Calorimetric and Spectroscopic Studies of the Interaction of *Manduca sexta* Apolipophorin III with Zwitterionic, Anionic, and Nonionic Lipids†

Yuanpeng Zhang, Ruthven N. A. H. Lewis, Ronald N. McElhaney, and Robert O. Ryan*

Department of Biochemistry and Lipid and Lipoprotein Research Group, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7

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ABSTRACT: The nature of the interaction of apolipophorin III (apoLp-III) from the insect *Manduca sexta* with a variety of zwitterionic and anionic phospholipids and with several nonionic glycolipids was investigated by differential scanning calorimetry (DSC) and ^{31}P-NMR spectroscopy. Monoglucosyldiacylglycerol, phosphatidylethanolamine, and phosphatidic acid (pH 7.2) appear to exhibit the weakest interaction with apoLp-III. DSC studies revealed that the gel/liquid-crystalline phase transition of these lipids is little affected by the binding of apoLp-III. Diglucosyldiacylglycerol, phosphatidylcholine (PC), phosphatidylserine, and phosphatidic acid (pH $\simeq$8.8) seem to exhibit somewhat stronger interactions with apoLp-III. The binding of apoLp-III to these lipids induces the formation of lipid domains which melt less cooperatively and at higher temperatures than do the pure lipid dispersions, while having little effect on the melting enthalpy of lipid hydrocarbon chains. Phosphatidylglycerol (PG) and phosphatidic acid (pH >9.3) appear to exhibit the strongest interactions with apoLp-III. The binding of apoLp-III to these lipids severely disrupts their bilayer structure, resulting in marked reductions in the cooperativity and enthalpy of the gel/liquid-crystalline phase transition of the lipids. Studies of binary mixtures of PC and PG indicate that such bilayer-disrupting interactions only occur in the presence of nonphysiologically high concentrations of PG. The binding of apoLp-III to binary mixtures of diacylglycerol and zwitterionic phospholipid has little effect on the chain-melting or the bilayer/nonbilayer phase transitions of these lipids, but it appears to promote the retention of water at the surface of the lipid aggregate. Our results indicate that the binding of apoLp-III to lipid bilayers is mediated primarily through polar and/or ionic interactions at the lipid bilayer surface. Our results also suggest that the interaction of apoLp-III with lipid bilayers promotes the hydration of their surfaces, a property which is consistent with the proposed in vivo functions of this protein.

In living systems, lipid transport is facilitated by lipoproteins. Much is now known about the structure and the function of the diverse array of mammalian lipoprotein particles. These large complexes are generally composed of a spherical nonpolar core of cholesterol ester and triacylglycerols surrounded by a monolayer of phospholipids, cholesterol, and apolipoproteins [see Shen et al. (1977)]. In insects, lipid transport is carried out in similar ways, but only one lipoprotein, lipophorin, serves to meet the varied lipid transport requirements (Shapiro et al., 1988; Ryan, 1990). An important aspect of lipophorin function is its role in lipid metabolism during flight [see Beenakkers et al. (1985) and Van der Horst (1990)]. In response to hormonal stimulation, adult high-density lipophorin (HDLp-A)[1] is converted to LDLp by the uptake of diacylglycerol (the transport form of neutral lipid in insects). Concomitant with DAG loading of lipophorin, there is a reversible association of multiple copies of apolipophorin III (a water-soluble apolipoprotein, M_r 18 000) with the LDLp particle (Kawooya et al., 1984; Wells et al., 1987). It has been postulated that binding of apoLp-III effectively covers the surface of DAG-enriched lipophorin particles with polar moieties, thereby maintaining their aqueous solubility and stabilizing them with respect to aggregation, fusion, etc. (Shapiro et al., 1988). Thus, in association with LDLp, DAG can be delivered to the flight muscles to be hydrolyzed by a lipophorin lipase and metabolized as an energy source (Wheeler et al., 1984). With the resulting depletion of DAG from the LDLp particle, apoLp-III is released, thereby regenerating HDLp-A. Since the HDLp-A and apoLp-III can re-form new LDLp particles, they can function as a DAG shuttle (Van Heusden et al., 1987; Ryan, 1990).

The reversible association of apoLp-III with lipid surfaces and its dual existence in lipid-associated and lipid-free forms raise fundamental questions about its structure and conformation as well as the nature of its interaction with lipid. At this time, however, there is little structural information on the lipid-bound forms of apoLp-III because most structural data have been obtained with the soluble, lipid-free form. In the case of *Manduca sexta*, apoLp-III is a single-chain, nonglycosylated polypeptide containing 166 amino acids (Cole et al., 1987) of which 27 are negatively charged, 29 are

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* Address correspondence to this author.

[1] Abbreviations: DSC, differential scanning calorimetry; ^{31}P-NMR, phosphorus nuclear magnetic resonance; apoLp-III, apolipophorin III; HDLp-A, adult high-density lipophorin; LDLp, low-density lipophorin; L/P, lipid/protein ratio; T_m, gel lamellar to fluid lamellar phase transition temperature; ΔH, transition enthalpy; $\Delta T_{1/2}$, transition width (measured at half-height); PC, phosphatidylcholine; PE, phophosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; DEPE, dielaidoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; DMPA, dimyristoylphosphatidic acid; DMPS, dimyristoylphosphatidylserine; DLPE, dilauroylphosphatidylethanolamine; MGDG, monoglucosyldiacylglycerol; DGDG, diglucosyldiacylglycerol; DAG, diacylglycerol; L_α, fluid lamellar phase; L_β, gel lamellar phase; L_c, crystalline phase; H_{II}, reversed hexagonal phase; PBS, phosphate-buffered saline.

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positively charged, and 28% of the total are hydrophobic. In solution, this apoLp-III exists as a monomeric species which adopts a primarily α-helical conformation (Kawooya et al., 1986; Ryan et al., 1993). In the case of *Locusta migratoria* apoLp-III, the three-dimensional structure has been determined (Holden et al., 1988; Breiter et al., 1991). Such studies indicate that lipid-free apoLp-III contains a bundle of five long α-helices, in which the hydrophobic residues face inward and the hydrophilic residues are oriented toward the aqueous environment. Amino acid sequence comparisons indicate that there is a 50% sequence homology between *M. sexta* and *L. migratoria* apoLp-III's but that the two proteins differ with respect to the percentages of charged and nonpolar residues present (Kanost et al., 1988). Despite these differences, it has also been shown that *M. sexta* and *L. migratoria* apoLp-III's are functionally indistinguishable (Van der Horst et al., 1988). Thus, it seems probable that both proteins possess similar amphipathic helix folding motifs which contribute to their function as lipid-binding proteins.

There has been considerable speculation about the structure of lipid-bound apoLp-III and about the mechanism by which it associates with lipid. For example, it has been suggested that the protein may undergo a dramatic conformational change when it interacts with a lipid surface [see Breiter et al. (1991)]. Such ideas are based on the results of monolayer film studies, which indicate that the molecular exclusion area of apoLp-III at the air/water interface is considerably greater than that expected of a globular protein of comparable size [see Kawooya et al. (1986)]. Thus, it was suggested that the solution structure of apoLp-III may be unstable when the protein is adsorbed to a lipid interface, resulting in a conformational change which expands the area of interfacial contact. From an examination of the three-dimensional structure of apoLp-III, some workers envisage that the putative conformational change would result in the exposure of the hydrophobic faces of the amphipathic α-helices, which in the absence of lipid would normally be sequestered in the interior of the helix bundle (Breiter et al., 1991). On the other hand, recent monolayer film studies have indicated that the molecular exclusion area occupied by apoLp-III in a mixed lipid/apoLp-III monolayer is actually less than that observed in lipid-free apoLp-III monolayers (Demel et al., 1992). However, it is still unclear whether binding of apoLp-III to a lipid surface actually results in a decrease in the area occupied by the protein, since the calculations of the latter authors were based on the assumption that the lipid/apoLp-III interaction did not affect the mean molecular areas of the lipids themselves. Interestingly, however, Demel and co-workers also showed that the high α-helical content of the protein is retained even when it is adsorbed to lipid monolayers. In considering plausible mechanisms for the binding of apoLp-III and lipids, some workers have speculated that apoLp-III may initially bind to lipid surfaces via one of its ends before unfolding onto the surface (Kawooya et al., 1986; Wells et al., 1987), while others have suggested that a region of the *L. migratoria* apoLp-III constituting "hinged" loops between helices 1 and 2 and between helices 3 and 4 is responsible for initiating interaction with lipid, with subsequent opening of the bundle via the hinges (Breiter et al., 1991). Resolution of these issues requires not only more definitive data on the structure and conformation of apoLp-III when bound to lipid surfaces but also a more thorough characterization of the nature of apoLp-III/lipid interactions.

