Articles

Effect of Diacylglycerol Content on Some Physicochemical Properties of the Insect Lipoprotein, Lipophorin. Correlation with the Binding of Apolipophorin-III†

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ABSTRACT: Diacylglycerol (DG) is the main lipid component of the insect lipoprotein lipophorin. In order to study the effect of DG content on the structure and properties of lipophorin and to analyze the role of DG in the binding of apolipophorin-III (apoLp-III), an exchangeable apolipoprotein, we developed a method that allows the modification of the DG content of lipoproteins. This method employs *sn*-1,2-dioctanoyl glycerol (diC_8-DG). The degree of incorporation of diC_8-DG was determined by including [^{14}C]-diC_8DG in the incubation and subsequent purification of the diC_8-DG-loaded lipophorins in a KBr gradient. The efficiency of diC_8-DG loading of lipophorin is time dependent, but high levels of loading are obtained in relatively short periods of time (100% in 3–4 h). For DG loading up to about 15% (w/w), the efficiency and rate of diC_8-DG loading are independent of the presence of apoLp-III. DG loading above 15% (w/w) in the absence of apoLp-III resulted in aggregation of the particles. Lipophorin particles enriched up to about 30% with diC_8-DG were obtained by this procedure. When lipophorin particles were loaded with diC_8-DG in the presence of apoLp-III, it was observed that binding of apoLp-III was proportional to the amount of diC_8-DG incorporated into the lipophorin particle, indicating that the only requirement for apoLp-III binding to lipophorin is an increased DG content. A decrease in the degree of order of the lipid phase of the lipoproteins was observed by anisotropy of fluorescence of diphenylhexatriene as the content of diC_8-DG was increased. A comparison of the lipid order of artificially loaded lipophorins, in the presence and absence of apoLp-III, with the lipid order of natural lipophorins, which differ in their DG content, and mammalian lipoproteins clearly showed that DG has a extraordinary perturbing effect on the lipid order of lipoproteins. It is suggested that this lipid-disordering effect of DG plays a prominent role in reorganizing the lipoprotein surface in such a manner as to permit apoLp-III to bind.

Lipophorin is the main lipoprotein found in the hemolymph of insects; it transports phospholipids (PL),[1] diacylglycerol (DG), hydrocarbons (HC), sterols, and free fatty acids among insect tissues. Depending on the metabolic state of the insect, the lipid content of lipophorin varies between 35% and 65% (wt %). Lipophorins have been isolated from the hemolymph of several insect species, and many of these studies have recently been summarized (Shapiro *et al.*, 1988; Law & Wells, 1989; Van der Horst, 1990; Ryan, 1990; Soulages & Wells, 1994). Most lipophorin particles contain one molecule each of two different apolipoproteins—apoLp-I, molecular mass ≈ 250 kDa, and apoLp-II, molecular mass ≈ 80 kDa (Shapiro *et al.*, 1984; Surtholt *et al.*, 1992). In some developmental stages and under certain physiological conditions, lipophorin is loaded with increasing amounts of DG and a third, exchangeable apolipoprotein, apolipophorin-III (apoLp-III) (MW = 18–20 kDa), binds to the lipoprotein particle (Bennakkers *et al.*, 1985; Wells *et al.*, 1987).

It has been determined that PL resides on the surface of the lipoprotein (Katagiri, 1985) and HC seems to be located in the interior of the particle unexposed to water (Katagiri *et al.*, 1985). The location of DG in the lipophorin particle is currently a matter of speculation, although the distribution of this lipid between the lipoprotein core and the surface is probably dependent on the lipid composition and DG content of the lipophorin (Soulages & Brenner, 1991; Soulages & Wells, 1994). Because DG is a major lipid component of lipophorin, it is important to understand how this lipid is organized in the lipoprotein particle and the possible effects that a change in the DG content of lipophorin might have on the structure and properties of the lipoprotein. In order to achieve this objective, it is necessary to obtain lipophorins that differ in their content of DG. Although lipophorins with different amounts of DG can be obtained from insects in different metabolic states, these lipophorins will also differ in the quantity of other lipids present, such as PL, and/or in the amount of apoLp-III associated with the lipophorin. It has been possible to obtain lipophorins with different amounts of DG by incubating two different types of lipophorin with a lipid-transfer particle (LTP) which is found in the hemolymph of some insects (Ryan *et al.*, 1986; Ryan, 1990). However, LTP also transfers PL and HC (Ryan *et al.*, 1986; Singh & Ryan, 1991), and so, more than one change in the lipid composition of lipophorin occurs during LTP-mediated lipid loading of lipophorins.

