyl)nonanoyl)-sn-glycero-3-phosphorylcholine; M/M, molar ratio of phospholipid to protein; T_m, gel to liquid crystalline phase transition temperature; ΔH, enthalpy of the gel to liquid crystalline phase transition; Δωₓ, width of phase transition at half-maximal change; LCAT, lecithin:cholesterol acyltransferase; PLA₂, phospholipase A₂.
phosphorylcholine (L-DMPC-ether), its D-isomer, and D-DMPC as nonhydrolyzable substrate analogues of L-DMPC. We report the characterization of L- and D-DMPC-ether and D-DMPC as adequate structural analogues of the natural L-isomer. Using the same R-HDL, some comparative studies of lecithin:cholesterol acyltransferase with bee venom and Naja melanoleuca A2 have been performed to test the suitability of nonhydrolyzable PCs as functional analogues of PCs.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

Racemic mixtures of alkyl ether lecithins have been previously demonstrated to be suitable analogues for studies of phosphatidylcholine structure (26, 27, 29, 30) and for the interaction of phospholipases (5, 6) with phospholipid bilayers. The differences between the physicochemical behavior of ether and ester phospholipids have been shown to be small (26, 27). Multilamellar liposomes of L- and D-DMPC and L- and D-DMPC-ether have similar ΔH, Tm, and Δt values. The ether analogues have slightly higher ΔH and Tm values, which indicates slightly better acyl chain packing (29). A number of criteria for the association of apo-A-I with D-DMPC and L- and D-DMPC-ether indicate that they are good structural analogues of DMPC. These criteria include 1) similar effects of an apoprotein on the thermal transition of the lipids, 2) induction of the same α-helical structure and tryptophan environment in apo-A-I, and 3) formation of stable, isolatable, lipid protein complexes having essentially the same stoichiometry, morphology, and hydrodynamic parameters. These results are consistent with the association of apolipoproteins with phospholipids being mainly determined by nonsterospecific hydrophobic interactions.

The effect of D-DMPC and L- and D-DMPC-ether on the lecithin:cholesterol acyltransferase reaction (Fig. 4) indicates that in this system they are good functional analogues of DMPC. For the ether analogues, a linear relationship between activity and the percentage of acyl donor in R-HDL is observed. Using the method of analysis of Verger and co-workers (7, 30), a linear relationship can be expected if 1) the affinity of the enzyme for the L-DMPC/ether PC mixtures is the same for R-HDL containing only L-DMPC, 2) the intrinsic catalytic rate constant of the enzyme (kcat) is the same for the mixed substrate as for pure substrate, and 3) the affinity of the inhibitor lipid for the active site is identical to that of the substrate lipid. According to this analysis, the ether PCs are good competitive inhibitors of the lecithin:cholesterol acyltransferase reaction. The variation of Vmax and the lack of variation of Km with the percentage of substrate in the R-HDL indicate that the inhibition occurs in the catalytic step and not due to changes in binding specificity. This observation suggests that the measured Km values are mainly the dissociation constants for the binding of the enzyme to the phospholipid surface. This conclusion is also supported by the observation that Km is independent of temperature even though there is a high activation energy for the enzymatic reaction. Thus, ether lecithins should permit the independent measurement of physical factors that regulate binding and catalysis.

We have compared the effect of the thermal phase transition on the activity of lecithin:cholesterol acyltransferase and bee venom and N. melanoleuca phospholipase A2. A comparison of these two types of enzymes is important because they all catalyze the cleavage of fatty acyl groups at a phospholipid/water interface. As observed by Jonas and Matz (22), there is no effect of the gel → liquid crystalline transition on the lecithin:cholesterol acyltransferase reaction when 100% L-DMPC-containing R-HDL are used. However, for both phospholipases A2, there is a pronounced change in enzymatic activity at Tt where the activation energy doubles when fluid phase lipid is replaced by gel phase lipid. We speculate that the differences in the behavior of these enzymes may be that they recognize substrate molecules that are in different locations in the R-HDL or that they have different rate-limiting steps. In model lipoproteins, it has been demonstrated that the phospholipid molecules adjacent to the apoprotein do not undergo a thermal phase transition, whereas the remainder of phospholipids have physical properties similar to those of multilamellar liposomes (14). The absence of a discontinuity in the lecithin:cholesterol acyltransferase reaction at Tt suggests that the PCs that are directly adjacent to the activator apoprotein may be the ultimate lecithin:cholesterol acyltransferase substrate.

