Identification and Localization of Two Distinct Microenvironments for the Diacylglycerol Component of Lipophorin Particles by ^{13}C NMR†

Jianjun Wang,‡ Hu Liu,§ Brian D. Sykes,‡ and Robert O. Ryan*,§

Protein Engineering Network of Centres of Excellence and Lipid and Lipoprotein Research Group, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

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ABSTRACT: ^{13}C nuclear magnetic resonance spectroscopy of lipoproteins, isolated from the insect *Manduca sexta,* has been employed to probe the microenvironment of diacylglycerol (DG), their major neutral lipid component. Natural abundance ^{13}C NMR spectra of high density lipophorin exhibited several well-separated resonances derived from its lipid moiety, including those for the carbonyl carbon atoms of phospholipid and DG fatty acyl chains in the region of 175−180 ppm. To verify the assignment of the DG acyl chain carbonyl carbon resonances, di[1-^{13}C]oleoylglycerol high density lipophorin was isolated after instilling a bolus of tri[1-^{13}C]oleoylglycerol into the midgut of larvae fed a fat-free diet. ^{13}C NMR spectra of the isolated lipoprotein revealed a specific and dramatic enrichment of resonances at 175.5 ppm. Expansion of this region revealed two resonances separated by 0.08 ppm. These were assigned as 1,2- and 1,3- isomers of DG, the latter presumably arising from spontaneous acyl chain migration of 1,2-DG following lipoprotein isolation. On the basis of compositional and structural analysis of this lipoprotein, it is postulated that these DG species are localized predominantly in the hydrophobic core of the particle. By contrast, natural abundance ^{13}C NMR spectra of the DG-rich, low density lipophorin (LDLp) subspecies revealed two additional resonances, separated by 0.2 ppm, that were tentatively assigned as 1,2- and 1,3-DG present at the surface of the particle. The verify this assignment, experiments employing phospholipase C, to convert lipophorin surface associated phospholipid into DG, were performed. The data revealed that loss of the phospholipid acyl chain carbonyl carbon resonance correlated with the appearance of two additional DG acyl chain carbonyl resonances (separated by 0.2 ppm) possessing chemical shifts similar to those observed in LDLp. Interestingly, the resonance assigned as 1,2-DG at the surface, predominanted immediately after phospholipase C hydrolysis, but its intensity decreased with time. Concomitantly, there was a corresponding increase in the resonance assigned as 1,3-DG, consistent with an interconversion of these isomers through acyl chain migration. Taken together, the results provide the first direct experimental evidence that DG molecules present at the surface monolayer and the particle core, respectively, can be distinguished. Thus it should be possible to design experiments to evaluate exchange between these two locations as well as the role of surface associated DG in binding of the exchangeable apolipoprotein, apolipophorin III.

NMR spectroscopy has been extensively employed in studies of mammalian lipoproteins (Hamilton & Morrisett, 1986). The inherent advantages of ^{13}C NMR spectroscopy, which include high resolution and readily assignable resonances, provide a powerful tool for obtaining structural information. For example, it has been shown that unesterified cholesterol molecules in human lipoproteins partition between the surface and core of particles (Lund-Katz & Phillips, 1984, 1986). Using [4-^{13}C]cholesterol, ^{13}C NMR spectra of human low density lipoprotein (LDL) revealed that about two-thirds of unesterified cholesterol molecules are located on the surface of LDL. Like cholesterol in human lipoproteins, diacylglycerol (DG;[1] the major neutral lipid component of insect lipoproteins) has been postulated to partition between the hydrophobic core and the surface monolayer in response to hormone-stimulated lipoprotein conversions (Kawooya et al., 1991; Wang et al., 1992). Direct evidence of such a spatial localization, however, has not been demonstrated by any biochemical or biophysical means.

Insect lipoproteins, lipophorins, have structural characteristics similar to those of human apolipoprotein B containing lipoproteins. The integral apolipoproteins, apolipophorin-I and apolipophorin-II, function in an analogous manner to human apoB in maintaining a stable particle matrix (Shapiro et al., 1984; Blacklock & Ryan, 1994). On the other hand, lipophorins also have unique properties, perhaps most notably the presence of DG, not triacylglycerol and cholesteryl ester, as their major core lipid. Importantly, lipophorin particles have the capacity to accept or donate DG, forming different subspecies which have distinct compositions, structures, and morphologies (Prasad et al., 1986b; Ryan et al., 1992).