Differential scanning calorimetry is a very powerful technique for studying thermodynamic aspects of lipid/protein interactions and has been widely used to study the interactions between both surface-associating and membrane-penetrating proteins with lipid bilayer membranes [see McElhaney (1986) and Boggs (1987)]. To date, the overwhelming majority of such studies have been on investigations of the interactions of proteins (or peptides) only with PC bilayers. However, such studies may only provide a limited perspective for our understanding of the principles of lipid/protein interactions, primarily because the nature of the interaction of any given protein with a lipid bilayer can vary widely with the type of lipid being studied [for an example, see Boggs and Moscaerllo (1978)]. In the case of the interaction of human apolipoproteins with lipid bilayers, studies so far have been limited to PC and PG (Epand et al., 1990; Surewicz et al., 1986; Aune et al., 1977; Jonas & Mason, 1981; Swaney & Chang, 1980) and, to a lesser extent, PS (Surewicz et al., 1986) and sphingomyelin (Swaney, 1983). These studies have shown that most apolipoproteins bind to acidic lipids more tightly than to zwitterionic lipids (Surewicz et al., 1986; Epand et al., 1990). Given the limited range of such studies, it is evident that a systematic program of study of a series of apolipoproteins with a wide array of lipids using a variety of physical techniques is required in order to fully characterize the interactions between apolipoproteins and lipids and to provide some basis for our understanding of the process. In this paper, we describe a detailed study of the interaction of *M. sexta* apoLp-III with a range of anionic, zwitterionic, and nonionic lipids. Specifically, we concentrate primarily on a characterization of the thermodynamic aspects of this interaction by DSC, and, where warranted, we use ^{31}P-NMR spectroscopy to examine the effect of the protein on the structural integrity of the lipid bilayer.

MATERIALS AND METHODS

The PC's and PE's used in this study were synthesized in this laboratory [for details, see Lewis et al. (1987) and Lewis and McElhaney (1993)], and all other phospholipids were obtained from Avanti Polar Lipids Inc. The DAG's dipalmitin and diolein were obtained from Sigma Chemical Co. Samples of dielaidoyl-MGDG and dielaidoyl-DGDG were purified from polar lipid extracts of *Acholeplasma laidlawii* B cultured to be acyl chain homogeneous in elaidic acid [for experimental details, see Silvius and McElhaney (1978) and Monck et al. (1992)]. Adult *M. sexta* were obtained from a laboratory colony reared as described elsewhere (Prasad et al., 1986). Hemolymph was obtained and apoLp-III was purified according to the procedure of Wells et al. (1985).

The lipid and apoLp-III vesicle suspensions were prepared for DSC as follows. With samples such as PC and mixtures of PC and PG, 1–2 mg of lipid was dissolved in chloroform and dried by slow evaporation under a stream of nitrogen so that the lipid forms a thin film on the inner surface of a glass tube. After removal of the last traces of solvent in vacuo overnight, PBS (100 mM PO_4, 150 mM NaCl, and 10 mM EDTA, pH 7.2) or an apoLp-III solution in PBS was added to obtain the desired lipid:protein ratio, and the hydrated lipid vesicle preparations were prepared by vortexing the mixture at temperatures above the T_m of the lipid. With samples such as PE and some samples of anionic lipids, this methodology was not satisfactory because the lipid tended to adhere to the sides of the test tube, thereby incurring considerable losses. In such cases, the lipid was initially lyophilized from benzene to form a loose powder, and vesicle suspensions were then prepared by adding PBS or apoLp-III solutions to the lipid in a glass tube followed by vortexing above the T_m of the lipid. In some cases, apoLp-III/lipid mixtures were also made by the addition of the protein solution to preformed lipid vesicles

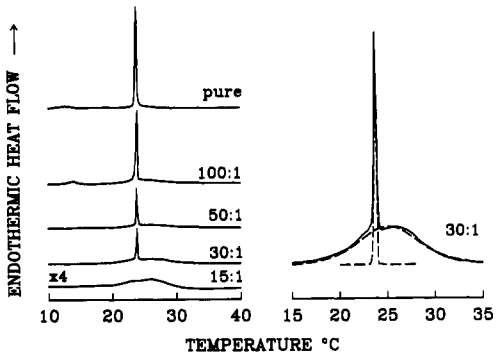

FIGURE 1: DSC heating thermograms of DMPC and DMPC/apoLp-III mixtures. The thermograms in the left panel were obtained at the L/P ratios indicated. An enlarged view of the DSC thermograms of the DMPC/apoLp-III mixture (30:1) is shown in the right panel to illustrate the fine structure of the thermograms observed and our estimates of the shapes of the component peaks (dashed lines).

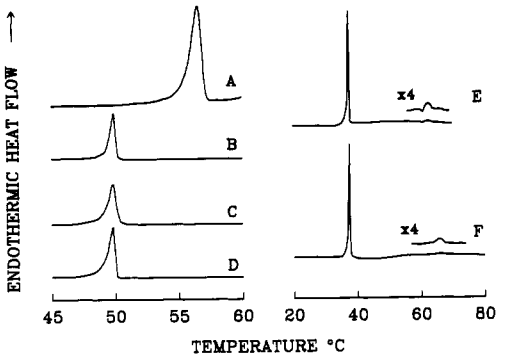

FIGURE 2: DSC thermograms of DMPE (left panel) and DEPE (right panel) and their mixtures with apoLp-III (L/P = 50:1). (A) DMPE sample after extensive low-temperature incubation. (B) Pure fully hydrated DMPE sample. (C) DMPE/apoLp-III (50:1) sample after low-temperature incubation for 1 week. (D) DMPE/apoLp-III (50:1) sample. (E) Pure fully hydrated DEPE sample. (F) DEPE/apoLp-III (50:1) sample.

that were prepared by hydration of the dried lipid (or lipid mixture) with PBS. DSC measurements were performed with a Microcal MC-2 high-sensitivity differential scanning calorimeter operating at heating rates of either 11 °C/h (pure lipids and samples which gave very sharp transitions) or 27 °C/hr (samples exhibiting broad transitions). Under the conditions employed, the thermal and electronic properties of this calorimeter are not limiting, and thus the DSC curves presented have not been corrected for the finite response time of the instrument.

Samples (containing typically 15 mg of phospholipid) were prepared for ^{31}P-NMR spectroscopy by methods which were essentially similar to that described for DSC except that an EDTA buffer (10 mM Tris, 10 mM EDTA, and 50 mM NaCl, pH 7.5) was used instead of PBS. ^{31}P-NMR spectra were recorded with a Varian Unity 300 spectrometer operating at 121.42 MHz for ^{31}P. The spectra were obtained using the single-pulse direct excitation techniques and postprocessing data handling procedures previously reported (Lewis et al., 1988).

RESULTS

Studies of the Interaction of ApoLp-III with Zwitterionic Phospholipids. (i) Interactions with Phosphatidylcholine Bilayers. Figure 1 shows the high-sensitivity DSC heating endotherms exhibited by aqueous dispersions of pure DMPC and by various DMPC/apoLp-III mixtures. As expected, pure DMPC exhibits endothermic transitions at 12.5 and 23.9 °C, which correspond to the pretransition and the highly cooperative gel/liquid-crystalline (chain-melting) phase transition of this lipid, respectively [see Marsh (1990) and references cited therein]. In the presence of low to moderate levels of apoLp-III, the observed chain-melting transition endotherm is composed of a sharp peak centered at 23.9 °C superimposed on a broader endotherm centered at 26.5 °C (see Figure 1, right panel). The peak temperatures and transition widths of both the sharp and broad components remain unchanged at all lipid:protein ratios studied. Interestingly, the total enthalpy change observed (≅5.8 kcal/mol of PC) is also insensitive to changes in the lipid:protein ratio. However, the relative contribution of the broad component to the total enthalpy change increases, and the relative contribution of the sharp component decreases, with increasing protein content, and only the broad component is present at lipid:protein ratios near and below 15:1 (see Figure 1). We thus suggest that the sharp component arises from domains of DMPC which are not interacting with the protein whereas the broad component originates from those lipid domains which are perturbed by interaction with apoLp-III. This assignment is supported by the effect of apoLp-III on the pretransition of the lipid. We find that an endothermic transition corresponding to the pretransition is calorimetrically detectable at all lipid:protein ratios at which the sharp component is observed although the enthalpy of the pretransition decreases with decreases in the lipid:protein ratio. Since the pretransition of a PC bilayer is very sensitive to the presence of impurities, it is normally abolished when relatively small amounts of such "impurities" are present [see McElhaney (1982) and references cited therein]. Thus, the persistence of the pretransition in the presence of relatively high apoLp-III concentrations is consistent with the existence of domains of pure DMPC in which interactions between hydrocarbon chains are not significantly perturbed by interaction with protein. Indeed, the fact that apoLp-III has little or no effect on the enthalpy of the overall chain-melting phase transition further suggests that its interaction with PC bilayers does not significantly alter the energetics of hydrocarbon chain packing in both the gel and liquid-crystalline states.

(ii) Interactions with Phosphatidylethanolamine Bilayers. The left panel in Figure 2 shows the DSC endotherms obtained in our studies of the interaction of apoLp-III with DMPE bilayers.[2] Unlike the PC's, aqueous dispersions of fully hydrated PE samples do not easily disperse into homogeneous suspensions. However, our DSC studies indicate that fully hydrated dispersions were formed by our experimental protocol because the gel/liquid-crystalline phase transition temperature (49 °C, see curve B) and associated enthalpy change (5.9 kcal/mol) observed are comparable with those previously measured for fully hydrated dispersions of this lipid [see Marsh (1990) and Lewis and McElhaney (1993) and references cited therein]. Interestingly, when mixed with apoLp-III ($L/P \cong$ 50), DMPE readily disperses into homogeneous fully hydrated dispersions. However, from the DSC data shown in Figure 2 (curve D), it is evident that the transition temperatures, transition widths, and enthalpy changes observed in the apoLp-III/DMPE dispersions are similar to those exhibited by dispersions of the pure lipid. Although interaction of apoLp-III with DMPE has no discernible effect on the properties of the gel/liquid-crystalline phase transition of this lipid, the relative ease with which these PE samples disperse when the protein is present clearly indicates that the protein does interact with the lipid. Thus, our DSC results suggest that interaction

[2] A similar pattern of behavior was also observed in studies of the interaction of apoLp-III with DLPE bilayers.

of apoLp-III with PE bilayers is considerably less perturbing of bilayer lipid/lipid interactions than is the case with PC bilayers.