Although it is clear that loading lipophorin with DG leads to binding of apoLp-III (Bennakkers *et al.*, 1985; Wells *et al.*, 1987), the changes in the lipoprotein surface which induce apoLp-III binding are unknown. It has been shown, in various

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[1] Abbreviations: DG, diacylglycerol; PL, phospholipid; HC, hydrocarbon; diC_8-DG, *sn*-1,2-dioctanoin; AKH, adipokinetic hormone; TLC, thin-layer chromatography; apoLp-III, apolipophorin-III.

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artificial systems, that apoLp-III can bind to PL (Kawooya *et al.*, 1986; Wang *et al.*, 1992; Zhang *et al.*, 1993) and/or DG (Kawooya *et al.*, 1986; Demel *et al.*, 1992); however, the lipid(s) recognized by free apoLp-III on the lipoprotein surface remain(s) to be identified.

Here, we describe a simple method for modifying the amount of DG in lipophorins which uses dioctanoin, a short-acyl-chain DG. This method permits the incorporation of DG in such a manner that the extent of the loading can be easily controlled. Using this method, we have determined how DG content affects the structure and properties of lipophorins and how DG content relates to apoLp-III binding.

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained from the following sources: diphenylhexatriene, Molecular Probes, Eugene, OR; *sn*-1,2-dioctanoin and lipid standards, Avanti Polar Lipids, Inc., Alabaster, AL; [1-^{14}C]octanoic acid, ARC, St. Louis, MO; con A-sepharose, phenylsepharose, and high molecular weight markers, Pharmacia LKB, Inc., Piscataway, NJ; alkaline phosphatase Type XXX, trifluoroacetic acid, and trifluoroacetic anhydride, Sigma Chemical Co., St. Louis, MO; silica gel 60 precoated plates, J.T. Baker, Phillipsburg, NJ; adipokinetic hormone, Peninsula Labs, Belmont, CA; BCA protein reagent, Pierce, Rockford, IL.

Insects and Hemolymph Collection. Animals were reared as previously described (Prasad *et al.*, 1986). Hemolymph from larval insects was collected through an incision in the second proleg directly into bleeding solution (100 mM potassium phosphate buffer, pH = 6.5, containing 2 mM EDTA, 1 mM diisopropyl phosphorofluoridate, and 5 mM glutathione). The hemolymph from adult insects was obtained by decapitation of insects after the injection of 1 mL of bleeding solution. In order to isolate low-density lipophorin, adult insects were injected with 100 pmol of AKH (Wells *et al.*, 1987), and hemolymph was collected after 90 min.

Purification of Lipophorin and Apolipophorin-III. Lipophorin was purified by ultracentrifugation in a KBr gradient at 50 000 rpm, 5 °C for 16 h, using a VTi 50 rotor (Shapiro *et al.*, 1984). Lipophorin of density 1.14 g/cm^3 was obtained from the hemolymph of fifth instar feeding larvae. Lipophorins of densities 1.17 and 1.11 g/cm^3 were obtained from insects that were in the first and second day of the prepupal stage, respectively. Lipophorin of density 1.17 was purified by two ultracentrifugation steps to eliminate non-lipoprotein contaminants. Densities were measured by refractometry. Lipophorins were stored at 4 °C in the KBr solution from the density gradient and desalted in a PD10 column, equilibrated in 50 mM potassium phosphate buffer, pH 6.8, just before use. ApoLp-III was purified from the hemolymph of adult insects as previously described (Wells *et al.*, 1985), stored in 0.1 M ammonium bicarbonate, and desalted into 50 mM potassium phosphate buffer, pH 6.8, before use using a Sephadex-25 column.

Synthesis of [^{14}C]-sn-1,2-Dioctanoin. Dioctanoyl phosphatidic acid was prepared using the method of Kanda and Wells (1981) for the preparation of short-acyl-chain lecithins, with the modification of replacing glycerol phosphorylcholine with *sn*-glycerol-3-phosphate. Briefly, 45 mg of glycerol phosphate was dissolved in 300 μL of trifluoroacetic acid. Seven hundred micromoles of octanoic acid containing 5.5 × 10^8 dpm of [^{14}C]octanoic acid was dissolved in 120 μL of trifluoroacetic anhydride and immediately added to the solution of glycerol phosphate. The mixture was vortexed for a few seconds and then kept, with stirring, for 30 min at room temperature. After the addition of 2 mL of methanol, the reaction mixture was dried under N_2. The residue was dissolved in chloroform and loaded onto a small silicic acid column. The column was washed with chloroform until no radioactivity was detected in the eluate. Phosphatidic acid was eluted with methanol. *sn*-1,2-Dioctanoin was obtained by enzymatic hydrolysis of phosphatidic acid using calf intestinal phosphatase. Phosphatidic acid was suspended in 600 μL of phosphatase buffer (50 mM Tris–HCl, pH = 8.2, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine). Two hundred units of phosphatase was added and the reaction allowed to proceed for 30 min at 37 °C. Another 100 units of phosphatase was added, and the reaction mixture was incubated for an additional hour. The incubation mixture was acidified by addition of 300 μL of 0.1 N HCl, and the lipids were extracted with 4 mL of chloroform:methanol, 2:1. The reaction products were dried, redissolved in chloroform, and subjected to chromatography on a column of silicic acid equilibrated with chloroform. [^{14}C]-diC$_8$-DG was eluated with chloroform, and its purity was ascertained by TLC on precoated silica gel 60 plates employing hexane:diethyl ether: acetic acid (70:30:3) as the developing solvent mixture. Although the efficiency of the enzymatic hydrolysis was only about 40%, the product was pure 1,2-[1-^{14}C]dioctanoin.

Electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) was carried out in 3.5–15% acrylamide gradient slab gels, and the proteins were stained with Coomassie Blue R-250. The content of apoLp-III in lipophorin samples was determined by densitometric scanning of the wet gels employing a LKB Ultroscan XL densitometer. Native gel electrophoresis of lipophorin samples was performed in 2.5–15% polyacrylamide gels (Nichols *et al.*, 1986) at 4 °C for 30 h at 130 V in a Tris–borate buffer, pH 8.8. Size calibration was performed with Pharmacia high molecular weight markers (thyroglobulin, 11.6 nm; ferritin, 10.2 nm; catalase, 8.2 nm; lactate dehydrogenase, 6.9 nm) employing a linear least-squares fitting to the relation 1/diameter vs R_f. R_f values were calculated from the migration distance of lactate dehydrogenase.

Fluorescence Measurements. Lipophorin (150 μg/mL) in 50 mM potassium phosphate buffer, pH 6.8, containing 0.1 M NaCl was labeled by the addition of a 3-μL solution of diphenylhexatriene (DPH) in tetrahydrofuran. The suspension was incubated until fluorescence intensity reached a plateau. The concentration of DPH was determined by its absorbance in hexane (Shinitzky & Barenholz, 1974), and the molar ratio of DPH to lipophorin was kept at 3:1. Protein was determined with the BCA reagent employing BSA as the standard. Anisotropy of fluorescence of diphenylhexatriene was measured in a Perkin-Elmer Model MPF 2A spectrofluorometer. Excitation and emission wavelengths were 365 nm (slit width, 8 nm) and 440 nm (slit width, 12 nm), respectively. Incident scattered light was eliminated through a 390-nm cutoff filter. Unlabeled samples were used as reference blanks which served to correct for light-scattering contributions to the fluorescence signal: the contribution of light scattering was no more than 4% of the fluorescence signal. The polarization ratio is $P = GI_{\parallel}/I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ refer to intensities emitted parallel and perpendicular, respectively, to the vertically polarized excitation light and G is a factor for instrumental correction (Azumi & Glynn, 1962). Steady-state fluorescence anisotropy (r_s) was calculated using the equation: $r_s = P - 1/P + 2$, where P is the polarization ratio defined above.

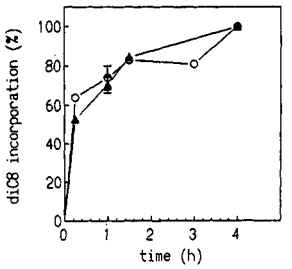

FIGURE 1: Incorporation of diC_8-DG into lipophorin. The efficiency of the diC_8-DG loading was determined from the recovery of radioactivity and protein after incubation of lipophorin (d = 1.11 g/cm^3, 5.4 mg of protein) with [^{14}C]-diC_8 (2 mg) in the absence (○) or presence (▲) of apoLp-III for the indicated times and separation of the loaded lipophorin in a KBr gradient. The data show the average of four determinations for 1 h, duplicate determinations for 4 h, and single values for the remaining times.

Diacylglycerol Loading of Lipophorin. *sn*-1,2-Dioctanoin (neat), containing [^{14}C]-diC_8-DG at the desired specific activity, was injected with a microsyringe into a rapidly mixing solution (2–4 mL) containing lipophorin at a concentration of about 2 mg/mL. When included, apoLp-III was present at a molar ratio of 20:1 relative to lipophorin. The loading reactions were incubated for the desired times at room temperature in a Lab-line Orbit Envirom shaker. The incorporation of diC_8-DG was stopped by dilution of the incubation mixture with cold KBr solution (0.3 g/mL). The resulting solution (total volume, 20 mL) was overlayered with 50 mM potassium phosphate buffer, pH 6.8, and centrifuged at 50 000 rpm for 16 h in a VTi 50 rotor. The gradient was fractionated, and the optical density at 280 nm, the refractive index, and the radioactivity were determined in each fraction. When the purpose of the experiment was to determine the extent of apoLp-III binding, fractions containing lipophorin were pooled and subjected to another ultracentrifugation step to eliminate unbound apoLp-III.