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* Address correspondence to this author at 328 Heritage Medical Research Centre, University of Alberta, Edmonton, AB T6G 2S2. Telephone: (403) 492-5153. Fax: (403) 492-3383.

‡ Protein Engineering Network of Centres of Excellence.

§ Lipid and Lipoprotein Research Group.

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[1] Abbreviations: apoLp-III, apolipophorin III; DG, diacylglycerol; HDLp-W2, high density lipophorin wanderer 2; HDLp-W1, high density lipophorin wanderer 1; HDLp-A, adult high density lipophorin; LDLp, low density lipophorin; SDS, sodium dodecyl sulfate; PL-C, phospholipase C.

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During energy demanding flight, large amounts of DG are transferred to pre-existing high density lipophorin (HDLp; 300 DG molecules per particle) forming a larger, low density lipophorin (LDLp) which contains over 1000 DG molecules per particle. Owing to its smaller, less polar, head group compared to phospholipids, DG molecules present in the surface monofilm will destabilize the monolayer unless additional surface components are provided. Such extra surface components are not provided as phospholipid, which remain constant at about ~140 molecules per particle during the HDLp-LDLp transformation (Ryan et al., 1986). Rather, DG-enriched LDLp particles are stabilized by association of the exchangeable, amphipathic apolipophorin-III (apoLp-III; Wells et al., 1987; Singh et al., 1992; Blacklock & Ryan, 1994). ApoLp-III is an abundant 18 000 Da hemolymph protein usually found in a lipid-free state. X-ray crystallography of lipid-free *Locusta migratoria* apoLp-III has revealed that the protein adopts a five-helix bundle organization in which the hydrophobic faces of its amphipathic α-helices orient toward the center of the bundle while their hydrophilic faces project toward the solvent (Breiter et al., 1991). It has been proposed that the helix bundle opens upon interaction with lipid surfaces (Kawooya et al., 1986; Breiter et al., 1991; Zhang et al., 1993; Wientzek et al., 1994). On the basis of ^{31}P NMR and compositional data, it has been hypothesized that partitioning of DG to the surface of LDLp influences apoLp-III binding (Wang et al., 1992). The recent observation that phospholipase C (PL-C) induced conversion of lipoprotein-associated phospholipid into DG induces a stable association of apoLp-III supports this concept (Liu et al., 1993; Singh et al., 1994). The present study was undertaken in an effort to obtain direct spectroscopic evidence for the existence of surface DG in apoLp-III-containing lipophorin particles.

MATERIALS AND METHODS

Materials. Phospholipase C from *Bacillus cereus* (grade I; <0.05% sphingomyelinase activity) was purchased from Boehringer Mannheim, Laval, PQ. Tri[1-^{13}C]oleoylglycerol was purchased from Cambridge Isotopes Laboratories, Andover, MA. Linolenic acid methyl ester was obtained from Sigma Chemical Co., St. Louis, MO. Larval and adult *Manduca sexta* were obtained from a continuing laboratory colony reared on a wheat germ based diet according to Prasad et al. (1986b). Lipophorins were isolated from freshly collected hemolymph of larval or adult *M. sexta* by density gradient ultracentrifugation (Shapiro et al., 1984). The subspecies HDLp-W2 and HDLp-W1 were isolated from prepupal larvae as described by Prasad et al. (1986b) while HDLp-A and LDLp were isolated from 1-day-old adult moths according to Ryan et al. (1986). In order to ensure homogeneous lipophorin preparations, samples were centrifuged a second time. ApoLp-III was purified from adult moth hemolymph as described by Wells et al. (1985).

Protein content was determined with the bicinchoninic acid assay (Pierce Chemical Co.), using bovine serum albumin as standard. SDS−PAGE was performed in 4−20% acrylamide gradient slab gels and stained with Coomassie brilliant blue. PL-C assays were performed as described by Liu et al. (1993).

[^{13}C]DG Enrichment of HDLp-L. Fat-free diet was prepared according to the method described by Prasad et al. (1986a). Standard laboratory diet (United States Biochemical, Cleveland, OH) was extracted with $CHCl_3$/methanol (3:1) in a Soxhlet extraction apparatus for 16 h. The lipid-extracted diet was dried in a fume hood for 24 h and kept in the dark. Prior to experiments the lipid extracted diet was mixed with 10 mg of cholesterol and 10 mg of linolenic acid methyl ester per 100 g of diet. Mid-third instar larvae were chosen and shifted from regular diet feeding status to fat-free diet. Before those caterpillars fed fat-free diet matured to the prepupal stage, a 45 μL bolus of tri[1-^{13}C]oleoylglycerol oil was delivered to the mid gut through a plastic microtube. Five hours later the caterpillars were bled, and lipophorin was isolated by density gradient ultracentrifugation.