To further characterize the interaction of apoLp-III with PE bilayers, the effect of apoLp-III on the formation of the L_c phase of DMPE and on the formation of the H_{II} phase of DEPE was examined. It is well-known that when pure samples of short-chain PE's such as DMPE are extensively incubated at low temperatures, the lipids form one or more highly ordered crystal-like phases which melt directly to the liquid-crystalline phase (i.e., they exhibit L_c/L_α phase transitions) at temperatures well above those of the normal gel/liquid-crystalline (i.e., L_β/L_α) phase transitions [see Lewis and McElhaney (1993) and references cited therein]. As shown in Figure 2 (curve A), the endothermic transition observed after pure DMPE is incubated at low temperatures occurs some 6 °C above the normal L_β/L_α phase transition, indicating that this thermotropic event is the L_c/L_α phase transition [see Lewis and McElhaney (1993) and references cited therein]. However, in the presence of apoLp-III (see curve C), the L_c phase of this lipid does not form even after prolonged periods of low-temperature incubation or several cycles of freezing and thawing [see Lewis and McElhaney (1993) for details of the effects of the freeze–thaw cycles]. Given these results, it is clear that apoLp-III does interact with DMPE bilayers and that its interaction inhibits the process(es) of nucleation and growth of the quasi-crystalline phases formed by this lipid. Since L_c phase formation involves headgroup and interfacial dehydration as well as the formation of an extended hydrogen-bonding network in the headgroup and interfacial regions of these lipid bilayers [see Lewis and McElhaney (1993)], the above observations are very significant because they relate directly to the proposed biological function of this protein (see Discussion).

Evidence for relatively weak interactions between apoLp-III and PE bilayers was also obtained in our studies of DEPE and its mixtures with apoLp-III. As shown in Figure 2 (curve E), the phase transitions exhibited by pure DEPE bilayers are a highly cooperative gel/liquid-crystalline phase transition centered at 37 °C and a weakly energetic lamellar/inverted hexagonal phase transition at 63 °C. (There are no reports of the formation of an L_c phase by this lipid.) As was observed with DMPE, the properties of the gel/liquid-crystalline phase transition of DEPE remain unchanged in the presence of apoLp-III. However, there is evidence for changes in the properties of the lamellar to H_{II} phase transition of the lipid when apoLp-III is present. As shown in Figure 2 (curve F), the L_α/H_{II} transition exhibited by the DEPE/apoLp-III mixture is less energetic than that of the pure lipid and is also shifted to higher temperatures by about 3 °C. It has been suggested that the lamellar to H_{II} phase transition of hydrated lipid bilayers may be mediated by interbilayer headgroup contact (Cullis et al., 1980; Allen et al., 1990) and involve a small decrease in bilayer hydration (Cevc & Marsh, 1987). Given the protein-mediated increase in the L_α/H_{II} transition temperature observed here, we suggest that interaction between apoLp-III and the surface of DEPE bilayers stabilizes the lamellar phase with respect to the H_{II} phase by inhibiting interbilayer headgroup contact and headgroup dehydration. Such a possibility is directly relevant to the proposed physiological role of apoLp-III and will be explored further in the Discussion.

Studies of the Interaction of ApoLp-III with Anionic Lipids. (*i*) *Interactions with Phosphatidylglycerol Bilayers.* DSC thermograms of mixtures of DMPG and apoLp-III are shown in Figure 3. As expected, the pure lipid exhibits a pretransition

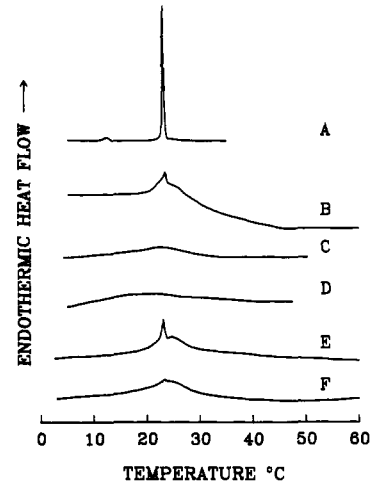

FIGURE 3: DSC thermograms exhibited by DMPG and DMPG/apoLp-III mixtures. Data are presented for (A) pure DMPG, (B) DMPG/apoLp-III (200:1), (C) DMPG/apoLp-III (100:1), (D) DMPG/apoLp-III (50:1), (E) DMPG/apoLp-III (50:1), and (F) DMPG/apoLp-III (50:1). The samples giving rise to thermograms A–D above were prepared by hydration of the lyophilized lipid sample with a solution of apoLp-III in PBS. The samples giving rise to thermograms E and F were prepared by the addition of apoLp-III to preformed aqueous dispersions of the lipid. Thermograms E and F correspond to the first and third DSC scans obtained after the addition of the protein.

at 12.5 °C and a chain-melting phase transition at 23.0 °C. Unlike the zwitterionic phospholipids, however, the interaction of apoLp-III with these negatively charged bilayers is manifest by dramatic changes in the thermotropic phase behavior of these lipids even at relatively low protein concentrations. As is evident from Figure 3, the presence of even low concentrations of the protein results in considerable broadening of the L_β/L_α transition of the lipid, which becomes virtually undetectable at lipid:protein ratios near 50:1. Moreover, in contrast to suspensions of pure DMPG, the apoLp-III-containing PG samples are transparent. This observation, and the dramatic effects of the protein on the thermotropic phase behavior of this lipid, suggests that its interaction with apoLp-III results in a major change in the structure of the lipid aggregate. This possibility was explored by investigating the effect of the addition of the protein to preformed multilamellar vesicles of DMPG by DSC, and by characterizing the resulting lipid/protein mixture by ^{31}P-NMR spectroscopy. As illustrated in Figure 3, the first scan (scan E) obtained after the addition of the protein to preformed DMPG multilamellar vesicles consists of a sharp peak centered at the T_m of the pure lipid (23.0 °C) and a broader component centered at higher temperatures (25 °C). With subsequent heating scans (scan F), the sharp component eventually disappears, and the area under the broad component progressively decreases. Thus, the initial interaction of apoLp-III with preformed PG bilayers may be very similar to the seemingly stable association observed when the protein interacts with PC bilayers. However, the initial association of apoLp-III with PG bilayers is thermodynamically unstable with respect to some other associated state in which interactions between the hydrocarbon chains of the lipid molecules have been severely perturbed. That the interaction of apoLp-III with these negatively charged bilayers does eventually result in a drastic change in the structure of the lipid aggregate was confirmed by the ^{31}P-NMR spectroscopic experiments described below.

The left panel in Figure 4 shows the ^{31}P-NMR spectra of samples of pure DMPG and of a DMPG/apoLp-III mixture (L/P = 50:1). Pure lipid exhibits the typical powder pattern

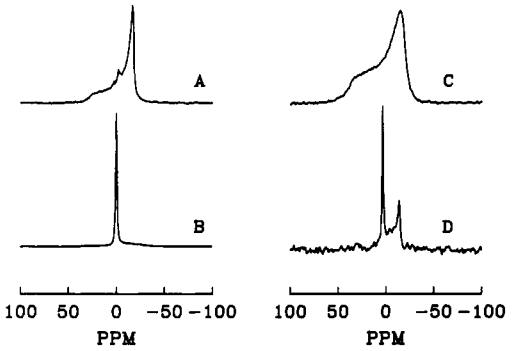

FIGURE 4: Proton-decoupled ^{31}P-NMR spectra of mixtures of apoLp-III with DMPG (left panel) and DMPA (right panel). Spectra are shown of (A) pure DMPG at 30 °C, (B) DMPC/apoLp-III (50:1) at 30 °C, (C) DMPA/apoLp-III (50:1) at pH 7.2, and (D) DMPA/apoLp-III (50:1) at pH 9.4. Spectra C and D were acquired at 25 °C and were obtained with the same DMPA/apoLp-III sample. Spectrum C was obtained by processing 8000 transients whereas spectrum D was obtained by processing 25 000 transients.

expected of phospholipid molecules undergoing axially symmetric motion within a lipid bilayer. However, it is evident that the ^{31}P-NMR spectroscopic features of the DMPG/apoLp-III mixture differ radically from those of the pure lipid. In the presence of the protein, the axially symmetric powder pattern collapses to a single resonance near 2 ppm downfield. The appearance of this resonance is consistent with the assembly of the phospholipid molecules into structures in which the motion of the phosphate headgroup is isotropic on the ^{31}P-NMR time scale. Thus, in principle, the isotropic signal shown in Figure 4 may be indicative of either the solubilization of the phospholipids into "true" isotropic solution, the reassembly of the phospholipids into micellar aggregates, the formation of structures with cubic symmetry (i.e., cubic phases), or the formation of phospholipid/protein aggregates which are tumbling fast on the ^{31}P-NMR time scale. Of the above possibilites, the solubilization of these phospholipids into true monomeric solution in any aqueous medium is highly unlikely, and the formation of a cubic phase can be eliminated by the fact that the lipid/protein mixture forms a transparent, homogeneous aqueous dispersion. We thus conclude that association of apoLp-III with DMPG radically destabilizes its bilayer structure, resulting either in the formation of a mixed protein/lipid micelle or in the formation of some other protein/lipid aggregate which permits isotropic motion of the lipid phosphate headgroups on the time scale of the ^{31}P-NMR experiment.