RESULTS

Lipophorin Loading. Lipophorin samples were loaded with diC_8-DG by injection of microliter amounts of pure dioctanoin into the lipoprotein solution. The incorporation of diC_8-DG into lipophorin was time dependent, as shown in Figure 1, which represents an experiment using 2 mg of diC_8-DG and 5.4 mg of lipophorin (d = 1.11 g/cm^3); up to 100% efficiency of loading can be achieved in a few hours. At this level of DG, the time course is independent of the presence of apoLp-III. The efficiency of diC_8-DG incorporation into the lipophorin particle was also measured at different ratios of diC_8-DG to lipophorin, employing a constant amount of lipophorin. Within the range of 0.027–0.197 mg of diC_8-DG/mg of lipophorin, an average incorporation of (70.4 ± 3.6)% (n = 4) was observed. We have been able to incorporate dioctanoin in amounts which represent up to 30% of the lipophorin particle weight. Thus, the levels of loading that can be achieved with the artificial DG compare very well with the maximum amounts of DG that are found in natural lipophorins: low-density lipophorin from adult *Manduca sexta* contains up to 47% DG.

Figure 2 shows how the density of lipophorin changes with the time of incubation of lipophorin with DG in the presence or absence of apoLp-III. This experiment was performed with lipophorin with a density of 1.11 g/cm^3; however, similar results were obtained with lipophorin with a density of 1.17 g/cm^3. With increasing time of incubation, the amount of DG incorporated increases, and this leads to the expected

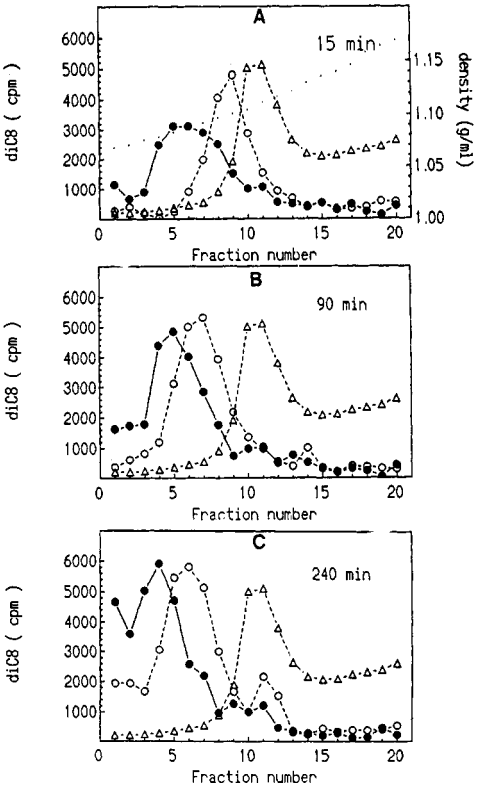

FIGURE 2: Changes in the density of lipophorin after incubation with diC_8-DG. Lipophorin, d = 1.11 g/cm^3 (5.4 mg of protein), was incubated with [^{14}C]-diC_8 (2 mg) in the absence (●) or presence (○) of apoLp-III during 15 min (panel A), 90 min (panel B), and 240 min (panel C). After the separation of the lipoprotein by centrifugation in a KBr gradient, the radioactivity, the optical density, and the density along the gradient were determined. The profile of optical density at 280 nm is shown for control, unloaded lipophorins (△). The profiles of optical density at 280 nm for diC_8-loaded lipophorins matched the profiles of radioactivity but, for the sake of clarity, were omitted.

decrease in the density of the lipophorin. For the sake of clarity, we have not included the profiles of lipophorin distribution for diC_8-DG-loaded lipophorins in Figure 2. However, it is worth noting that, in all samples, the pattern of lipophorin distribution along the density gradient, as judged by SDS-PAGE and optical density, matched the pattern of radioactivity distribution, indicating that diC_8-DG was incorporated into the lipoprotein particles. In the absence of apoLp-III, incorporation of diC_8-DG in amounts greater than 15% of the loaded-lipophorin particle weight produced increasing aggregation, as judged by sample turbidity. In addition, ultracentrifugation of lipophorin samples loaded in the absence of apoLp-III and containing over 15% diC_8-DG resulted in a progressive decrease in the recovery of lipoprotein because aggregated lipophorin adhered to the walls of the centrifuge tubes.