^{13}C NMR Studies. Before the ^{13}C NMR experiments, lipophorin samples were dialyzed against 50 mM phosphate buffer, pH 7.4, 150 mM NaCl, and 1 mM Ca^{2+} for 24 h. Lipophorin sample concentrations are indicated in the figure legends. Fourier transform ^{13}C NMR experiments were carried at 75.4 MHz with a Varian UNITY 300 spectrometer with proton decoupling. D_2O was used as an internal lock and shim signal. Using a 10 mm NMR tube, 2.5 mL of the lipoprotein sample was mixed with 0.5 mL of D_2O at room temperature and placed into the NMR tube. 2,2-Dimethyl-2-silapentane-5-sulfonate was used as external chemical shift reference since it is insensitive to temperature and pH variations (Wishart & Sykes, 1994). ^{13}C NMR spectra were obtained at different temperatures (5, 25, and 37 °C). Before data were acquired, the samples were equilibrated for at least 30 min at a given temperature. The spectral width was 18 000 Hz. All spectra were processed using the line-broading parameter equal to 1.0 Hz.

For each lipophorin sample, except HDLp-W2, three ^{13}C NMR spectra were taken at a given temperature, which corresponded to (1) lipophorin itself, (2) lipophorin with apoLp-III and (3) lipophorin with defined amounts of apoLp-III and PL-C. PL-C treated HDLp-W2 samples were examined in the absence of apoLp-III. For the [^{13}C]DG-enriched HDLp-L sample, good quality spectra were obtained after 1000 scans, whereas for natural abundance spectra of lipophorin samples 15 000−30 000 scans were collected. For the time course of phospholipase C digestion of HDLp-W2, 1800 scans (about 1 h) were collected at each time point.

RESULTS

Lipophorin ^{13}C NMR Spectral Resonances. Natural abundance ^{13}C-NMR spectra of the lipophorin subspecies, HDLp-W1, contains several well-separated resonances (Figure 1, panel A). Like apolipoprotein B of human lipoproteins, the apoprotein moieties of lipophorin particles do not give rise to detectable resonances (Hamilton & Morrisett, 1986). Thus, all observed resonances were derived from the lipid component of the particles. Assignment of resonances was based on chemical shift values in comparison with corresponding ^{13}C NMR studies of human lipoproteins (Hamilton & Morissett, 1986). The major resonances observed in HDLp-W1 are (1) acyl chain carbonyl carbons from DG and phospholipid (175−180 ppm), (2) unsaturated fatty acyl carbon atoms (130−134 ppm), (3) amino trimethyl carbons from the choline moiety of phosphatidylcholine (54−60 ppm), (4) acyl chain CH_2 carbons (~30−36 ppm), and (5) methyl carbons (~17 ppm).