(ii) Interactions with Phosphatidylserine Bilayers. Typical DSC thermograms obtained in our studies of DMPS/apoLp-III mixtures are presented in Figure 5. As expected from previous studies, pure DMPS exhibits a main phase transition at 37.5 °C with an enthalpy of some 7.8 kcal/mol [see Marsh (1990)]. We also observed a small peak (ΔH = 0.5 cal/mol) near 32 °C. To our knowledge, this peak has not been reported previously, and its nature is currently unidentified. In the presence of the protein, the DSC thermograms consist of two component peaks. One of these is qualitatively similar to that of the pure lipid, and the other is somewhat broader and centered at a slightly higher temperature (38.0 °C) which is independent of the protein concentration. As was the case with PC/apoLp-III mixtures, the total enthalpy of the main phase transition is apparently unaffected by the presence of the protein, and the contribution of the higher temperature peak to the total enthalpy change increases with increasing protein concentration. The interactions of apoLp-III with PC and PS bilayers are thus generally similar despite their different charges, although some differences exist. As shown

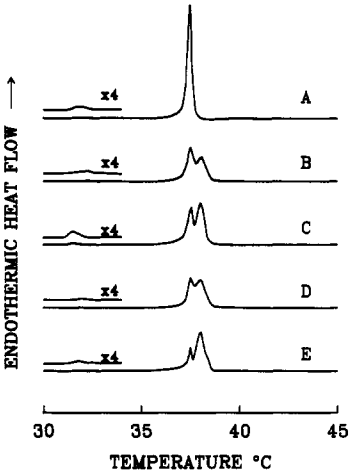

FIGURE 5: DSC thermograms illustrating the behavior of DMPS and DMPS/apoLp-III mixtures. Note the small gel phase transition observed near 32 °C. Thermograms are shown for the following: (A) Pure DMPS. (B) DMPS/apoLp-III (85:1), first heating scan. (C) DMPS/apoLp-III (85:1), third heating scan. (D) DMPS/apoLp-III (50:1), first heating scan. (E) DMPS/apoLp-III (50:1), third heating scan.

in Figure 5, the difference between the peak temperatures of the pure and perturbed lipid domains (≅0.5 °C) is considerably smaller than that observed with the PC/apoLp-III system (2.6 °C), and the width of the "perturbed lipid" peak ($\Delta T_{1/2}$ ≅ 0.8 °C) is considerably smaller than that observed with the PC/apoLp-III system ($\Delta T_{1/2}$ ≅ 9 °C). Moreover, unlike the PC/apoLp-III system, there is significant hysteresis in the interaction between apoLp-III and these PS bilayers. Thus, at any given lipid:protein ratio, the shape of the DSC thermograms differs in successive runs of the same sample. As shown in Figure 5, in successive heating scans of the same sample, the higher temperature peak obviously grows at the expense of the lower temperature peak. From this, we conclude that the kinetics of interaction between apoLp-III and these PS bilayers are slower than that observed with PC bilayers, since the two systems approach equilibrium at markedly different rates. As is also the case with the PG/apoLp-III system, the rate at which the system approaches equilibrium is dependent upon the way in which the samples are prepared. Thus, equilibrium is achieved more quickly when samples are prepared by direct hydration of lyophilized lipid with a solution of apoLp-III in PBS than is the case when a solution of apoLp-III is added to preformed DMPS dispersions. However, regardless of how the samples are prepared, there are no significant differences between the DSC thermograms exhibited by any given mixture once equilibrium is achieved.

(iii) Interactions with Phosphatidic Acid Bilayers. Characterization of the interaction between apoLp-III and PA bilayers is complicated by the fact that the thermotropic phase behavior of this lipid and its mixtures with apoLp-III is pH-sensitive. For these experiments, samples were initially prepared at pH 7.2 by hydrating the lyophilized lipid with PBS (or apoLp-III in PBS). DSC measurements were initially performed on the freshly prepared samples and then repeated with the same samples after titration to pH 8.8, to pH 9.3, and finally back to pH 7.2. We find that with each pH change the initially adjusted pH is unstable and gradually decreases. Therefore, in these experiments, the pH was constantly monitored and adjusted until a seemingly stable value was achieved (at least 15 min) and then remeasured after the DSC experiment.

DSC thermograms typical of the thermotropic behavior observed with the DMPA/apoLp-III mixtures are presented

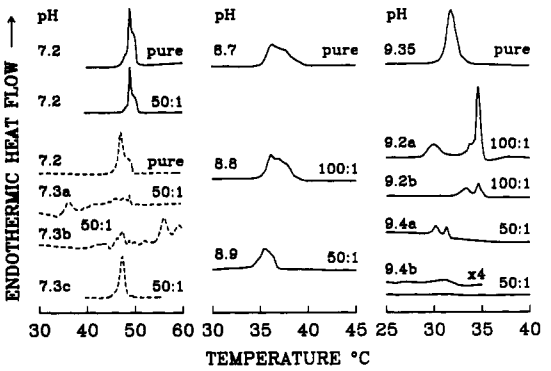

FIGURE 6: DSC thermographs of DMPA and DMPA/apoLp-III mixtures. The data were obtained at the lipid:protein ratios indicated and at nominal pH values near 7.2 (left panel), 8.8 (middle panel), and 9.3 (right panel). The solid lines indicate the initial DSC thermograms obtained or those obtained upon first titration to the nominal pH. The dashed lines indicate the thermograms obtained when the samples were back-titrated from pH 9.3 to pH 7.2. The pH values next to each thermogram were measured at the end of the DSC experiments with the subscripts a and b indicating the first and second DSC scans, respectively. The thermograms labeled c was obtained after sample 7.3b was stored at room temperature for 1 week.

in Figure 6. At pH 7.2, pure DMPA exhibits a complex chain-melting phase transition centered at 49.1 °C with an enthalpy of 6.6 kcal/mol, in agreement with previous measurements [see Blumé and Eibl (1979)]. Complex chain-melting transitions previously reported for DMPA bilayers have been ascribed to small pH variations at the gel/liquid-crystalline phase transition temperature [see Boggs (1987)]. We find that at pH 7.2 the thermotropic phase properties of DMPA are essentially unaffected by the presence of protein. When titrated to near pH 8.8, DMPA exhibits a broad DSC thermogram which appears to be a summation of two peaks with maxima near 36.3 and 37.5 °C (see Figure 6) and a total enthalpy of 6.8 kcal/mol. In the presence of apoLp-III, there are protein concentration-dependent decreases in the width of the DSC thermogram and concomitant decreases in the peak maxima (e.g., T_m = 35.5 °C at L/P = 50:1) and associated enthalpy change (ΔH = 5.4 kcal/mol at L/P = 100:1 and 4.5 kcal/mol at L/P = 50:1). Further changes in the thermotropic phase behavior of these systems also occur when the samples are titrated to near pH 9.3 (see Figure 6). Under these conditions, pure DMPA exhibits a single-component transition centered near 31.9 °C with an enthalpy of 6.8 kcal/mol. This endotherm is also narrower than that observed at pH 8.8. The difference between this result and that observed near pH 8.8 is probably attributable to the near-complete dissociation of the second ionizable proton of the phosphate headgroup [see Boggs (1987)]. Given this, the broad, multicomponent transition observed at pH 8.8 can be attributed to incomplete dissociation of the phosphate proton. At pH 9.3, dramatic changes in the thermotropic phase behavior of the PA/apoLp-III mixtures are observed. As is the case with the other negatively charged lipids studied, the shape of the DSC thermograms changes with successive DSC scans (see Figure 6, scans pH 9.2a,b and pH 9.4a,b), indicating that under these conditions the interaction between apoLp-III and the lipid is a relatively slow process which only attains equilibrium after considerable exposure to higher temperatures. With successive scans of any given sample (i.e., as the sample approaches equilibrium), there is a marked diminution in the size of the thermograms observed, a process which becomes more pronounced as the protein content is increased. Thus, for example, after equilibrium was attained, endothermic transitions of markedly reduced enthalpy (≅2–3 kcal/mol) are clearly evident at L/P near 100:1 but are barely discernible at L/P near 50:1 (see Figure 6, scans pH 9.2b and pH 9.4b). Interestingly, at pH 9.3, DMPA/apoLp-III mixtures appear as cloudy dispersions prior to the initial DSC scans but are transparent and relatively viscous when examined at the end of the DSC experiment. This change in the properties of the sample and the marked reduction in the enthalpy of the DSC endothermic peaks are remarkably similar to those observed with the DMPG/apoLp-III mixtures and suggest that the bilayer structure may have been similarly destabilized. Titration of the pH of these DMPA/apoLp-III samples back to pH 7.3 does not readily restore the thermotropic phase properties of the sample. Unlike the pure lipid, the properties of which were easily pH-reversible, the initial DSC scans of such samples (see Figure 6, pH 7.3a and pH 7.3b) produce DSC programs with undefined and irreproducible peaks. However, after having been stored at room temperature for 1 week, the sample exhibits a major heating endothermic transition at temperatures comparable to that expected of the pure lipid under comparable pH conditions (see Figure 6, pH 7.3c), and its thermotropic phase behavior essentially reverts to that observed when the sample was initially dispersed at pH 7.2. This observation clearly indicates that pH-dependent changes in the properties of these lipid/protein mixtures are reversible but that the kinetics of the process(es) are very slow.

