Lipoprotein lipase-catalyzed hydrolysis of phosphatidylcholine of guinea pig very low density lipoproteins and discoidal complexes of phospholipid and apolipoprotein: effect of apolipoprotein C-II on the catalytic mechanism

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Abstract To elucidate the mechanism by which apolipoprotein C-II (apoC-II) enhances the activity of lipoprotein lipase (LpL), discoidal phospholipid complexes were prepared with apoC-III and di[^{14}C]palmitoyl phosphatidylcholine (DPPC) and containing various amounts of apoC-II. The rate of DPPC hydrolysis catalyzed by purified bovine milk LpL was determined on the isolated complexes. The rate of hydrolysis was optimal at pH 8.0. Analysis of enzyme kinetic data over a range of phospholipid concentrations revealed that the major effect of apoC-II was to increase the maximal velocity (V_{max}) some 50-fold with a limited effect on the Michaelis constant (K_m). V_{max} of the apoC-III complex containing no apoC-II was 9.2 nmol/min per mg LpL vs. 482 nmol/min per mg LpL for the complex containing only apoC-II. The effect of apoC-II on enzyme kinetic parameters for LpL-catalyzed hydrolysis of DPPC complexes was compared to that on the parameters for hydrolysis of DPPC and trioleoylglycerol incorporated into guinea pig very low density lipoproteins ($VLDL_p$) which lack the equivalent of human apoC-II. Tri[^{3}H]oleoylglycerol-labeled $VLDL_p$ were obtained by perfusion of guinea pig liver with [^{3}H]oleic acid. Di[^{14}C]palmitoyl phosphatidylcholine was incorporated into the $VLDL_p$ by incubation of $VLDL_p$ with sonicated vesicles of di[^{14}C]palmitoyl phosphatidylcholine and purified bovine liver phosphatidylcholine exchange protein. The rates of LpL-catalyzed hydrolysis of trioleoylglycerol and DPPC were determined at pH 7.4 and 8.5 in the presence and absence of apoC-II. In the presence of apoC-II, the V_{max} for DPPC hydrolysis in guinea pig $VLDL_p$ increased at both pH 7.4 and pH 8.5 (2.4- and 3.2-fold, respectively); the value of K_m did not change at either pH (0.23 mM). On the other hand, the kinetic value of K_m for triacylglycerol hydrolysis in the presence of apoC-II decreased at both pH 7.4 (3.05 vs. 0.54 mM) and pH 8.5 (2.73 vs. 0.62 mM). These kinetic studies suggest that apoC-II enhances phospholipid hydrolysis by LpL in apoC-III-DPPC discoidal complexes and $VLDL_p$ mainly by increasing the V_{max} of the enzyme for the substrates, whereas the activator protein primarily causes a decrease in the apparent K_m for triacylglycerol hydrolysis.—**Shirai, K., T. J. Fitzharris, M. Shinomiya, H. G. Muntz, J. A. K. Harmony, R. L. Jackson and D. M. Quinn.** Lipoprotein lipase-catalyzed hydrolysis of phosphatidylcholine of guinea pig very low density lipoproteins and discoidal complexes of phospholipid and apolipoprotein: effect of apolipoprotein C-II on the catalytic mechanism. *J. Lipid Res.* 1983. **24:** 721–730.

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Lipoprotein lipase (LpL) catalyzes the hydrolysis of triacylglycerols in the triglyceride-rich lipoproteins, chylomicrons, and very low density lipoproteins (VLDL) (ref. 1 for review). A characteristic feature of LpL is that its activity is enhanced by apolipoprotein C-II(apoC-II) (2). ApoC-II is a 78 amino acid residue peptide (3) present in triglyceride-rich lipoproteins and in high density lipoproteins (HDL). In addition to triacylglycerol, LpL, isolated either from post-heparin plasma or milk, catalyzes the hydrolysis of the primary acyl bond of phosphatidylethanolamine and phosphatidylcholine in triglyceride-rich lipoproteins (4–7), artificial triacylglycerol-phospholipid emulsions (8), and sonicated phospholipid vesicles (9–11); phospholipase activity is also enhanced by apoC-II. Limited information is available as to the mechanism by which apoC-II enhances the activity of LpL. Fitzharris et al. (12) and Matsuoka et al. (13) have shown that apoC-II decreases the ap-

Abbreviations: LpL, lipoprotein lipase; VLDL, very low density lipoproteins (d < 1.006 g/ml); $VLDL_p$, VLDL isolated from guinea pig liver perfusate; apoC-II and apoC-III, apolipoproteins from VLDL; BSA, bovine serum albumin; DPPC, dipalmitoyl phosphatidylcholine; V_{max}, maximal velocity; K_m, Michaelis constant.

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parent K_m for hydrolysis of VLDL triacylglycerol with little change in V_{max}.

The purpose of the present study was to determine the effect of apoC-II on the kinetic parameters for phospholipid hydrolysis by LpL. Discoidal complexes of phospholipid and apolipoprotein and guinea pig VLDL were used as substrates. Discoidal complexes containing dipalmitoyl phosphatidylcholine (DPPC) and apoC-III, another apolipoprotein constituent of VLDL and HDL, were prepared with various amounts of apoC-II. The rates of DPPC hydrolysis of these complexes catalyzed by LpL and the enzyme kinetic paramenters were determined and compared to those of VLDL-phospholipids. The results of these studies show that the effects of apoC-II on the kinetic paramenters for DPPC hydrolysis and for trioleoylglycerol hydrolysis by LpL are different in that apoC-II mainly affects the V_{max} for phospholipid hydrolysis whereas the K_m is affected for triacylglycerol hydrolysis. A kinetic model is proposed that accommodates the diverse observations reported here for apoC-II.