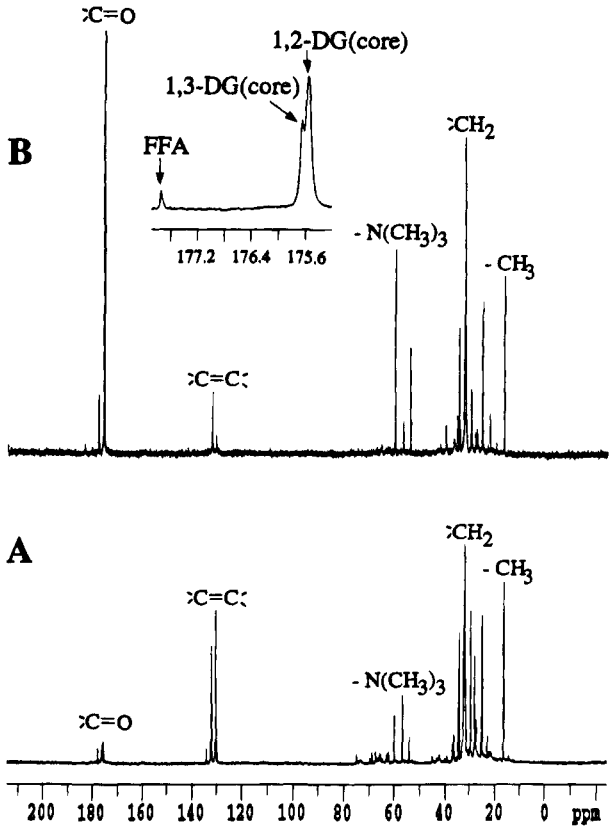

FIGURE 1: (Panel A) Natural abundance ^{13}C NMR spectrum of HDLp-W1 (170 mg of protein). (Panel B) ^{13}C NMR spectrum of ^{13}C-DG enriched HDLp-L (21 mg of protein). Samples were dissolved in 50 mM phosphate, pH 7.0, and mixed with 0.5 mL of D_2O (final volume = 3 mL) and analyzed by NMR. (Inset) Expanded carbonyl carbon region of [^{13}C]DG-enriched HDLp-L. FFA = free fatty acid; DG = diacylglycerol. Spectra were obtained at 37 °C as described in Materials and Methods.

DG Acyl Chain Carbonyl Carbon Resonance Assignment. To unambiguously assign the DG carbonyl carbon resonances, the acyl chain carbonyl carbons of the DG moiety of lipophorin were enriched with ^{13}C by instilling a bolus of tri[1-^{13}C]oleoylglycerol to the midgut of larvae fed a fat-free diet (Prasad et al., 1986a). Animals reared on this diet form a very high density lipophorin which is DG deficient. When triolein is supplemented, however, DG is produced which is then transferred onto pre-existing circulating lipophorin particles, decreasing their density to form a mature HDLp particle (Prasad et al., 1986a). The spectrum of [^{13}C]-DG-enriched HDLp revealed a significant increase in resonance intensity only in the carbonyl carbon region (Figure 1, panel B). When this region (175−180 ppm) was expanded (Figure 1, panel B inset), three resonances were observed. The resonance at 177.7 ppm was assigned as the carboxyl carbon of free fatty acid derived from the hydrolysis of labeled triolein. The lack of detectable resonances in the phospholipid acyl chain carbonyl region (176.0−176.4 ppm) of the spectrum indicates that no specific enrichment of this lipid class occurred upon bolus feeding of tri[1-^{13}C]oleoylglycerol. This result is consistent with the fact that <2% of radioactivity was recovered in phospholipid (versus 96% in DG) after instilling [^{3}H]triolein into the gut of animals fed a fat-free diet (Prasad et al., 1986a).

Conceivably, the two resonances observed in the DG acyl chain carbonyl region (175.55 and 175.63 ppm, respectively) could represent the carbonyls of the *sn*-1 and *sn*-2 acyl chains of *sn*-1,2-DG. Previous studies have shown that, in organic solvents, the acyl chain carbonyls of *sn*-1,2-DG resonate at different frequencies separated by 0.2−0.4 ppm (depending on the solvent employed; Hamilton et al., 1991a; Soulages et al., 1994; Table 1). By contrast, when present in sonicated phospholipid vesicles (Hamilton et al., 1991a) or in intact lipophorin (Soulages et al., 1994), separation between the *sn*-1 and *sn*-2 carbonyl carbon resonances of DG is not observed. In aqueous systems, however, distinct resonances are observed for 1,2-DG and 1,3-DG (Hamilton et al., 1991a,b). Consistent with the previously reported occurrence of 1,3-DG in lipophorin as a result of spontaeous acyl chain migration of *sn*-1,2-DG (Van der Horst, 1990), thin layer chromatography of lipophorin lipid extracts used in the present studies revealed the presence of both DG isomers. In their study of the conversion of *sn*-1,2-DG into *sn*-1,3-DG via acyl chain migration in sonicated phospholipid vesicles, Hamilton et al. (1991a,b) showed that, as this process occurs, a relative decrease in 1,2-DG is accompanied by the irreversible appearance of a new resonance ~0.15 ppm downfield (higher ppm). On the basis of these considerations we conclude that, in HDLp, the two resonances dramatically enriched upon feeding insects tri[1-^{13}C]-oleoylglycerol correspond to 1,2-DG (175.55 ppm) and 1,3-DG (175.63 ppm).