The DSC studies of the DMPA/apoLp-III mixtures are indicative of a complex, pH-dependent interaction between the protein and PA bilayers and hint at the possible destabilization of the bilayer structure at elevated pH. This possibility was investigated by ^{31}P-NMR spectroscopy. We find that aqueous dispersions of DMPA exhibit ^{31}P-NMR spectra consistent with the maintenance of a stable phospholipid bilayer over the pH range examined in this study. In the presence of apoLp-III (Figure 4, right panel), there is little or no evidence for significant destabilization of the bilayer structure below pH 9. However, a major change in the spectroscopic properties of the sample occurs when the mixture is titrated to near pH 9.4. We find that once titrated to pH 9.4 and equilibrated by heating to temperatures near 50 °C, there is a dramatic decrease in the intensity of the ^{31}P-NMR resonance. Thus, despite the acquisition of a larger number of transients, the signal to noise ratio of the spectrum recorded at pH 9.4 is very poor compared with that recorded for the same sample at pH 7.2 (see Figure 4, right panel). This suggests that at pH 9.4 the NMR signal observed originates from a small fraction of the ^{31}P nuclei present in the sample because most of the ^{31}P nuclei are not "NMR visible" under our experimental conditions. Moreover, the NMR-visible signal observed at pH 9.4 consists of a sharp isotropic signal (≅2 ppm downfield) superimposed on a considerably broader axially symmetric powder pattern. Evidently these signals originate from residual populations of fast tumbling phospholipid/protein aggregates (or micelles) and bilayer structures, respectively. From our unsuccessful attempts to detect the bulk of the ^{31}P nuclei of the sample, we concluded that a drastic change in the relaxation properties of those ^{31}P nuclei had made their detection impractical. It has been demonstrated previously that immobilization of phosphate groups can result in dramatic decreases in the spin–lattice relaxation rates of the ^{31}P nucleus (Withers et al., 1985). Together, the loss of "NMR visibility" under our experimental conditions coupled with the fact that the sample appears as a viscous transparent "solution" indicates that once equilibrium is achieved the phosphate headgroups of the lipid are effectively immobilized in long-lived complexes with the protein. We

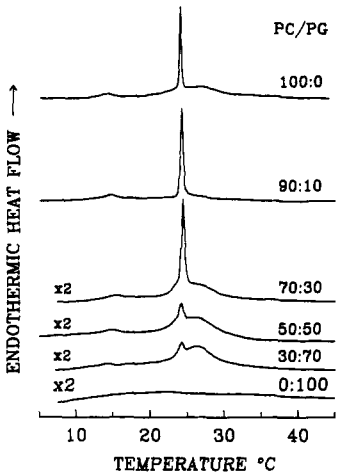

FIGURE 7: DSC thermograms of mixtures of apoLp-III with binary mixtures of DMPC and DMPG. The thermograms shown were all obtained with an overall lipid:protein ratio of 50:1 and at the DMPC:DMPG molar ratios indicated. The data were obtained from fully equilibrated samples prepared by the hydration of chloroform-dried films of the various lipid mixtures with apoLp-III in PBS.

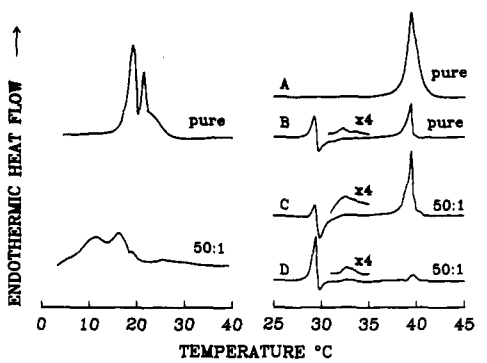

FIGURE 8: Effect of apoLp-III on the thermotropic phase behavior of dielaidoyl-DGDG (left panel) and dielaidoyl-MGDG (right panel). The DSC thermograms shown were obtained at the lipid:protein ratios indicated. MGDG/apoLp-III thermograms B and D were recorded immediately after samples are cooled to low temperatures whereas thermograms A and C were recorded after low-temperature incubation of samples for 1 day.

suggest that under such conditions the ^{31}P spin–lattice relaxation times are very long[3] with the result the NMR detection using standard direct excitation techniques is impractical. The formation of lipid/protein aggregates with immobilized phosphate headgroups by these PA/apoLp-III mixtures is in marked contrast to the fast-tumbling aggregates (or micelles) that are formed by the PG/apoLp-III mixtures described above.

(iv) Interactions with Binary Mixtures of Phosphatidylcholine and Phosphatidylglycerol. Negatively charged lipids are significant components (up to 30 mol %) of virtually all cell membranes. Given this, the demonstration that the structure of some negatively charged lipid bilayers can be disrupted upon interaction with apoLp-III raises questions about the stability of natural cell membranes in the presence of this protein. To investigate this further, we studied the interaction of apoLp-III with bilayers composed of binary mixtures of zwitterionic and negatively charged lipids. Lipid bilayers were made from mixtures of DMPC and DMPG (ranging from low to high levels of the negatively charged component) at a constant lipid:protein ratio of 50:1 (see Figure 7). The results suggest that, at this lipid:protein ratio, the lipid bilayer can contain up to 70 mol % negatively charged lipid before there is significant damage to its integrity. At low to moderate levels of anionic phospholipid (i.e., <70 mol %), the DSC thermograms indicate that the pattern of thermotropic phase behavior observed is essentially similar to that observed when apoLp-III interacts with DMPC bilayers. Over this range of anionic lipid content, the DSC thermograms exhibit a sharp component centered at the chain-melting phase transition temperature of the given "pure" lipid mixture[4] and a broader component centered at higher temperatures. In addition, the presence of apoLp-III has little effect on the total chain-melting enthalpy measured, which is essentially similar to that measured for the respective "pure" lipid mixtures. We find, however, that the contribution of the sharp component to the total enthalpy change decreases as the anionic lipid content of the bilayer increases (see Figure 7). In the higher range of anionic lipid content (>70 mol %), our data suggest extensive disruption of the bilayer structure, as evidenced by a marked reduction in the transition enthalpy and by clearing of the dispersions. Thus, in the high range of anionic lipid content, the behavior of the system is essentially similar to that described above for the DMPG/apoLp-III mixtures. Given the above results and the fact that natural biomembranes rarely contain more than 30–35 mol % of anionic lipids, it seems unlikely that interactions of natural biomembranes with apoLp-III will significantly destabilize their bilayer structure in vivo, even if this protein could gain access to the bilayer surface.

Studies of the Interaction of ApoLp-III with Uncharged Lipid Bilayers. From the data presented above, it is clear that interactions between apoLp-III and lipid bilayers are affected by the charged groups on the bilayer surface. Thus, to evaluate whether charged group participation is necessary for such interactions, the effect of apoLp-III on the thermotropic phase behavior of uncharged glucolipid bilayers was examined. Bilayers composed of DGDG and MGDG were chosen for this study because in many plants and microorganisms these groups of lipids, respectively, appear to be the functional equivalents of the PC and PE present in animal cell membranes. The heating thermogram exhibited by pure DGDG consists of two peaks with maxima at 19.2 and 21.5 °C (see Figure 8). It is generally found that the thermotropic phase behavior of glycolipids can be very complex because it is often the case that their hydrated gel phases are very unstable and tend to form one or more crystalline phases when cooled to temperatures below T_m [see Mannock et al. (1990) and references cited therein]. Thus, with dielaidoyl-DGDG, the two peaks shown in Figure 8 correspond to the melting of two different crystalline forms of the lipid and not to the melting of the hydrated gel (i.e., L_β) phase (unpublished experiments from this laboratory). Nevertheless, it is clear that the protein does interact with this lipid since the thermotropic phase behavior of the DGDG/apoLp-III mixture (L/P = 50:1) differs significantly from that of the pure sample. As shown in Figure 8 (left panel), DGDG/apoLp-III mixtures exhibit two broad transitions centered at 11.5 and 16.5 °C. Unlike the pure lipid, these transitions probably correspond to the melting of the hydrated gel (i.e., L_β) phase of the lipid. Thus, it would appear that formation of crystalline forms of this lipid is inhibited by interaction with apoLp-III.

In the case of the dielaidoyl-MGDG, the gel/liquid-crystalline phase transition temperature of the pure lipid occurs

[3] From our attempts at optimizing conditions for detecting this population of ^{31}P nuclei, we estimate that the spin–lattice relaxation times may be as high as or greater than 30 s.