The possible interaction of apoLp-III with DiC_8-DG was investigated under identical conditions to those employed in lipophorin-loading experiments but omitting the lipophorin. Under these conditions, apoLp-III does not seem to interact with the lipid and, after ultracentrifugation, the diC_8-DG is found as an oily layer on the top of the gradient.

In the presence of apoLp-III, the incorporation of diC_8-DG into lipophorin resulted in the binding of the exchangeable apolipoprotein to lipophorin. As shown in Figure 3, the binding of apoLp-III to lipophorin of density 1.11 g/cm^3 did not occur until the degree of diC_8-DG loading reached a level of 10% of the lipophorin weight, under which conditions the sum of

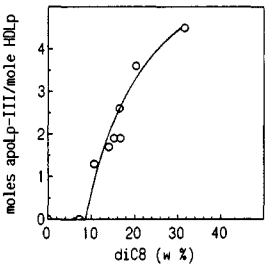

FIGURE 3: Binding of apoLp-III to diC_8-DG-loaded lipophorins. Samples were loaded with DG in the presence of apoLp-III. In order to eliminate possible contamination by unbound apoLp-III, the loaded lipophorins were centrifuged twice in KBr gradients. Then, the apoLp-III was determined by densitometry of Coomassie blue stained gels (SDS-PAGE).

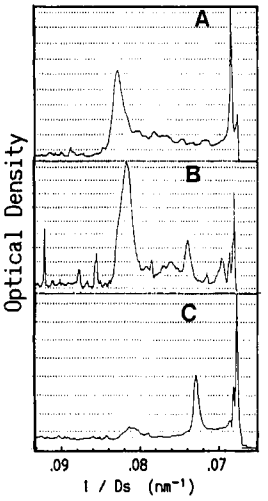

FIGURE 4: Changes in the lipophorin size and particle distribution observed by gradient gel electrophoresis in polyacrylamide gels under native conditions: (A) control lipophorin (d = 1.11 g/cm^3) and (B) and (C) lipophorins loaded with diC_8-DG in the presence of apoLp-III and containing 12.3% and 20.3% of diC_8-DG, respectively. Stokes diameters (D_s) were calculated from the calibration curve, D_s^{-1} vs R_f, obtained with molecular weight markers electrophoresed in the same gel as the lipoprotein samples.

the content of natural and artificial DG was about 28% (w/w). This result is consistent with the fact that apoLp-III is not found in natural lipophorins whose DG content is lower than 25%. The number of molecules of apoLp-III which bind to lipophorin increased as the content of diC_8-DG increased. However, we have not been able to produce particles with as much apoLp-III as that which occurs in natural low-density lipophorin (Wells *et al.*, 1985).

Size of Lipophorin Particles. Control and DiC_8-DG-loaded lipophorins were analyzed by nondenaturing gradient gel electrophoresis in 3.5–15% gels. The densitometric scans of typical gel patterns are shown in Figure 4 for three lipophorin preparations: control lipophorin (d = 1.11 g/cm^3) and two diC_8-DG-loaded lipophorins containing 12.3% and 20.3% diC_8-DG. The control lipophorin showed two major bands with nonhydrated diameters of 12.1 nm (59.2%) and 12.8–13 nm (32.8%) and one minor band of 14 nm (7.9%). Upon loading of lipophorin with diC_8-DG in the presence of apoLp-III, both an increase in the size of the identifiable peaks and a different pattern of size distribution were observed. The major changes observed in those lipophorins loaded in the presence of apoLp-III can be summarized as a decrease in the amount of the subfraction of 12.1 nm which is replaced by particles with apparent diameters of 13.5–13.7 nm. The heterogeneity of particle sizes is not an exclusive characteristic of the lipophorin whose densitogram is shown in Figure 4. A similar pattern