MATERIALS AND METHODS

Purification of bovine milk LpL

LpL was purified from skimmed bovine milk by affinity chromatography on heparin-Sepharose (14) according to a modification of Kinnunen (15) as described previously (16). Enzyme activity was determined using Triton X-100-emulsified tri[^{14}C]oleoylglycerol. The standard assay mixture contained 0.378 μmol of unlabeled trioleoylglycerol, 0.067 μCi of tri[^{14}C]-oleoylglycerol (50 mCi/mmol, New England Nuclear), 0.02% Triton X-100, 2% fatty acid-free bovine serum albumin (Sigma, Fraction V), apoC-II (2 μg), 0.1 M Tris–HCl, pH 8.0, and various amounts of enzyme in a final volume of 0.25 ml. Incubation was performed for 30 min at 37°C. Released [^{14}C]oleic acid was extracted by the method of Belfrage and Vaughan (17). The specific activity of the purified enzyme was 200 μmol free fatty acid released/min per mg LpL.

Preparation of apoC-III-DPPC discoidal complexes containing various amounts of apoC-II

Ten mg of L-α dipalmitoyl phosphatidylcholine (Applied Science) and 4 μCi of di[^{14}C]palmitoyl phosphatidylcholine (100 mCi/mmol, New England Nuclear) were dissolved in chloroform and were evaporated under nitrogen gas in a 8 × 100 mm glass tube. ApoC-II and apoC-III$_2$ were dissolved in 6 M guanidine-HCl, 10 mM Tris–HCl, pH 8.0, to give 10 mg/ml and then dialyzed against a standard buffer containing 0.9% NaCl, 10 mM Tris–HCl, pH 7.4, and 0.1% sodium azide. Apolipoprotein solutions (**Table 1**) were prepared containing various amounts of apoC-II and apoC-III to give 10 mg of total apoproteins in 3.5 ml of standard buffer, and were added to the DPPC. After incubation at 42°C for 24 hr with gentle shaking, the density of each mixture was adjusted to 1.15 g/ml by the addition of solid KBr (this density was based on pilot experiments and on the data shown in Fig. 1). The mixtures were then centrifuged in a Beckman Type 50 Ti rotor for 18 hr at 48,000 rpm at 15°C. The top 1.0 ml of each fraction which contained the apoprotein-DPPC complexes was removed, and was dialyzed against standard buffer. The content of apoC-II and apoC-III in the isolated complexes was determined by specific radioimmunoassays (18, 19).

Electron microscopy

For electron microscopic observation, apolipoprotein-DPPC complexes were negatively stained with 2% phosphotungstic acid, pH 7.4. A Phillips EM 300 microscope operating at an accelerated voltage of 60 KV was employed. The instrument was calibrated with a carbon grating replica 28,800 lines/inch (E. F. Fullam, Inc., New York). Preparations were initially observed at a magnification of 42,000×; particle size was determined by examination of the prints processed to a final magnification of 105,000×; 30 particles were counted and measured.

Determination of the rate of DPPC hydrolysis of the apolipoprotein-DPPC complexes by LpL

The incubation mixtures contained the apolipoprotein-DPPC discoidal complexes (105 μg of phospholipid), 2% fatty acid-free bovine serum albumin (BSA), 0.1 M Tris–HCl, pH 7.4, heparin (10 μg), and LpL (5 μg) in a final volume of 0.2 ml. The incubation was performed at 37°C for 60 min; all assays were linear with respect to time. The enzyme reaction was termi-

TABLE 1. Composition of apoC-II/apoC-III-DPPC discoidal complexes[a]

Preparation	ApoC-II	ApoC-III	Mol Ratio (Total apoC:DPPC)
	μg/mg DPPC		
1	0	645	1:19.6
2	4.1	563	1:22.5
3	9.2	609	1:18.7
4	11.2	504	1:22.0
5	38.0	494	1:23.8
6	73.0	657	1:16.6
7	874	0	1:13.3

[a] The apolipoprotein-DPPC complexes were prepared and isolated by ultracentrifugal flotation as described in Materials and Methods. ApoC-II and apoC-III concentrations were determined by radioimmunoassay.

nated and lipids were extracted by the addition of 0.3 ml of H_2O, 1.25 ml of MeOH, and 0.625 ml of $CHCl_3$ following the method of Bligh and Dyer (20). After shaking at 0°C for 30 min, 0.625 ml of $CHCl_3$ and 0.75 ml of H_2O were added and the samples were shaken at 0°C for 30 min. After centrifugation at 3000 rpm for 10 min, the $CHCl_3$ phase was removed and evaporated to dryness with nitrogen. The lipids were redissolved in $CHCl_3$ and applied to thin-layer plates Silica gel 60 (EM Reagents). The thin-layer plates were developed in chloroform–methanol–water 70:30:4; lipids were visualized with iodine vapor and were scraped from the plates and the radioactivity was determined.

Preparation of guinea pig liver perfusate VLDL ($VLDL_p$) containing tri[^{3}H]oleoylglycerol and di[^{14}C]palmitoyl phosphatidylcholine

Guinea pig VLDL were prepared from liver perfusate as described previously (12). Briefly, livers (10–12 g) of adult female albino guinea pigs (300–400 g) were perfused in a recycling system with 65 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 3% (w/v) delipidated BSA and 100 mg % glucose. After a 20-min equilibration period, an oleic acid albumin complex that contained 0.3 g of delipidated BSA, 150 μmol of oleic acid, and 25 μCi of [^{3}H]oleic acid in 10 ml of standard buffer was added to the perfusate. After 4 hr, the perfusion was terminated and the perfusate was centrifuged at perfusate density (d 1.006 g/ml) in a Beckman Type 50 Ti rotor for 18 hr at 48,000 rpm at 4°C. The top layer containing the $VLDL_p$ was collected. The density of this fraction was adjusted to 1.019 g/ml by addition of KBr and the density-adjusted solution was layered under a solution of KBr (d 1.006 g/ml); the mixture was centrifuged at 48,000 rpm for 18 hr. The purified $VLDL_p$ (top layer) were removed and dialyzed exhaustively against 0.05 M Tris–HCl, pH 7.4, containing 0.9% NaCl. Approximately 90% of the [^{3}H]oleic acid was incorporated into triacylglycerol.