The rate-limiting step in the lecithin:cholesterol acyltransferase reaction is not known. As shown in Table 3, the turnover number (moles of substrate/min/mol of enzyme) of lecithin:cholesterol acyltransferase is considerably less than either of the phospholipases. This is not due to our preparation of lecithin:cholesterol acyltransferase or these two phospholipases A2; other investigators observe a similar specific activity for lecithin:cholesterol acyltransferase (31). Additionally, similar turnover numbers have been found for other phospholipases; the Naja naja naja phospholipase A2 has a turnover number of 50,000 (32). Although both lecithin: cholesterol acyltransferase and phospholipase A2 form an acyl-enzyme intermediate, this process presumably occurs by different mechanisms; in the latter case, there is no requirement for Ca2+. The chemical nature of the reaction of lecithin:cholesterol acyltransferase suggests that it must go through an acyl-enzyme intermediate (33); since lecithin:cholesterol acyltransferase is inhibited by chemical modification by compounds such as phenylmethylsulfonyl fluoride (34, 35), a serine may perform the nucleophilic function in ester hydrolysis analogous to the active center serine in the serine proteases. In the phospholipase A2 reaction, a water molecule may be the nucleophile (7). Thus, the rate-limiting step for lecithin:cholesterol acyltransferase may be deacylation of the enzyme by the acyl acceptor, a process that is not affected by the physical state of the phospholipid.

Acknowledgments—We acknowledge many helpful discussions with Dr. Louis C. Smith. We wish to thank Sarah Myers-Fossett and Marjorie Sampel for preparing the manuscript, Susan Kelly for providing the line drawings, and Susan She for giving technical assistance. We wish to acknowledge the contribution of Dr. S. S. Newaz of Synthon, Inc. in the production of the diether DMPC and Dr. Roger D. Knapp for the nonlinear least squares computer program for analysis of Km and Vmax.

**REFERENCES**


---

Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–5, and Tables 1–3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9600 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-1272, cite the authors, and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.


Figure 1: DSC heating curves of L-DMPC (A) and L-DMPC-ether (B) liposomes. All samples were scanned at 2.5°C/min at a sensitivity of 1.0 mV/°C. Lecithin, cholesterol and 10% (w/w) bovine serum albumin were solubilized in 10 mM Tris, pH 7.2, containing 1% SDS. DSC heating curves of DMPC (C) and DMPC-ether (D) recombinants with apoA-I. The pooled samples from Sepharose CL-6B column chromatography (Figure 2) were concentrated by vacuum filtration using a 25,000 molecular weight cutoff cellulose bag from Schleicher and Schuell, Inc. (Keene, NH). All samples were scanned at 2.5°C/min at a sensitivity of 0.5 mV/°C. The enthalpies were calculated from the area under the DSC peaks and were used as an enthalpy standard for temperature calibration measurements. The data are summarized in Tables 1 and 2.

Table 1: Physical Properties of L- and D-DMPC and L- and D-DMPC-ether Liposomes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>L-DMPC</th>
<th>L-DMPC/DMPC</th>
<th>L-DMPC-ether</th>
<th>L-DMPC-ether/DMPC</th>
<th>L-DMPC</th>
<th>L-DMPC/DMPC</th>
<th>L-DMPC-ether</th>
<th>L-DMPC-ether/DMPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol lipids/mol protein</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
</tr>
<tr>
<td>T0 (°C)</td>
<td>26.5</td>
<td>27.0</td>
<td>26.5</td>
<td>27.0</td>
<td>26.5</td>
<td>27.0</td>
<td>26.5</td>
<td>27.0</td>
</tr>
<tr>
<td>ΔH (kJ/mol)</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
</tr>
</tbody>
</table>

The characterization of apolipoprotein-phospholipid recombinants: Addition of apoA-I to the two isomers of DMPC and DMPC-ether at the transition temperature of the lipid produced spontaneous assembly of the components into lipid-protein complexes, with concomitant clarification of liposomal turbidity. The complexes, which were isolated by gel filtration on Sepharose CL-6B, had similar elution profiles and elution volumes (Table 2). All four complexes gave similar hydrodynamic sizes and, according to the data, measured by gel filtration, the hydrodynamic size of the apoA-I/L-DMPC-ether complex was similar to that of the apoA-I/L-DMPC complex. The distribution of macromolecular species in the L- and D-DMPC-ether complexes was studied by negative staining electron microscopy (Fig. 2). The micrographs show reconstituted formation and essentially identical dimensions for the two recombinants. Analysis of the thermal properties of the D-DMPC shows that those containing DMPC-ether (Fig. 1) have transition temperatures that are slightly higher than those containing DMPC (Table 2). No significant difference in height was observed (Table 2). Addition of 2 mol % of MNPAC to each of the complexes produced a small depression in the melting temperature. The presence of 6 mol % co-esterification in an apoA-I/L-DMPC-ether recombinant broadened the thermal transition (ΔT) and lowered the transition enthalpy (ΔH). The implication of these observations is that L- or D-DMPC and L- or D-DMPC-ether have similar thermal and physical properties, both of which are only slightly affected by the addition of the pyrenyl label, lecithin. The circular dichroism of apoA-I in H2O containing L-DMPC and L-DMPC-ether recombinants was identical, suggesting that in both lipids the protein secondary structure was the same. Table 2 shows the results of fluorescence measurements for apoA-I and apoA-I/DMPC-ether. The fluorescence spectra and CD spectra were identical with apoA-I/DMPC-ether being substituted for DMPC. On the basis of the thermal, spectroscopic, and physical properties of the apoA-I recombinants, we conclude that phospholipid/apoA-I recombinants containing L- or D-DMPC-ether or D-DMPC are structurally similar if not identical to those previously characterized for L-DMPC (15,17,18,35).