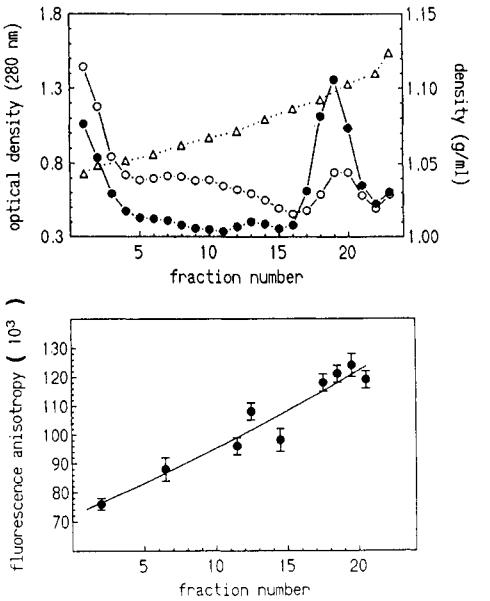

FIGURE 5: Upper panel: Distribution of adult *M. sexta* lipoproteins in a KBr gradient before (●) and after (○) the injection of adipokinetic hormone. The density of the fractions is shown by (△). Lower panel: Change in the anisotropy of fluorescence of DPH in the lipophorin particles isolated from the fractions of the KBr gradient shown in the upper panel. The differences between the values of anisotropy of fluorescence of DPH in lipophorins obtained from control or AKH-treated insects were within the experimental error and were averaged.

of size heterogeneity was observed in the lipophorin of density 1.17 g/cm^3. Although the pattern is different from those of the larval lipophorins, lipophorins from the adult *M. sexta* also showed size heterogeneity when analyzed by gradient gel electrophoresis (data not shown).

Fluorescence Depolarization. The lipid order in native lipophorins and lipophorins loaded with dioctanoin was studied with the fluorescent probe DPH. The localization of DPH in lipoproteins has been previously studied (Ben-Yashar & Barenholz, 1991; Sklar *et al.*, 1980), and those studies showed that DPH resides in regions that are buried away from the aqueous medium. The results obtained with DPH should then be seen as a consequence of the lipid order found in the nonpolar regions of the lipophorins. The lipid core of lipophorins is thought to contain, as its main components, long-chain aliphatic HC (Katagiri *et al.*, 1985) and DG (Pattnaik *et al.*, 1979; Shapiro *et al.*, 1988; Soulages & Brenner, 1991). We have studied the lipid order in three larval native lipophorins, which do not contain apoLp-III, and six lipoprotein fractions obtained from the hemolymph of adult insects which do contain apoLp-III. The highest degrees of lipid order were found in larval high-density lipophorins which have the lowest lipid and DG content. Among the larval lipophorins, those of d = 1.17 g/cm^3 (35% lipid) showed the highest lipid order, r_s = 0.165, followed by those of densities 1.14 and 1.11 g/cm^3 which contain 37.3% and 46.9% lipid and gave values of anisotropy of fluorescence of 0.154 and 0.145, respectively. The main differences in the lipid composition of these three larval lipophorins are the PL to DG molar ratios which are 1.2, 0.92, and 0.92 for the lipophorin fractions with densities of 1.17, 1.14, and 1.11 g/cm^3, respectively (Prasad *et al.*, 1986).

Lipophorin fractions of different densities and DG content were also obtained by ultracentrifugation of hemolymph from adult insects in a KBr gradient (Figure 5A). Each fraction contains variable amounts of DG and apoLp-III (Wells *et al.*,

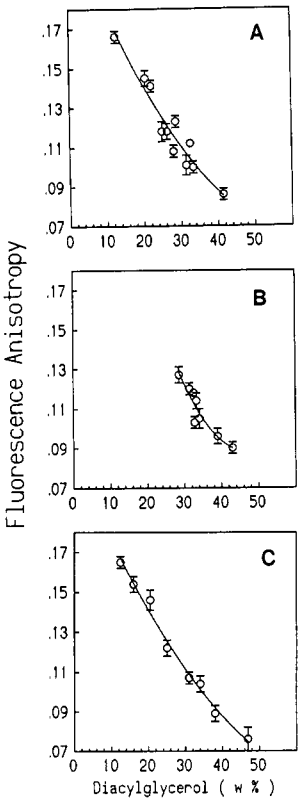

FIGURE 6: Modification of the lipophorin–lipid order by the content of DG in the lipoprotein. Curves show the change in the anisotropy of fluorescence of DPH in lipophorins loaded with different amounts of diC_8-DG in the absence of apoLp-III (panel A) or in the presence of apoLp-III (panel B) and naturally occurring lipophorins that differ in the content of DG (panel C). To calculate the wt % of lipophorin DG, the content of both natural DG and incorporated diC_8-DG was taken into account. Error bars represent the experimental standard deviations.

1987). From the top to the bottom of the density gradient, the content of DG decreases from 47% to 25% and the number of molecules of apoLp-III/mol of lipophorin decreases from 14 to 2. Accompanying these changes, a continuous increase in the anisotropy of fluorescence is observed (Figure 5B).