DPPC was incorporated into tri[^{3}H]oleoylglycerol-labeled $VLDL_p$ using di[^{14}C]PPC vesicles and bovine liver phospholipid exchange protein by the method of Jackson, Wilson, and Glueck (21). DPPC (20 mg) and di[^{14}C]PPC (30 μCi) were dissolved in chloroform and evaporated in vaccuo. Standard buffer was added to the lipid to give 6 mg of phospholipid/ml and vesicles were prepared by sonication at 42°C for 15 min using a Heat Systems Ultrasonics, Inc., Cell Disruptor (Model W-225R). Tri[^{3}H]oleoylglycerol-labeled $VLDL_p$ (6.9 mg of phospholipid) were incubated with di[^{14}C]PPC vesicles (15 mg; 3.25×10^6 cpm/mg) in the presence of bovine liver phospholipid exchange protein (50 μg) and 0.2% fatty acid-free BSA in 16 ml of standard buffer. After incubation at 37°C for 2 hr, radiolabeled VLDL were separated by ultracentrifugation (d 1.006 g/ml). The radioactivity of the labeled $VLDL_p$ was 1.3×10^6 cpm of tri[^{3}H]oleoylglycerol/mg triacylglycerol and 2.7×10^5 cpm of di[^{14}C]PPC/mg phospholipid.

Determination of di[^{14}C]PPC and tri[^{3}H]oleoylglycerol hydrolysis in $VLDL_p$

The reaction mixtures contained various amounts of $VLDL_p$-triacylglycerol (0.5, 1.0, 1.5, 2.0, 2.5 mM), fatty acid-free BSA (50:1 weight ratio of BSA to $VLDL_p$-triacylglycerol), 0.1 M Tris–HCl, pH 7.4 or pH 8.5, LpL (0.15 μg), and various amounts of apoC-II in a final volume of 0.25 ml. To determine tri[^{3}H]oleoylglycerol hydrolysis, the enzyme reactions were performed at 37°C for 15 min and terminated by the addition of a chloroform–methanol–heptane extraction mixture as described by Belfrage and Vaughan (17). ^{3}H and ^{14}C were determined with a Beckman dual channel scintillation counter. To determine the hydrolysis of di[^{14}C]PPC, the incubation was carried out for 30 min. The reactions were terminated and lipids were extracted by the method of Bligh and Dyer (20). The extracted lipids were separated by thin-layer chromatography in a solvent system of chloroform–methanol–water 70:30:4 (v/v). The radioactivity of formed mono[^{14}C]PPC was determined in a Beckman Scintillation Spectrometer. The enzyme reaction increased linearly with 0.5 μg LpL up to 15 min for tri[^{14}C]oleoylglycerol and up to 30 min for di[^{14}C]PPC. Rates of hydrolysis were assessed at <12% depletion of the substrate lipids.

Analysis of kinetic parameters

The rates of DPPC hydrolysis in apolipoprotein-DPPC complexes, and DPPC and trioleoylglycerol hydrolysis in $VLDL_p$ were analyzed by the Lineweaver-Burk linear transform. The observed kinetic parameters, Michaelis constant (K_m), and maximal velocity (V_{max}) were calculated by a computerized weighted linear-least squares fit of the data, where weighting factors directly proportional to V_i^4 were used to prevent artificial biasing of the linear fit due to skewing of the residuals of $1/V_i$ at low V_i. This skewing results from transforming the native data set, V_i vs. S, to I/V_i vs. $1/S$. The rationale for weighting Lineweaver-Burk linear-least squares analysis of enzyme kinetic data has been presented by Cleland (22).

Preparation of apoC-II and apoC-III

ApoC-II and apoC-III_2 (containing 2 mol of sialic acid) were isolated from VLDL of patients with Type IV lipoprotein patterns as described by Brown, Levy, and Frederickson (23) with modifications as described previously (3).

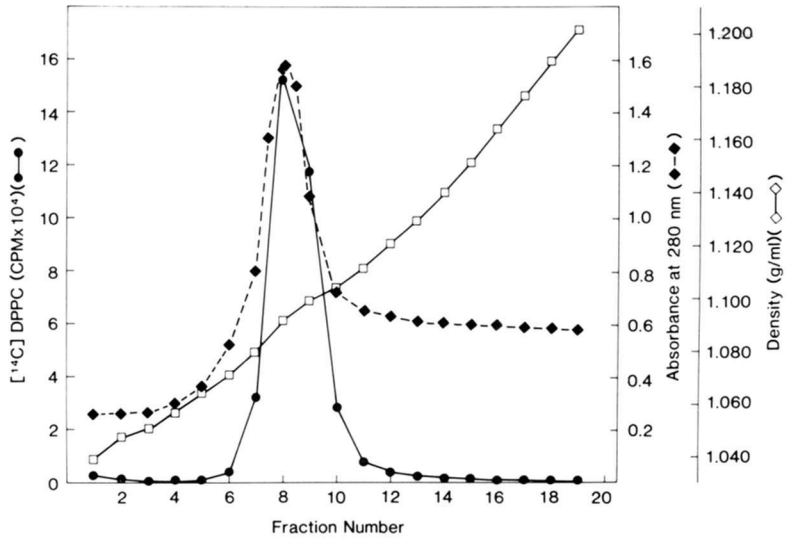

Fig. 1. Density-gradient ultracentrifugation of an apoprotein-DPPC complex. The lipid-protein complex was prepared with apoC-II (0.5 mg), apoC-III (9.2 mg), and DPPC (6.2 mg) containing 4 μCi of di[^{14}C]DPPC in a total volume of 7.0 ml of standard buffer as described in Materials and Methods. The complex was placed in the low density side of an ISCO Model 570 gradient former. The high density side contained 7.0 ml of a d 1.37 g/ml KBr solution in standard buffer. After formation of the gradient, the tubes were centrifuged in a Beckman SW-40 Ti rotor at 40,000 rpm for 60 hr at 23°C. After centrifugation, 0.75-ml fractions were collected with an ISCO Model 640 density-gradient fractionator. Sample densities were determined using a Bausch and Lomb (Abbe-3L) refractometer.

Analytical procedures

Triglycerides were analyzed using a Triglyceride Kit based on the enzymatic (glycerol kinase, pyruvate kinase, lactate dehydrogenase) determination of glycerol. Phospholipid-phosphorus was measured by the method of Bartlett (24). Protein concentrations were determined by a modification of the method of Lowery et al. (25) using 0.1% sodium dodecyl sulfate to clarify the samples; BSA was the standard.