Table 2: Physical Properties of apoA-I recombinants with L- and D-DMPC and L- and D-DMPC-ether.

<table>
<thead>
<tr>
<th>Sample</th>
<th>apoA-I</th>
<th>L-DMPC/DMPC</th>
<th>L-DMPC-ether</th>
<th>L-DMPC-ether/DMPC</th>
<th>L-DMPC</th>
<th>L-DMPC/DMPC</th>
<th>L-DMPC-ether</th>
<th>L-DMPC-ether/DMPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol lipids/mol protein</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
<td>1:100</td>
</tr>
<tr>
<td>T0 (°C)</td>
<td>26.5</td>
<td>27.0</td>
<td>26.5</td>
<td>27.0</td>
<td>26.5</td>
<td>27.0</td>
<td>26.5</td>
<td>27.0</td>
</tr>
<tr>
<td>ΔH (kJ/mol)</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Figure 2: Negative staining electron micrographs of apoA-I/DMPC and apoA-I/DMPC-ether recombinants. The magnification is 150,000x and the bar represents a length of 100 nm.
Kinetics of LCAT on Model Lipoproteins: the rates of cholesterol ester formation catalyzed by LCAT were measured in a series of R-HDL containing lecithin/cholesterol ratio (1:0.5/1.0). Typical substrate saturation curves for reconstituted lipoproteins containing 100% L-DOPA and 50% L-DOPA/50% DOPC were shown in Figure 1. Apparent $K_m$ and $V_{	ext{max}}$ values were determined by a non-linear least squares fit to the data.

In R-HDL containing 100% L- or B-DOPA both lipids are utilized as LCAT substrates. However, DOPA is more active than L-DOPA. Figure 2 shows $K_m$ and $V_{	ext{max}}$ values for R-HDL containing of linear combinations of L-DOPA and DOPA. $K_m$ value in Figure 3 is constant. For DOPA-depleted and L-DOPA-depleted with DOPA content constant. With D-DOPA, maximal values of $V_{	ext{max}}$ were found at approximately 0% L-DOPA in R-HDL. Even though there was a linear variation in $V_{	ext{max}}$ with increasing substrate concentration, there was essentially no difference in the $K_m$ values within a given substrate inhibitor series (Fig. 3). These results suggest that the non-hydrolysable substrate analogues are inhibitors of the catalytic step of LCAT but not of the binding step.

Temperature Dependence of LCAT and Phospholipase A: Reaction: The temperature Dependence of LCAT on R-HDL containing 5% cholesterol and 0.1% DOPA/2% DOPC was measured (Figure 4). At 31° and 37°, the $V_{	ext{max}}$ values for 50% L-DOPA are essentially identical (Table 4). Figure 4 shows that the Arrhenius plots are linear in the temperature range of the thermal phase transition. The Arrhenius plots for 50% and 90% lecithin/cholesterol phospholipids, on substrate containing 30% DOPA-phospholipid [0/30/6 M/M] and apol-13/1MDP, were measured (Fig. 5). As 37° and 41°, the $V_{	ext{max}}$ values for the reaction are unaffected by the addition of phospholipase A, with activation energies of 17 kcal/mole and 11 kcal/mole for fluid and gel phase lipids, respectively.

Table 3: Kinetic parameters for LCAT and lecithin vesicles in R-HDL containing L-DOPA and DOPA.

![Figure 1: Substrate saturation curves for the formation of cholesterol ester catalyzed by LCAT.](image)

![Figure 2: Temperature dependence of LCAT on R-HDL containing 5% cholesterol and 0.1% DOPA/2% DOPC.](image)

![Figure 3: Arrhenius plots for LCAT with R-HDL containing L-DOPA 0.1% and DOPA.](image)