The effect of DG alone on the anisotropy of fluorescence was studied in lipophorins that were loaded with different amounts of diC_8-DG in the absence of apoLp-III. The results of this study (Figure 6A) indicate that DG by itself provokes major changes in the order of the lipid phase of the lipoprotein particles. Figure 6B shows the effect of diC_8-DG on the lipid order of lipophorins loaded in the presence of apoLp-III. A slight increase, about 5%, in the anisotropy of fluorescence of apoLp-III-containing lipophorins is observed when compared to that of lipophorins without apoLp-III. Thus, binding of apoLp-III does not seem to affect significantly the lipid order of the artificially loaded lipophorins. Figure 6C presents a composite of anisotropy of fluorescence data for natural lipophorins and also shows that there is an increase in lipid disorder which correlates with the increase in lipophorin DG content.

DISCUSSION

The present study describes a simple and reproducible method to load insect lipoproteins with DG. Even though we have utilized an artificial, short-acyl-chain DG, the properties of the loaded lipoproteins resemble, in many respects, the properties of natural lipophorins. Thus, we have shown that diC_8-DG-loaded lipophorins bind apoLp-III and that the effect of diC_8-DG content on the lipid order of lipophorins is similar to that observed in natural lipophorins which differ in their DG content.

Effect of DG Content on the Properties of Lipophorin. As revealed by these studies, DG content has two major effects on lipophorin: decreased lipid order and increased particle size. The close correlation of the anisotropy of fluorescence with the content of DG observed in natural or artificially loaded lipophorins suggests that DG is the lipoprotein component which produces the large lipid disorder observed in naturally or artificially DG-loaded lipophorins. The data also show that, as probed by DPH, apoLp-III has a negligible effect on the lipid order of the lipoproteins. In this regard, a comparison of the values of anisotropy of fluorescence obtained for four different human lipoproteins at 25 °C might help to clarify the particular effect of DG on lipid order. The following approximate values have been reported for human VLDL, LDL, HDL_2, and HDL_3, respectively: 0.115, 0.270, 0.205, and 0.220 (Ben-Yashar & Barenholz, 1991). The lipid order is dependent on the nature of the lipid phase and the nature of lipid–protein interactions. Moreover, a larger ordering effect of the protein on the lipid component is to be expected when the protein to lipid ratio is increased. According to the lipid to protein ratio, most of the lipophorins should be comparable to HDL_2 and HDL_3, but these human lipoproteins obviously have a larger lipid order than the lipophorins. On the other hand, on the basis of the similarities of the properties of apoLp-I, apoLp-II, and apoB (Kashiwazaki & Ikai, 1985; Kawooya *et al.*, 1989), lipophorins could be compared to VLDL and LDL. In the later case, it is observed that, even though LDL has a higher lipid to protein ratio than any lipophorin, lipophorins have a higher degree of lipid disorder than LDL. When compared to VLDL, which has 92% lipid, it is also observed that any lipophorin with more than 25% DG has a higher lipid disorder.

The comparison of the anisotropy of fluorescence of DPH observed in lipophorins with diverse DG contents or between lipophorins and human lipoproteins, where DG is virtually absent, further confirms the extraordinary lipid disordering effect of DG.

The size heterogeneity found in lipophorins observed by electrophoresis under native conditions is an experimental fact that does not conform with the widely accepted, but unproven, suggestion that in most stages of the life cycle of insects, or at least in each density fraction, only one lipophorin species is present. The presence of three lipophorin subspecies has been routinely found. We do not yet know the nature of the particle heterogeneity or its physiological significance.

Role of DG in Promoting ApoLp-III Binding to Lipophorin. It has been proposed that the function of apoLp-III is to bind to hydrophobic "patches" on the surface of lipophorin induced by DG loading (Kawooya *et al.*, 1986; Wells *et al.*, 1987). The data presented clearly support this hypothesis because, in the absence of apoLp-III, DG loading leads to lipoprotein aggregation, whereas in the presence of apoLp-III, stable DG-loaded lipoproteins with bound apoLp-III are formed. ApoLp-III binds spontaneously to DG- or phosphatidylcholine-coated polystyrene beads (Kawooya *et al.*, 1986) and to monolayers of 1,2-DG, 1,3-DG, and PC (Demel *et al.*, 1992) and PL liposomes (Wang *et al.*, 1992; Zhang *et al.*, 1993). However, the requirements for binding of apoLp-III to lipophorin are unknown. The loading of lipophorin with DG can be accomplished by incubating fat bodies with lipophorin and purified apoLp-III in the presence of adipokinetic hormone and Ca^{2+} (Van Heusden *et al.*, 1984; Chino *et al.*, 1989).

Under these conditions, binding of apoLp-III to the DG-loaded lipophorin is observed. However, due to the absolute requirement for fat body in this in vitro system, it could not be determined whether other factors, in addition to DG, are required to promote binding of apoLp-III to lipophorin. The present report shows that incorporation of DG into lipophorin is sufficient to promote binding of apoLp-III to lipophorin. The fact that larval lipophorin can bind apoLp-III when loaded with diC_8-DG also indicates that there is nothing special about adult lipophorins in promoting binding of apoLp-III.