[4] The DMPC/DMPG mixtures used all exhibit sharp chain-melting phase transitions centered near 24 °C. After overnight incubation at 0–4 °C, most of these membranes also form an L_c phase which, depending on the lipid composition, decomposes to the gel phase at temperatures between 10 and 18 °C (data not presented). In the presence of apoLp-III, the L_c phases of these lipid mixtures are not formed.

at 29 °C, and a weak lamellar to inverted hexagonal phase transition is observed at 33 °C (unpublished experiments from this laboratory). However, like the corresponding DGDG, the gel phase of this lipid is unstable, and when incubated at low temperature, it readily converts to a crystalline form which melts at temperatures near 39 °C (see Figure 8, curve A). With this lipid, however, its crystalline phase tends to form more slowly than is the case with DGDG. Thus, when a sample is reheated immediately after being cooled to temperatures below the T_m, transitions attributable to the melting of both the crystalline form and any residual gel phase lipid are often observed in the same DSC thermogram (see curve B). In the presence of apoLp-III, we find that the observed thermotropic phase behavior is essentially similar to that of the pure lipid. As observed in our studies of PE/apoLp-III mixtures, the properties of the gel/liquid-crystalline phase transition of the lipid are unaffected by the presence of apoLp-III, and the lamellar to H_{II} phase transition is broadened and shifted to slightly higher temperatures in the presence of the protein (see curve D). Unlike the PE/apoLp-III mixtures, we find that apoLp-III does not prevent crystallization of the lipid at temperatures below T_m. Thus, as shown by curve C, peaks corresponding to the melting of crystalline forms of the MGDG are clearly visible. However, we find that the rate of crystallization of the lipid is affected by the presence of the protein. When samples of pure lipid and the lipid/protein mixture are cooled and immediately reheated, the extent of conversion to the crystalline form of the lipid is always greater in the absence of protein (compare curves B and D). We thus conclude that there is also some interaction between apoLp-III and MGDG bilayers.

Studies of the Interaction of ApoLp-III with Mixtures of Phospholipid and Diacylglycerol. Diacylglycerols (typically 12–46% by weight) and zwitterionic phospholipids are the major lipid components of lipophorin particles (Wang et al., 1992). Given that diacylglycerol at the concentrations typically found in lipophorins can radically alter the phase preferences of phospholipids (Epand, 1985; DeBoeck & Zidovetzhi, 1989), mixtures of apoLp-III with lipid systems composed of phospholipid and diacylglycerol (viz., DMPC/dipalmitin, DPPC/dipalmitin, and DEPE/diolein) were examined to study interactions between apoLp-III and lipophorin particles. We find that with both PC-containing lipid mixtures, relatively broad endothermic transitions are observed and these are apparently unaffected when the lipids were mixed with apoLp-III. Thus, these DSC results provide no insights into the nature of any interactions between apoLp-III and these particular mixtures. However, some insights into the probable effects of such interactions were obtained from ^{31}P-NMR spectroscopic studies of these systems. For example, with the DEPE/diolein system (PE:diolein ratio of 73:27), DSC scans obtained in the presence and absence of apoLp-III revealed two heating endothermic transitions centered at 27 and 34 °C. The two transitions are probably attributable to the differential melting of the two lipid components. In a ^{31}P-NMR spectroscopic experiment, we find evidence that at 40 °C the phospholipid components of both the protein-containing and protein-free mixtures are not assembled into normal lipid bilayers. As shown in Figure 9, the spectra obtained are narrower than that obtained with fluid bilayers (for a comparison, see the bilayer spectra shown in Figure 4), and of reversed asymmetry. This type of ^{31}P-NMR powder pattern is typical of inverted hexagonal phases of hydrated phospholipids. Interestingly, when the samples are cooled to temperatures near 15 °C, we find that in the absence of apoLp-III, resonances arising from the ^{31}P nuclei of the sample are not detected under our experimental conditions. This is probably because the spin–lattice relaxation times of ^{31}P nuclei are so long (≫100 s, our estimates) that recording of a spectrum was impractical with the available instrumentation. Similar results have been reported when the stable L_c phases of the *n*-saturated diacyl-PE's are formed (Xu et al., 1988; Lewis & McElhaney, 1993) and are believed to be the result of the formation of dehydrated crystalline structures in which the phosphate groups have been immobilized (Lewis & McElhaney, 1993). We find, however, that in the presence of apoLp-III, resonances arising from the ^{31}P nuclei are observed when the samples are cooled to temperatures below T_m and that the ^{31}P-NMR spectrum obtained constitutes a broad powder pattern typical of that exhibited by phospholipid bilayers in the gel phase (see Figure 9). Under the experimental conditions described here, axially symmetric ^{31}P-NMR powder patterns such as these can only be observed when the phosphate groups are fully hydrated. Thus, we suggest that interaction of apoLp-III with the PE/DAG mixture can inhibit the dehydration of at least some of the polar headgroups of the phospholipid component of this mixture. The above result implicitly suggests that apoLp-III can interact with the surface of "model lipophorin particles" such as the PE/DAG mixtures described and affects hydration/dehydration phenomena at the surface of such particles. This pertains directly to the proposed in vivo functions of this protein and will be explored further under Discussion.

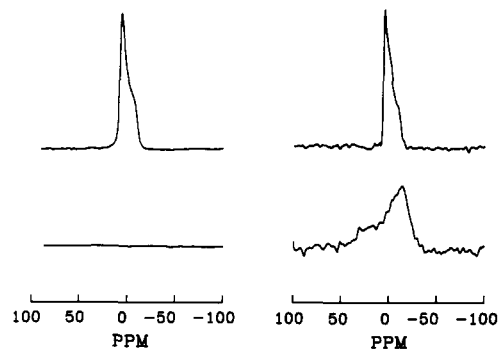

FIGURE 9: Proton-decoupled ^{31}P-NMR spectra of DEPE/diolein (left panel) and its mixtures with apoLp-III (right panel). The data shown were acquired at 40 °C (top) and 15 °C (bottom) and correspond with the melted and unmelted states of the lipid, respectively. The inferior signal-to-noise of the spectra obtained with the protein-containing samples is a reflection of a considerably smaller sample size.

DISCUSSION

These studies indicate that *M. sexta* apoLp-III can interact with all of the lipids and lipid mixtures studied and that one or more of the physical properties of any given assembly of lipids is affected by interaction with the protein. Our observations are consistent with the interpretation that interactions between apoLp-III and lipids are mediated primarily by charged and polar headgroup interactions and, as would be expected, the strength of such interactions varies with the nature of the lipid polar headgroup. Judging from the effects of apoLp-III on the thermotropic phase behavior and organization of the various lipid assemblies studied, we can arrange these lipid classes in a graded series in which the degree of perturbation of their bilayers, and possibly the relative strengths of their interactions with apoLp-III, increases in the following order:

$$\text{MGDG} < \text{PE} < \text{PA}_{\text{pH 7.2}} < \text{DGDG} \cong \text{PC} < \text{PS} < \text{PA}_{\text{pH 8.8}} < \text{PG} < \text{PA}_{\text{pH 9.3}}$$

With lipids such as MGDG and PE, there is no discernible

effect on apoLp-III on the gel/liquid-crystalline phase transitions of the lipids, and the only observable effects of interaction with apoLp-III appear to be an inhibition (or retardation) of the formation of both the L_c and H_{II} phases of these lipids. Given that the formation of the L_c and H_{II} phases of lipids is affected by factors such as interbilayer contact (Cullis et al., 1980; Allen et al., 1990) and changes in hydration and hydrogen-bonding interactions at the polar/apolar interfacial regions of the lipid bilayers (Cevc & Marsh, 1987; Lewis & McElhaney, 1990, 1993), one can easily envisage how apoLp-III can retard or even inhibit such phenomena by binding (even weakly and reversibly) to the surface of these lipid bilayers.

With lipids such as PC, PS, and DGDG, apoLp-III significantly perturbs their gel/liquid-crystalline phase transition, suggesting a significantly stronger interaction than observed with bilayers composed of PE or MGDG. For these lipids, the common characteristic of their interaction with apoLp-III is by the formation of protein-associated lipid domains which undergo less cooperative gel/liquid phase transitions at slightly higher temperatures than do the pure lipid. In general, these changes are not accompanied by significant changes in the total enthalpy of the lipid chain-melting phase transition. This effect is comparable with that proposed for so-called type I proteins which are expected to interact with lipid membranes exclusively through polar and/or electrostatic forces at the bilayer surface [see Papahadjopoulos et al. (1975) and McElhaney (1986)]. Thus, it seems unlikely that the observed effects of apoLp-III can be attributed to significant penetration into the hydrophobic domains of these lipid bilayers. Given the evidence for moderate interactions between apoLp-III and the surface of these lipid bilayers, one would also expect that such interactions with the protein should also retard or inhibit the formation of the L_c phases of this group of lipids. For the most part, we find that interaction of these lipids with apoLp-III does inhibit L_c phase formation, particularly with those lipids for which L_c phase formation involves the formation of an extended hydrogen-bonding network involving the polar headgroups and/or substantial changes in headgroup and interfacial hydration.