RESULTS

Fig. 1 shows the density-gradient ultracentrifugation profile of a phospholipid-apoprotein complex prepared with apoC-II (0.5 mg), apoC-III (9.2 mg), and DPPC (6.2 mg). With these conditions of protein excess, all of the lipid was associated with protein. The complex banded at a mean flotation density of 1.090 g/ml and had a protein:DPPC molar ratio of 1:21.4.

Since the data in Fig. 1 indicate only one type of lipid-protein complex was formed, the complexes used in the enzyme kinetic experiments described below were isolated in a fixed-angle rotor at d 1.15 g/ml. Electron micrographs of negatively stained apoC-III-DPPC complexes isolated at d 1.15 g/ml revealed stacked disc structures as shown in **Fig. 2A** with an average disc thickness (± standard error) of 53.1 ± 0.8 Å. ApoC-II/apoC-III-DPPC complexes also appeared as stacked discs (Fig. 2B) with a disc thickness of 53.3 ± 1.1 and a diameter of 165.0 ± 3.2 Å, respectively. Table 1 gives

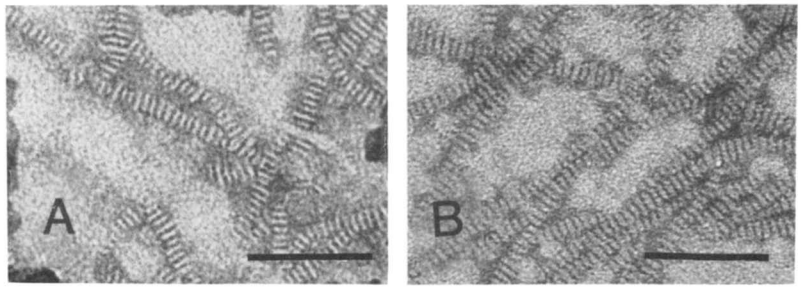

Fig. 2. Electron micrographs of (A) an apoC-III-DPPC complex (apoC-III/DPPC-0.645 w/w) and (B) apoC-II/apoC-III-DPPC complex (apoC-II + apoC-III/DPPC = 0.038 + 0.494 w/w). Samples were negatively stained with 2% phosphotungstic acid (pH 7.2). The bar represents 1000 Å.

the content of apoC-II/apoC-III and the mol ratio of total apoCs to DPPC in the isolated complexes.

The time course of the hydrolysis of DPPC (0.7 mM) in apoC-II/apoC-III-DPPC complexes catalyzed by purified bovine milk LpL is shown in **Fig. 3.** In the absence of apoC-II, DPPC in the apoC-III-DPPC complex was hydrolyzed at a rate of 6 nmol/min per mg LpL. With increasing amounts of apoC-II in the apoC-III-DPPC complexes, the rate of hydrolysis increased. The rate of DPPC hydrolysis in the apoC-II/apoC-III-DPPC complex containing 38.0 μg apoC-II/mg DPPC was 120 nmol/min per mg LpL, corresponding to a rate 20 times greater than that for the apoC-III-DPPC complex.

The dependence of the initial rate of the LpL-catalyzed hydrolysis of DPPC on pH is shown in **Fig. 4.** The optimal pH was approximately pH 8.0 for the apoC-II (38 μg/mg DPPC)/apoC-III-DPPC complex; the rate obtained for the apoC-II (4.1 μg/mg DPPC)/apoC-III-DPPC complex showed a broad optimum between pH 7.5 and 9.0.

Fig. 5A shows the initial rates at pH 8.0 of DPPC hydrolysis at various concentrations of the apoC-II/apoC-III-DPPC complexes. As the lipid concentration increased, the rates of hydrolysis catalyzed by LpL increased and reached a plateau. In these experiments, maximal extent of hydrolysis of substrate lipid was <12%. Lineweaver-Burk double reciprocal plots of the kinetic data are shown in Fig. 5B. The calculated apparent K_m and V_{max} values at each amount of apoC-II in the complexes are given in **Table 2.** The apparent

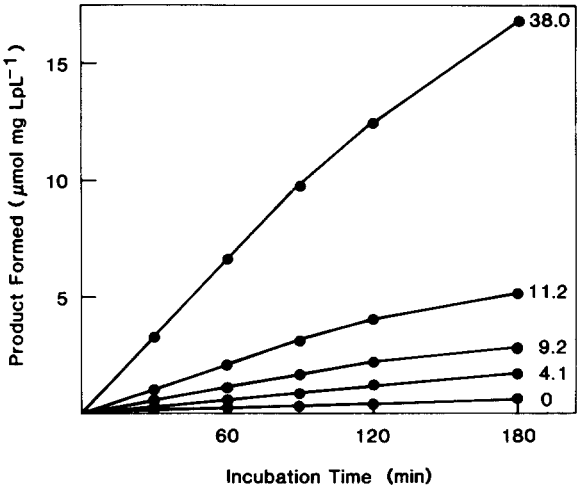

Fig. 3. Time course of the LpL-catalyzed hydrolysis of DPPC in complexes containing apoC-III-DPPC and amounts of apoC-II (μg/mg DPPC) as indicated. The reaction mixtures contained 0.7 mM DPPC of each apoprotein-phospholipid complex, 2% fatty acid-free BSA, 0.1 M Tris–HCl, pH 8.0, heparin (10 μg), and LpL (5 μg) in a final volume of 0.2 ml; the incubation temperature was 37°C. At the indicated times, samples were removed and the amount of product formed was determined as described in Materials and Methods.

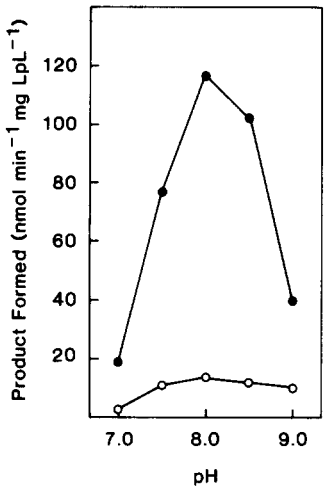

Fig. 4. Dependence of LpL-catalyzed hydrolysis of DPPC on pH. The reaction mixtures contained 0.7 mM DPPC of either apoC-II/apoC-III-DPPC complex (4.1 μg apo C-II/mg DPPC, – ○ – ○ –) or apoC-II/apoC-III-DPPC complex (38.0 μg apo C-II/mg DPPC, – ● – ● –), 2% fatty acid-free BSA, 0.1 M Tris–HCl, pH as indicated, heparin (10 μg), and LpL (5 μg) in a final volume of 0.2 ml. Incubations were performed for 60 min at 37°C. The amount of product formed was determined as described in Materials and Methods.