Lipophorin can be envisaged as containing a hydrophobic lipid core and an outer layer of PL and apolipoproteins. Despite the hydrophobic nature of DPH, it can be assumed that it will be distributed among the inner lipid core and the hydrophobic acyl chains of the lipids of the outer layer (Ben-Yashar & Barenolz, 1991). Thus, to a certain extent, the anisotropy of fluorescence of DPH will reflect the lipid order of the lipoprotein surface. If that is the case, the increase in the content of DG of lipophorins would imply an increase in the content of surface DG with concomitant surface disorder.

When compared to apoA-I, apolipophorin-III seems to interact weakly with lipid surfaces (Liu *et al.*, 1991). In addition, the denaturation of apoLp-III resembles that of apoA-IV which is the mammalian apolipoprotein with the lowest lipid affinity (Ryan *et al.*, 1993). We suggest that the particularly high perturbing effects of DG on lipid surfaces make the binding of apoLp-III and the stabilization of lipophorin possible. In this regard, the data of anisotropy of fluorescence and the fact that increasing aggregation of lipophorin is observed when the incorporation of diC_8-DG exceeds 15% in the absence of apoLp-III support our notion on the effects of DG. Thus, both the lipid disorder and the generation of defects with exposure of hydrophobic spots to the aqueous medium would constitute the driving force for the binding of apoLp-III to the lipophorin surface. It is believed that the major changes produced by DG on the properties of natural and artificial membranes (Das & Rand, 1986; Allan *et al.*, 1978; Ohki *et al.*, 1982; De Boeck & Zidovetzki, 1989; Ortiz *et al.*, 1992), such as changes in charge, morphology, fusion, and bilayer to nonbilayer transitions, are due to the cone-shaped geometry of the DG molecule which perturbs the packing of the polar groups of the lipids in the membrane surface. This perturbation would result from an increased hydration of the acyl chains and a concomitant destabilization due to the hydrophobic effect. Following this rationale, the relatively lower ability of diC_8-DG-loaded lipophorin to promote the binding of apoLp-III might be due to the lower superficial disorder generated by diC_8-DG than by natural DG. This possibility is supported by the smaller size and the saturation of the acyl chains of diC_8-DG, which result in a lower disproportion between the size of the polar and hydrophobic regions of the molecule. DiC_8-DG would have a more cylindrical shape than long-chain DG, and therefore, its presence on the surface would promote a lower disorder, or number of defects. The lower number of defects would mean the existence of a lower number of binding sites for apoLp-III and/or a lower binding affinity. In this regard, the relatively low affinity of apoLp-III for lipids, and a reduced binding affinity due to the properties of diC_8, might be affecting the estimation of the binding of apoLp-III to diC_8-loaded lipophorins. Thus, it is possible that after two ultracentrifugations in high-salt media, a partial dissociation of apoLp-III from the lipophorin surface had taken place.

We suggest that the binding of apoLp-III to lipophorins is dependent on the presence of a minimal amount of DG in the lipophorin particle. This suggestion is supported by the observation that the depletion of DG from the highly DG-loaded low-density lipophorin leads to the release of apoLp-III (Kawooya *et al.*, 1991), although it has also been reported that the depletion of DG does not promote the release of apoLp-III (Hiraoka & Katagiri, 1992), an observation we cannot explain. It has also been reported (Singh *et al.*, 1992) that the LTP-mediated transference of DG from low-density lipophorin to human LDL promotes the aggregation of LDL.

While the presence of a minimal amount of DG in lipophorin is necessary to promote apoLp-III binding, it does not follow that apoLp-III interacts exclusively with DG. The crystal structure of *Locusta migratoria* apoLp-III was recently elucidated (Breiter *et al.*, 1991) and showed the presence of five amphipathic α-helices. Sequence-based prediction of the secondary structure of apoLp-III has shown the presence of type A α-helices which have shown to interact with phospholipids (Segrest *et al.*, 1992). Thus, it is likely that on the surface of lipophorin, apoL-III interacts with both PL and DG. However, it is necessary to enrich the lipophorin with DG in order to allow the binding of apoLp-III. Perhaps the positive charges which border type A α-helices interact with the negatively charged phosphate groups in the phospholipids before the proposed conformational change in apoLp-III can occur (Breiter *et al.*, 1991). In this regard, the presence of DG in the surface may serve to disrupt interactions between phospholipid head groups that would shield the phosphate group from apoLp-III.

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