It is also evident that interactions of apoLp-III with some negatively charged lipids such as PG and $PA_{pH\ 9.3}$ not only markedly perturb the gel/liquid-crystalline phase transition but also can severely disrupt the organization of these lipid bilayers. The possibility that interactions between some apolipoproteins and negatively charged lipid bilayers may be strong enough to disrupt bilayer structure has been noted in previous studies of human apolipoproteins (Surewicz et al., 1988; Epand et al., 1990). Interestingly, however, our demonstration that interaction of apoLp-III with negatively charged PS or $PA_{pH\ 7.2-9.0}$ bilayers does not disrupt the integrity of those bilayers also indicates that a net negatively charged lipid bilayer per se may not itself constitute a sufficient condition for such strong "bilayer-disrupting" interactions to take place. Moreover, we also find that bilayer integrity can be maintained in the presence of apoLp-III even if the lipid bilayer contains a substantial quantity of negatively charged lipids which, in isolation, will not form stable bilayers when apoLp-III is present. These observations are significant in that they establish that the integrity of natural membranes can be maintained in the presence of high in vivo concentrations of apoLp-III even though these membranes may contain substantial quantities of negatively charged lipids. One should also note that negatively charged lipids are noticeably absent from lipophorin particles [see Ryan (1990) and references cited therein]. Since fast, reversible binding of apoLp-III to HDLp-A particles is considered to be an essential aspect of the "DAG shuttle" [see Van Heusden et al. (1987), Ryan (1990), and Surholt et al. (1991)], we can suggest that the absence of negatively charged lipids on lipophorin particles is consistent with such functions, given our evidence that apoLp-III tends to bind strongly to, and dissociate slowly from, some negatively charged lipid surfaces.

Table I: Relative Electrostatic, Hydrogen-Bonding, and Steric Contributions to the Screening of Bilayer Phosphate Groups[a]

lipid	electrostatic	H-bonding	steric
PE	***	**	**
PC	**	–	***
PS	*	*	***
PG	–	*	***
$PA_{pH\ 7-9}$	–	***	–
$PA_{pH\ >\ 9}$	–	–	–

[a] Abbreviations: (–) no effects; (*) weak effects; (**) moderate effects; (***) strong effects.

In principle, the varying strengths of the interactions of apoLp-III with different phospholipids can be satisfactorily rationalized by considering the nature of interactions between phospholipid molecules at the surface of the bilayer. The common feature of phospholipid bilayers is a high density of negatively charged phosphate groups next to the bilayer polar/apolar interface. Thus, if one accepts that electrostatic interactions between these phosphate groups and positively charged moieties on the surface of the protein constitute the basis of the interactions between apoLp-III and all of the phospholipid bilayers studied, then one can rationalize the differential interactions between the protein and individual phospholipid bilayers on the basis of differential screening/shielding of the negatively charged phosphate groups by the other moieties (e.g., choline, ethanolamine, serine, glycerol, etc.) present at the bilayer surface. With any of the moieties listed above, effective screening of negatively charged phosphate groups would be the cumulative effect of relatively strong contributions arising from their capacity for electrostatic and hydrogen-bonding interactions and smaller steric contributions related to their effective sizes.[5] The probable contributions of steric, electrostatic, and hydrogen-bonding effects to the overall "screening capacities" of the phospholipids used in this study are summarized in Table I. Given the information shown, one can rationalize the relatively weak apoLp-III/PE interactions observed on the basis of effective screening of the bilayer phosphates by electrostatic and intermolecular hydrogen-bonding interactions with the positively charged amino groups of the lipid. With PC bilayers, stronger interactions with the protein would be expected mainly because of weaker "screening" by the positively charged permethylated amino groups, which have no capacity to form hydrogen bonds. Similarly, at the other end of the scale, one can rationalize the strong interactions observed between apoLp-III and "poorly screened", negatively charged bilayers (such as PA at high pH[6]) by the fact that the strength of such interactions is progressively attenuated as the negatively charged phosphate groups are increasingly screened by the presence of uncharged (e.g., glycerol in PG bilayers) and zwitterionic groups (e.g., serine in PS bilayers or choline) or by pure hydrogen-bonding effects [e.g., PA bilayers at pH < 9; for a more detailed discussion of hydrogen-bonding effects in PA bilayers, see Boggs (1987)]. We do stress, however, that although

[5] This involves the intrinsic size of the group itself and its associated/bound water(s).

[6] Note that the fully deprotonated phosphate groups which form at high pH cannot form phosphate–phosphate hydrogen bonds.

electrostatic interactions are probably the dominant binding forces between apoLp-III and phospholipid bilayers, the fact that apoLp-III does interact with nonionic glycolipid bilayers indicates that lipid/apoLp-III interactions can also occur in the absence of such forces. Indeed, strong interactions between apoLp-III and uncharged lipid surfaces have been confirmed in recent monolayer film studies (Demel et al., 1992). With glycolipid bilayers such as MGDG and DGDG, hydrogen-bonding interactions between donor and acceptor groups on the surfaces of apoLp-III and the lipid bilayers probably form the basis of the lipid/protein interactions observed, and it is also probable that such forces contribute to the binding energy of the interaction between apoLp-III and phospholipid bilayers. Also, the fact that there is a hydrophobic component to the character of virtually all lipid polar headgroups is often overlooked [see Lewis and McElhaney (1992a)]. Given this, one should also consider the possibility that hydrophobic interactions may also be involved in the interaction of apoLp-III and most of the lipid bilayers studied. However, it would appear that hydrophobic forces are probably minor contributions to the interactions between apoLp-III and phospholipid bilayers though they may possibly play a bigger role in the interactions between apoLp-III and the nonionic glycolipid bilayers.

The demonstration that binding of apoLp-III can disrupt the structure of negatively charged bilayers such as PG and $PA_{pH > 9}$ provides some clues about the distribution of charged groups on the surface of the protein. Given the severity of the disruption of these bilayers by apoLp-III, it is very unlikely that the positively charged amino acid residues of apoLp-III are randomly distributed over its exposed hydrophilic surfaces. At this time, the three-dimensional structure of *M. sexta* apoLp-III has not yet been determined, but the current consensus is that it probably adopts an amphipathic helix folding motif similar to that adopted by *L. migratoria* apoLp-III [see Brieter et al. (1991)]. The latter conclusion is compatible with secondary structure predictions based on a hydropathy profile analysis of the amino acid sequence of *M. sexta* apoLp-III (Cole et al., 1987) and recent studies showing that *M. sexta* apoLp-III exists predominantly as α-helical monomers in solution (Kawooya et al., 1986; Ryan et al., 1993). Further support for the above also comes from our own helical wheel analyses of the amino acid sequence. From such studies, we conclude that the putative α-helical regions identified by Cole et al. (1987) should form amphipathic helices with laterally segregated hydrophobic and hydrophilic surfaces (unpublished results from this laboratory). Interestingly, as predicted from similar analyses of many apolipoproteins [see Segrest et al. (1992)], our analyses also suggest that there is a statistically significant concentration of positively charged groups at the interfaces between the hydrophobic and hydrophilic surfaces of those putative α-helical regions. Moreover, these positively charged groups tend to be "concentrated" in near-linear stretches along some surfaces of the putative α-helical regions of the protein. These observations are consistent with our conclusion that positively charged groups are not randomly distributed over the surface of apoLp-III (see above) and suggest that the lipid/apoLp-III interactions which eventually lead to the disruption of bilayer integrity are mediated by the binding of one or more regions of the protein in which there is a local concentration of positively charged amino acid residues. However, in assessing the probable effects of the binding of such regions of the protein, one should also note that studies of strongly basic proteins such as poly(L-lysine) and myelin basic protein have indicated that although they interact very strongly with anionic lipid bilayers, such interactions do not result in the disruption of those lipid bilayers. Indeed, it is often the case that binding of such proteins will stabilize the structure of negatively charged lipid bilayers [see McElhaney (1986) and references cited therein]. Given this, it seems unlikely that disruption of bilayer structure can be directly attributed to the binding of apoLp-III. Instead, we suggest that such interactions probably trigger changes in the conformation (i.e., secondary structure) and/or the folding motifs (i.e., tertiary structure) of the protein and that the disruption of bilayer structure results from interaction between lipids and the altered form of the protein. Such a suggestion is supported by previous studies which show that *M. sexta* apoLp-III is a highly surface-active molecule which can undergo major structural changes when located at the interfaces between nonpolar and aqueous media (Kawooya et al., 1986). We therefore suggest that the initial binding of apoLp-III to the negatively charged lipid surfaces provides the energy needed to locate the protein near to the polar/apolar interfacial region of the lipid bilayer and this may be the factor which triggers the structural changes which eventually result in substantial disruption of the lipid bilayer.