K_m values of the complexes containing various amounts of apoC-II varied by 3-fold; the apparent K_m values for the apoC-III-DPPC complex and for the apoC-II-DPPC complex were 0.14 and 0.51 mM, respectively. The apparent V_{max} values increased linearly as the amount of apoC-II increased in the apoC-III-DPPC complexes (**Fig. 6**). The values of V_{max} for the apoC-III-DPPC complex and for the apoC-II-DPPC complex were 9.2 and 482 nmol/min per mg LpL, respectively, corresponding to a 50-fold increase in V_{max}.

The effects of pH on the kinetic parameters V_{max} and K_m are presented in Table 2. At pH 7.4 and pH 8.5, the apparent K_m values for apoC-II/apoC-III-DPPC complexes varied by only 1.5-fold. However, the change in the values of V_{max} by increasing amounts of apoC-II (0 to 38 μg/mg DPPC) in the complexes at pH 8.5 was more prominent (8.2 to 131 nmol/min per mg LpL) than that at pH 7.4 (8.0 to 98.9 nmol/min per mg LpL).

Fitzharris et al. (12) reported that the major effect of apoC-II on trioleoylglycerol hydrolysis in guinea pig $VLDL_p$ catalyzed by purified bovine milk LpL was to decrease the apparent K_m by 3.5- to 4.5-fold at pH 7.4; apoC-II increased the apparent V_{max} by 1.3-fold. Matsuoka et al. (13) also reported that apoC-II mainly affects the apparent K_m with human apoC-II-deficient VLDL and purified bovine milk LpL. Therefore, in the next series of experiments, the influence of apoC-II on the kinetic parameters for both the LpL-catalyzed hydrolysis of triacylglycerol and phospholipid was assessed. $VLDL_p$ containing tri[^{3}H]oleoylglycerol and di[^{14}C]PPC were prepared as described in Materials and Methods.

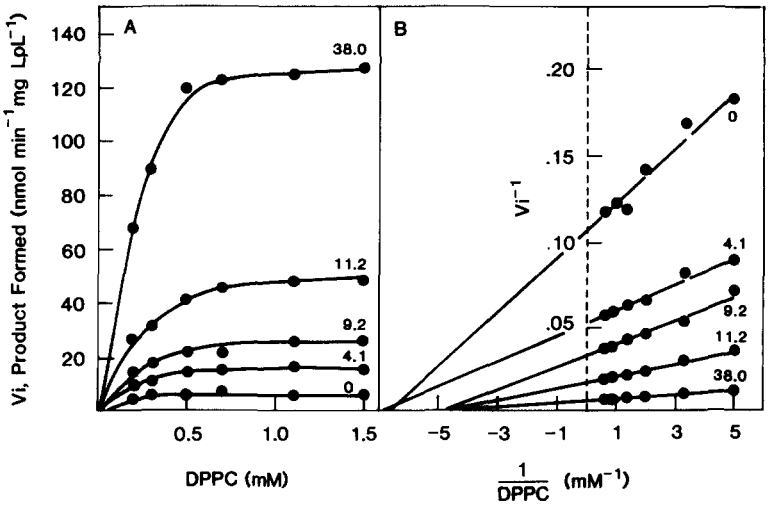

Fig. 5. A, Effect of substrate concentration on the LpL-catalyzed hydrolysis of DPPC in discoidal complexes containing apoC-III-DPPC and amounts of apoC-II as indicated. The reaction mixtures contained the indicated amounts of DPPC, fatty acid-free BSA (50 times more than the weight of DPPC in each case), 0.1 M Tris–HCl, pH 8.0, heparin (10 μg), and LpL (5 μg) in a final volume 0.2 ml. Incubations were performed for 60 min at 37°C. The amount of product formed was determined as described in Materials and Methods. B, Lineweaver-Burk double reciprocal plots for the LpL-catalyzed hydrolysis of DPPC. Plots of $1/V_i$ vs. 1/DPPC were constructed using the data in Figure 2A.

The lipid and protein compositions of $VLDL_p$ were nearly identical to those presented previously (12). Ninety percent of [^{3}H]oleic acid was incorporated into triacylglycerol, 5% was in phospholipids, and 0.5% was in cholesteryl ester. About 3–5% of the $VLDL_p$-associated radioactivity was present as unesterified [^{3}H]oleate. The LpL-catalyzed hydrolysis of di[^{14}C]PPC and tri[^{3}H]oleoylglycerol in guinea pig $VLDL_p$ was enhanced by the addition of apoC-II. The changes in the enzyme kinetic parameters by apoC-II were investigated at both pH 7.4 and pH 8.5. As is shown in **Table 3B,** in the presence of apoC-II (5 μg/mg triglyceride), the value of the K_m for trioleoylglycerol decreased 5.6-fold at pH 7.4 and 4.4-fold at pH 8.5. The value of V_{max} for trioleoylglycerol hydrolysis was not changed by the addition of apoC-II at pH 7.4. On the other hand, at pH 8.5, V_{max} values were increased 1.5-fold, consistent with the previous report (12). The effects of apoC-II on the

TABLE 2. Effect of pH on the kinetic parameters for the LpL-catalyzed hydrolysis of DPPC in apoC-II/apoC-III-DPPC complexes[a]

	pH 8.0		pH 7.4		pH 8.5	
ApoC-II	K_m[b]	V_{max}[c]	K_m[b]	V_{max}[c]	K_m[b]	V_{max}[c]
μg/mg DPPC						
0	0.14	8.4	0.39	8.0	0.52	8.2
4.1	0.13	16.5	0.38	18.6	0.50	23.5
9.2	0.21	35.1	0.38	29.2	0.41	32.9
11.2	0.20	60.1	0.40	53.7	0.53	72.3
38.0	0.19	155	0.27	98.9	0.39	131
73.0	0.38	409	ND[d]	ND	ND	ND
872	0.51	482	ND	ND	ND	ND

[a] The assay conditions were the same as those described in Fig. 3. The kinetic parameters were calculated from Lineweaver-Burk plots using a CLINFO computer as described in Materials and Methods. The standard error calculated from least squares fits in K_m and V_{max} was 5%.
[b] K_m = mM phospholipid.
[c] V_{max} = nmol product formed min^{-1} mg LpL^{-1}.
[d] ND, not determined.

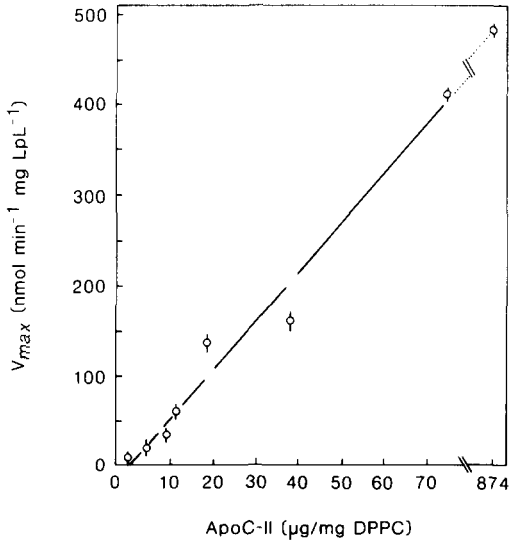

Fig. 6. The relationship between the values of V_{max} and the amount of apoC-II in apoC-III-DPPC complexes. V_{max} values were obtained as described in Materials and Methods. The value corresponding to 18.2 μg apoC-II/mg DPPC represents the complex isolated by density gradient ultracentrifugation (Fig. 1). The other values are those complexes isolated by ultracentrifugation at d 1.15 mg/ml (Table 1).

TABLE 3. Kinetic parameters for the LpL-catalyzed hydrolysis of di[^{14}C]PPC and tri[^{3}H]oleoylglycerol in guinea pig $VLDL_p$[a]

ApoC-II (μg/mg triglyceride)	pH 7.4		pH 8.5	
	K_m[b]	V_{max}[c]	K_m[b]	V_{max}[c]
A. di[^{14}C]PPC				
0	0.23	7.0	0.28	10.2
1	0.28	12.0	0.34	27.1
2	0.23	13.5	0.23	23.8
5	0.31	16.9	0.18	32.5
B. tri[^{3}H]oleoylglycerol				
0	3.05	152	2.73	175
1	1.11	155	0.88	225
2	0.61	145	0.82	270
5	0.54	150	0.62	265

[a] The assay conditions are described in Materials and Methods. The kinetic parameters were calculated from the Lineweaver-Burk plots using a CLINFO computer as described in Materials and Methods. All standard errors in K_m and V_{max} calculated from least squares fits were <5% (mean, 3.9%) and <5% (mean, 2.9%), respectively.

[b] K_m = mM phospholipid (A) or triacylglycerol (B).

[c] V_{max} = μmol product formed min^{-1} mg LpL^{-1}.

kinetic parameters for hydrolysis of DPPC in $VLDL_p$ were different from those for trioleoylglycerol in $VLDL_p$. As is shown in Table 3A, at both pH 7.4 and pH 8.5, the values of the apparent K_m for DPPC in $VLDL_p$ were not significantly changed by the addition of apoC-II compared with those for trioleoylglycerol. On the other hand, the values of V_{max} were increased 2.4-fold at pH 7.4 and 3.2-fold at pH 8.5 by the addition of apoC-II.

DISCUSSION

Discoidal complexes of apoC-III and DPPC containing various amounts of apoC-II were used as substrates for lipoprotein lipase. The rate of the LpL-catalyzed hydrolysis of DPPC was markedly enhanced by the activator protein. The increase in the rate of DPPC hydrolysis was mainly due to an apoC-II-induced increase in the apparent V_{max} of some 50-fold with only a 3-fold change in the apparent K_m for substrate phospholipid. If one assumes that the V_{max} value of 482 nmol/min per mg LpL is optimal with the complex containing only apoC-II, then it is possible to determine the amount of apoC-II that is required for maximal LpL activity. Extrapolation of the line in Fig. 6 to maximal V_{max} gives 86 μg apoC-II/mg DPPC. Assuming 200,000 daltons for a lipid-protein complex of dimensions 161 × 53 Å, a molecular weight that is consistent with that described by Massey et al. (26), then for maximal LpL activity the average composition of the complex would be 1 apoC-II, 8 apoC-III, and 154 DPPC.

ApoC-II had a similar but not as pronounced influence on the kinetic parameters for LpL-catalyzed hydrolysis of the phospholipid constituents of di[^{14}C]PPC/tri[^{3}H]oleoylglycerol-labeled $VLDL_p$. The apparent V_{max} values increased 2.4- to 3.2-fold in the presence of apoC-II. The enhancing effect of apoC-II on the V_{max} values in both systems was greater at alkaline pH values. These results are in agreement with observations by Fielding (27) that both V_{max} vs. pH and $1/K_m$ vs. pH were shifted to alkaline pH values by the activator protein. The influence of apoC-II on the kinetic parameters for phospholipid hydrolysis is distinct from that of apoC-II on triglyceride hydrolysis. In contrast to the results obtained for phospholipid hydrolysis catalyzed by LpL, apoC-II decreased the apparent K_m for hydrolysis of $VLDL_p$-triglyceride by 4.4- to 5.6-fold, dependent on the pH, but had little influence (1.5-fold increase at pH 8.5) on the V_{max} of the reaction. The latter results are consistent with those of Fitzharris et al. (12) obtained with guinea pig perfusate $VLDL_p$ as well as with those of other investigators who determined the effect of the activator protein on the kinetic parameters for triacylglycerol hydrolysis by LpL using artificial substrates of triacylglycerol emulsified with gum arabic (28), phospholipid (27), or BSA (29). Schrecker and Greten (28) reported a 5-fold decrease in the apparent K_m with gum arabic emulsified trioleoylglycerol and human post-heparin plasma LpL. ApoC-II also decreased the apparent K_m for triacylglycerol hydrolysis by purified bovine milk LpL using apoC-II-deficient human VLDL (13).