These studies also suggest that the binding of apoLp-III to lipid surfaces promotes retention of water at those surfaces. This conclusion was inferred from our observations that interaction of apoLp-III with lipid bilayers inhibits the formation of crystalline forms (i.e., L_c phases) of many lipids. Our ^{31}P-NMR spectroscopic studies of DAG/phospholipid mixtures also provide direct evidence in support of such a conclusion. That the binding of apoLp-III to a lipid surface should promote water retention at that surface should not be surprising given that such interactions inevitably involve at least a partial covering of those surfaces with polar moieties. We believe that this aspect of the apoLp-III/lipid interactions is of fundamental importance to the normal in vivo functioning of this and most other apolipoproteins. This is because it is very unlikely that the mixture of lipids normally found in lipophorin particles would form stable bilayer vesicles in aqueous dispersion. Owing to the high content of diacylglycerol, this mixture of lipids will, in isolation, probably form inverted nonlamellar aggregates when dispersed in water (Epand, 1985; DeBoeck & Zidovetzhi, 1989). Any large-scale in vivo formation of such structures would be inviable because they are very unstable with respect to aggregation and fusion, and contact with any cell membrane will almost certainly result in cell lysis. With the lipoprotein particles, this problem has been effectively overcome by the association of the lipids with apolipoproteins which stabilize the particles by covering the lipids with polar moieties. One can thus envisage that uptake of substantial quantities of DAG will upset the hydrophobic/hydrophilic balance at surfaces of those particles and binding of additional apolipoprotein molecules (in this case apoLp-III) will be required to stabilize the system [see Wang et al. (1992)]. We can also suggest that binding of apoLp-III to the surface of these particles and the concomitant hydration of the particle surface should also be biologically useful. This is because more effective hydration of the surface of the lipophorin particle would make lipid molecules such as DAG more accessible to metabolic enzymes present in the aqueous phase. The fact that the DAG from LDLp is more effectively hydrolyzed by lipophorin lipase than is the DAG from HDLp-A [see Van Heusden et al. (1986)] is consistent with the above suggestion.

Finally, our results clearly indicate that the interaction of apoLp-III with lipids covers a fairly broad spectrum of possibilities, some of which may or may not be directly relevant

to the normal in vivo functions of the protein. Moreover, the broad spectrum of observed effects is consistent with the concept that there are probably at least two (possibly three) different conformers of the protein which give rise to the different pattern of lipid/apoLp-III interactions observed. The structural properties of the protein when bound to the various lipids studied and the mechanism by which interaction with some forms of this protein can disrupt the integrity of lipid bilayers are unknown at present and are the focus of continuing study.

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REFERENCES

Allen, T. M., Hong, K., & Papahadjopoulos, D. (1990) *Biochemistry 29*, 2976–2985.

Aune, K. C., Gallagher, J. G., Gotto, A. M., & Morriset, J. D. (1977) *Biochemistry 16*, 2151–2156.

Beenakkers, A. M. T., Van der Horst, D. J., & Van Marrewijk, W. J. A. (1985) *Prog. Lipid Res. 24*, 19–67.

Blumé, A., & Eibl, H. (1979) *Biochim. Biophys. Acta 558*, 13–21.

Boggs, J. M. (1987) *Biochim. Biophys. Acta 906*, 353–404.

Boggs, J. M., & Moscarello, M. A. (1978) *J. Membr. Biol. 39*, 75–96.

Breiter, D. R., Kanost, M. R., Benning, M. W., Wesenberg, G., Law, J. H., Wells, M. A., Rayment, I., & Holden, H. M. (1991) *Biochemistry 30*, 603–608.

Cevc, G., & Marsh, D. (1987) in *Phospholipid Bilayers*, Wiley Interscience, New York.

Cole, K. D., Fernando-Warnakulasuriya, G. J. P., Boguski, M. S., Freeman, M., Gordon, J. I., Clark, W. A., Law, J. H., & Wells, M. A. (1987) *J. Biol. Chem. 262*, 11794–11800.

Cullis, P. R., de Kruijff, B., Hope, M. J., Nayar, R., & Schmid, S. L. (1980) *Can. J. Biochem. 58*, 1091–1100.

DeBoeck, H., & Zidovetzki, R. (1989) *Biochemistry 28*, 7439–7446.

Demel, R. A., Van Doorn, J. M., & Van der Horst, D. J. (1992) *Biochim. Biophys. Acta 1124*, 151–158.

Epand, R. M. (1985) *Biochemistry 24*, 7092–7095.

Epand, R. M., Segrest, J. P., & Anantharamaiah, G. M. (1990) *J. Biol. Chem. 265*, 20829–20832.

Holden, H. M., Kanost, M. R., Law, J. H., Wells, M. A., & Rayment, I. (1988) *J. Biol. Chem. 263*, 3960–3962.

Jonas, A., & Mason, W. R. (1981) *Biochemistry 20*, 3801–3805.

Kanost, M. R., Boguski, M. S., Freeman, M., Gordon, G. I., Wyatt, G. R., & Wells, M. A. (1988) *J. Biol. Chem. 263*, 10568–10573.

Kawooya, J. K., Keim, P. S., Ryan, R. O., Shapiro, J. P., Samaraweera, P., & Law, J. H. (1984) *J. Biol. Chem. 259*, 10733–10737.

Kawooya, J. K., Meredith, S. C., Wells, M. A., Kézdy, F. J., & Law, J. H. (1986) *J. Biol. Chem. 261*, 13588–13591.

Lewis, R. N. A. H., & McElhaney, R. N. (1990) *Biochemistry 29*, 7946–7953.

Lewis, R. N. A. H., & McElhaney, R. N. (1992a) in *The Structure of Biological Membranes* (Yeagle, P. L., Ed.) pp 73–155, CRC Press, Boca Roca, FL.

Lewis, R. N. A. H., & McElhaney, R. N. (1992b) *Biophys. J. 61*, 63–77.

Lewis, R. N. A. H., & McElhaney, R. N. (1993) *Biophys. J.* (in press).

Lewis, R. N. A. H., Mak, N., & McElhaney, R. N. (1987) *Biochemistry 26*, 6118–6126.

Lewis, R. N. A. H., Sykes, B. D., & McElhaney, R. N. (1988) *Biochemistry 27*, 880–887.

Mannock, D. A., Lewis, R. N. A. H., & McElhaney, R. N. (1990) *Biochemistry 29*, 7790–7799.

Marsh, D. (1990) *Handbook of Lipid Bilayers*, CRC Press Inc., Boca Raton, FL.

McElhaney, R. N. (1982) *Chem. Phys. Lipids 30*, 229–259.

McElhaney, R. N. (1986) *Biochim. Biophys. Acta 864*, 361–421.

Monck, M. A., Bloom, M., Lafleur, M., Lewis, R. N. A. H., McElhaney, R. N., & Cullis, P. R. (1992) *Biochemistry 31*, 10037–10043.

Paphadjopoulos, D., Moscarello, M., Eylar, E. H., & Isac, T. (1975) *Biochim. Biophys. Acta 401*, 317–335.

Prasad, S. V., Ryan, R. O., Law, J. H., & Wells, M. A. (1986) *J. Biol. Chem. 261*, 558–562.

Ryan, R. O. (1990) *J. Lipid Res. 31*, 1725–1739.

Ryan, R. O., Oikawa, K., & Kay, C. M. (1993) *J. Biol. Chem. 268*, 1525–1530.

Segrest, J. P., Jones, M. K., De Loof, H., Brouillette, C. G., Venkatachalapathi, Y. V., & Anantharamaiah, G. M. (1992) *J. Lipid Res. 33*, 141–166.

Shapiro, J. P., Law, J. H., & Wells, M. A. (1988) *Annu. Rev. Entomol. 33*, 297–318.

Shen, B. W., Scanu, A. M., & Kézdy, F. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 837–841.

Silvius, J. R., & McElhaney, R. N. (1978) *Can. J. Biochem. 56*, 462–469.

Surewicz, W., Epand, R. M., Pownall, H. J., & Hui, S.-W. (1986) *J. Biol. Chem. 261*, 16191–16197.

Surholt, B., Goldberg, J., Schulz, T. K. F., Beenakkers, A. M. Th., & Van der Horst, D. J. (1991) *Biochim. Biophys. Acta 1086*, 15–21.

Swaney, J. B. (1983) *J. Biol. Chem. 258*, 1254–1259.

Swaney, J. B., & Chang, B. C. (1980) *Biochemistry 19*, 5637–5644.

Van der Horst, D. J. (1990) *Biochim. Biophys. Acta 1047*, 195–211.

Van der Horst, D. J., Ryan, R. O., Van Heusden, M. C., Schulz, T. K. F., Van Doorn, J. M., Law, J. H., & Beenakkers, A. M. Th. (1988) *J. Biol. Chem. 263*, 2027–2033.

Van Heusden, M. C., Van der Horst, D. J., Van Doorn, J. M., Wes, J., & Beenakkers, A. M. Th. (1986) *Insect Biochem. 16*, 517–523.

Van Heusden, M. C., Van der Horst, D. J., Voshol, J., & Beenakkers, A. M. Th. (1987) *Insect Biochem. 17*, 771–776.

Wang, J., Liu, H., Sykes, B. D., & Ryan, R. O. (1992) *Biochemistry 31*, 8706–8712.

Wells, M. A., Ryan, R. O., Prasad, S. V., & Law, J. H. (1985) *Insect Biochem. 15*, 565–571.

Wells, M. A., Ryan, R. O., Kawooya, J. K., & Law, J. H. (1987) *J. Biol. Chem. 262*, 4172–4176.

Wheeler, C. H., Van der Horst, D. J., & Beenakkers, A. M. (1984) *Insect Biochem. 14*, 261–266.

Withers, S. G., Madsen, N. B., & Sykes, B. D. (1985) *Biophys. J. 48*, 1019–1026.

Xu, H., Stephenson, F. A., Lin, H., & Huang, C. (1988) *Biochim. Biophys. Acta 943*, 63–75.