In the classical Michaelis-Menten treatment of kinetic data, a decrease in the K_m of the enzyme for the substrate frequently corresponds to an increased affinity of the enzyme for substrate. An increase in the V_{max} is related to an increase in catalytic rate. When the substrate is not water-soluble, interpretation of the data is not straightforward. Verger and de Haas (30) expanded the Michaelis-Menten approach to include situations in which the substrate is a part of a large lipid interface. To provide a unique explanation for the different effects of apoC-II on the LpL-catalyzed triglyceride and phospholipid hydrolyses, the Verger and de Haas mechanism must be expanded to include LpL-apoC-II interactions:

$$\begin{array}{ccccc} E + I & \underset{k_a}{\overset{k_p}{\rightleftharpoons}} & E^* & \underset{K_m^*}{\overset{S}{\rightleftharpoons}} & E^*S \xrightarrow{k_{cat}} E^* + \text{products} \\ & & A \Big\downarrow\Big\uparrow K_A & & A \Big\downarrow\Big\uparrow \alpha K_A \\ & & E^*A & \underset{\alpha K_m^*}{\rightleftharpoons} & E^*SA \xrightarrow{\beta k_{cat}} E^*A + products \end{array}$$

In this mechanism there are four LpL complexes formed. E* is the complex formed when LpL binds at the substrate interface I, but in which LpL's active site is not yet bound to individual substrate molecules. E*S is the interfacial Michaelis complex, in which LpL's ac-

tive site is bound to triacylglycerol or phospholipid. E*A is an LpL-apoC-II complex formed at the interface, and E*SA is the interfacial Michaelis complex, apoC-II ternary complex. The mechanism assumes that there are two routes of association between LpL and apoC-II at the interface, one with dissociation constant K_A and the other with dissociation constant αK_A. The term α allows for the possibility that the interaction of LpL and apoC-II may be different when LpLs' active sites are free (E*) than when they are occupied by substrate (E*S) and is the degree to which K_m is reduced when LpL and apoC-II interact. Moreover, the catalytic rate constant for enzyme turnover from E*SA is βk_{cat}, accounting for the possibility that interaction between LpL and apoC-II changes k_{cat} by an amount β; β measures the potential increase in k_{cat} when apoC-II and LpL interact.

A steady-state kinetic derivation based on Equation 1 gives the following expressions for apparent V_{max} and apparent K_m:

$$V_{max}^{app} = \frac{k_{cat}E_T S(1 + \beta A/\alpha K_A)}{K_m^*(1 + A/K_A) + S(1 + A/\alpha K_A)} \quad \text{Eq. 2)}$$

$$K_m^{app} = \frac{k_d}{k_p}\frac{K_m^* S}{K_m^*(1 + A/K_A) + S(1 + A/\alpha K_A)} \quad \text{Eq. 3)}$$

$$K_m^* = \frac{k_{-1} + k_{cat}}{k_1} \quad \text{Eq. 4)}$$

where k_1 is the rate constant for formation of E*S from E* and k_{-1} is the rate constant for reversion of E*S to E*. E_T in Equation 2 is the total LpL concentration. These equations show that both apparent V_{max} and apparent K_m depend on apoC-II. Although the kinetic data presented in this paper cannot be used to determine the various important terms in Equations 2 and 3 (K_A, k_{cat}, α, β, etc.), the equations can accommodate the different effects of apoC-II on the LpL-catalyzed hydrolysis of phospholipid (primarily a V_{max} effect) and of triacylglycerol (primarily a K_m effect) detailed herein.

Consider the possibility that the association between LpL and apoC-II at the interface is much tighter when a substrate molecule is bound at LpL's active site (E*S) than when the active site is free (E*). In this case, $\alpha \ll 1$ and Equations 2 and 3 reduce to

$$V_{max}^{app} = \frac{k_{cat}E_T S(1 + \beta A/\alpha K_A)}{K_m^* + S(1 + A/\alpha K_A)} \quad \text{Eq. 5)}$$

and

$$K_m^{app} = \frac{k_d}{k_p}\frac{K_m^* S}{K_m^* + S(1 + A/\alpha K_A)} \quad \text{Eq. 6)}$$

since when $(1 + A/\alpha K_A) \gg 1$, $(1 + A/K_A) \sim 1$. Assuming that the interaction between LpL and apoC-II is much tighter when substrate is bound at the active site is equivalent to assuming that interaction of LpL and apoC-II at the interface increases the affinity of LpL for substrate (cf. Equation 1). The two possible routes are indistinguishable thermodynamically.

What information can Equations 5 and 6 provide us about the mechanism of apoC-II enhancement of LpL-catalysis? Consider the kinetic data for apoC-II effects on LpL-catalyzed hydrolysis of trioleoylglycerol in $VLDL_p$ in Table 3. At pH 7.4, K_m is decreased 5.6-fold but V_{max} is unchanged. These results are predicted by Equations 5 and 6. If $S(1 + A/\alpha K_A) \gg K_m$ and $\beta = 1.0$, then the apparent K_m and apparent V_{max} are expressed by Equations 7 and 8.

$$V_{max}^{app} = k_{cat}E_T \quad \text{Eq. 7)}$$

$$K_m^{app} = \frac{k_d}{k_p}\frac{K_m^*}{1 + A/\alpha K_A} \quad \text{Eq. 8)}$$

These equations suggest that apoC-II brings about a decrease in K_m by decreasing K_m^*, i.e., apoC-II increases the affinity of LpL for the triacylglycerol substrate in the $VLDL_p$ interface. This conclusion is supported by recent kinetic analysis of the LpL-catalyzed hydrolysis of apoC-II-deficient guinea pig and human VLDL (12, 13). As Equation 4 shows, K_m contains k_{cat}. However, apoC-II cannot decrease K_m by decreasing k_{cat} because this would require $\beta < 1.0$ and a seemingly paradoxical inhibition of substrate turnover by LpL. We have thus come, albeit by circumlocution, to experimental support for the assumption by which Equations 5 and 6 were deduced from Equations 2 and 3, that apoC-II increases the affinity of LpL's active site for the triacylglycerol substrate or, *pari passu*, that apoC-II has a greater affinity for E*S than for E* (cf. Equation 1).

At pH 8.5, addition of apoC-II causes an overall 4.4-fold decrease in the apparent K_m and a 1.5-fold increase in apparent V_{max} of LpL-catalyzed hydrolysis of $VLDL_p$ trioleoylglycerol (cf. Table 3). This combination of apoC-II effects is described by Equations 5 and 6 with $K_m < S(1 + A/\alpha K_A)$. The implication is that K_m increases from pH 7.4 to 8.5, so that at pH 8.5 K_m is of the same order of magnitude as S. Because the change in V_{max}/K_m brought about by apoC-II at pH 8.5 (6.6-fold) is similar to that at pH 7.4 (5.6-fold), β must be approximately 1.0 at pH 8.5. Therefore, the results for triacylglycerol hydrolysis at pH 8.5 are consistent with an apoC-II-mediated increase in the affinity of LpL's active site for the triacylglycerol substrate and/or with a greater affinity of apoC-II for E*S than for E*.

The effect of apoC-II on the LpL-catalyzed hydrolysis of DPPC incorporated into $VLDL_p$ is quite different than that of $VLDL_p$-trioleoylglycerol. As Table 3 shows, there is no clear trend in K_m as apoC-II is added, and the apparent K_m appears independent of apoC-II within experimental error. However, the apparent V_{max} is in-

creased by apoC-II 2.4-fold at pH 7.4 and 3.2-fold at pH 8.5. These results can be explained by assuming that $K_m \gg S(1 + A/\alpha K_A)$, in which case Equations 5 and 6 are reduced to Equations 9 and 10, respectively.

$$V_{max}^{app} = \frac{k_{cat} E_T S}{K_m^*} (1 + \beta A/\alpha K_A) \qquad \text{Eq. } 9)$$

$$K_m^{app} = \frac{k_d}{k_p} S \qquad \text{Eq. } 10)$$

Therefore, even though the concentration of substrate per unit surface area (S) is much greater for phospholipid than for triacylglycerol in $VLDL_p$, Equations 9 and 10 correctly describe the observed effects of apoC-II on the LpL-catalyzed hydrolysis of $VLDL_p$-DPPC, necessitating that K_m for DPPC is much greater than the K_m for triacylglycerol. This prediction of relative K_m values is intuitively satisfying since it predicts that the intrinsic affinity of LpL for triacylglycerol (LpL's preferred substrate) is much greater than for phospholipid. Moreover, the lack of an apoC-II effect on the apparent K_m of LpL-catalyzed hydrolysis of $VLDL_p$-DPPC means that apoC-II does not alter the equilibrium constant for binding of LpL at the particle interface, k_p/k_d (cf. equations 6 and 10). This interpretation of the kinetic data is consistent with recent findings showing that apoC-II does not increase the amount of enzyme bound to sonicated DPPC vesicles (31) to trioleoylglycerol emulsions (11), or to monomolecular films of 1,2-didecanoylglycerol (32). Correspondingly, the previously discussed (12) decrease in apparent K_m of LpL-catalyzed hydrolysis of $VLDL_p$-triacylglycerol brought about by apoC-II cannot be due to apoC-II-mediated increase in binding of LpL to the $VLDL_p$ interface because the two substrates, DPPC and triacylglycerol, are contained in the same particles. This conclusion is supported by experimental evidence: apoC-II does not increase the binding of radiolabeled LpL to guinea pig apoC-II-deficient $VLDL_p$ covalently attached to Sepharose.[4] It is also consistent with the measured dissociation constants for the binding of apoC-II to LpL which is bound to DPPC vesicles (33). Shirai, Jackson, and Quinn (33) reported that the dissociation constant of the apoC-II:LpL complex found at the vesicle surface is 0.13 μM. However, the affinity of apoC-II for the vesicle surface is 6.5 μM (34), again suggesting that apoC-II's affinity for the lipid surface is too weak to be kinetically significant.

Equation 9 predicts that V_{max} for $VLDL_p$-DPPC hydrolysis should vary linearly with apoC-II. However, such is not the case, presumably because addition of apoC-II also enhances LpL-catalyzed triacylglycerol hydrolysis and, thus, triacylglycerol competitively inhibits DPPC hydrolysis. This presumption is supported by the report that antibodies to LpL inhibit triacylglycerol and phospholipid hydrolyses equally, which is consistent with a single LpL active for both classes of substrates (35). However, the situation is different when DPPC is the only lipid contained in the substrate particles. Fig. 6 shows the data for the LpL-catalyzed hydrolysis of DPPC in apoC-II/apoC-III-DPPC discoidal complexes; the apparent V_{max} is increased 50-fold. The linear dependence of V_{max} on apoC-II concentration in the discoidal complexes is predicted by Equation 9, thereby lending further support to the mechanism proposed herein for apoC-II effects on LpL catalysis.

The effect of apoC-II on LpL-catalyzed phospholipid hydrolysis appears, in summary, to be exclusively a V_{max}-enhancing effect. As Equation 9 shows, the V_{max}-enhancing effect may arise from an increase in k_{cat} ($\beta > 1.0$) and a decreased K_m, or simply from a decreased K_m if $\beta = 1.0$. These two alternatives cannot be distinguished with the present data. ApoC-II enhancement of k_{cat} necessitates that interaction of LpL with apoC-II increases the rate of substrate turnover per se, a feature not indicated for LpL-catalyzed $VLDL_p$-trioleoylglycerol hydrolysis. However, since apoC-II also appears to cause a decreased K_m for DPPC hydrolysis, a common thread binds the mechanisms for apoC-II enhancement of LpL-catalyzed $VLDL_p$-DPPC and -trioleoylglycerol hydrolyses. Both mechanisms involve an increased affinity of LpL for lipid substrates in an interface, and/or greater affinity of apoC-II for the interfacial Michaelis complex (E*S) than for LpL at the interface but with active sites free (E*).

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