In HDLp DG is localized primarily in the particle core (Soulages & Brenner, 1991). By contrast, DG in LDLp is postulated to reside both in the core and within the surface monolayer, where it influences association of the exchangeable apolipoprotein, apoLp-III, with the particle surface (Blacklock & Ryan, 1994). Thus, it can be predicted that ^{13}C NMR spectra of LDLp may display additional heterogeneity in carbonyl resonance chemical shifts which reflect the existence of 1,2- and 1,3-DG in the particle core as well as at the particle surface. Figure 2 shows expanded natural abundance ^{13}C NMR spectra of the carbonyl region of the

Table 1: Carbonyl Carbon Chemical Shifts (ppm) of DG in Different Systems

	1,2-DG		1,3-DG	
	sn-1	*sn*-2	*sn*-1/3	$\Delta\delta^a$ (ppm)
CCl_4[b]	174.33	173.92	174.33	0.00
$CHCl_3$[b]	176.10	175.73	176.20	0.10
2:1 $CHCl_3/CH_3OH$[b]	176.78	176.56	176.97	0.19
vesicles (38 °C)[b]	175.61		175.76	0.15
lipophorin[c]	175.65[d]			
	175.56[e]			
HDLp-L (25 °C)[f]	175.55		175.63	0.08
HDLp-W2 (25 °C)	175.54		175.62	0.08
LDLp (core) (5 °C)	175.36		175.46	0.10
LDLp (surface) (5 °C)	175.30		175.50	0.20
LDLp (core) (25 °C)	175.55		175.65	0.10
LDLp (surface) (25 °C)	175.48		175.70	0.22
LDLp (core) (37 °C)	175.63		175.73	0.10
LDLp (surface) (37 °C)	175.54		175.77	0.23

[a] $\Delta\delta$ is the chemical shift difference between 1,3-DG and 1,2-DG in vesicles and lipophorins or between the *sn*-1 carbonyl chemical shift in *sn*-1,2-DG and the *sn*-1/3 carbonyls in *sn*-1,3-DG in organic solvents. [b] Data were extracted from Hamilton et al. (1991) with normalized by using DSS as the chemical shift standard. [c] Data were extracted from Soulages et al. (1994). Only one signal was observed (lipophorin DG) for lipophorin in this work. The chemical shifts were normalized by using DSS as the chemical shift standard. [d] Carbonyl chemical shift of [^{13}C]palmitic acid-enriched lipophorin. [e] Carbonyl chemical shift of [^{13}C]oleic acid-enriched lipophorin. [f] The chemical shift data in this work are referenced with DSS as the standard.

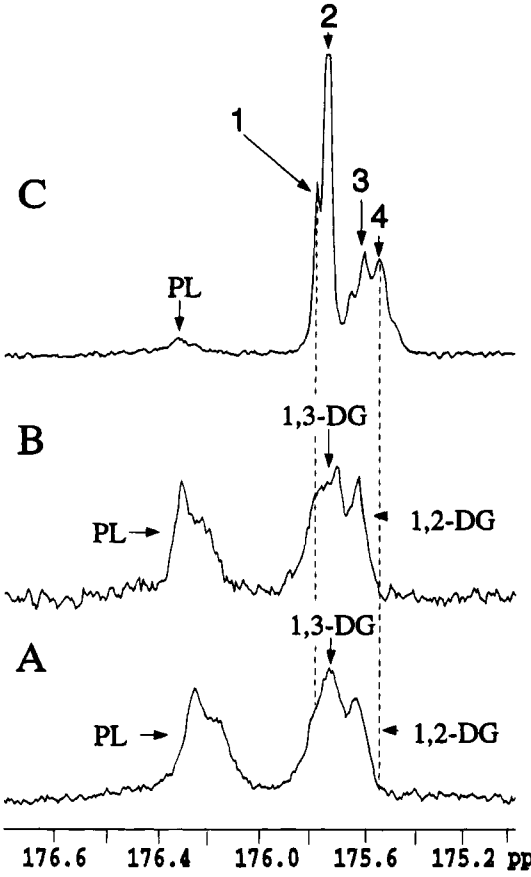

FIGURE 2: Natural abundance ^{13}C NMR spectrum of the carbonyl carbon region of lipophorin subspecies. (Panel A) HDLp-W1 (170 mg of protein); (panel B) HDLp-W2 (200 mg of protein); (panel C) LDLp (48 mg protein). Isolated lipoproteins were dialyzed against phosphate buffered saline and mixed with 0.5 mL of D_2O (final volume = 3 mL) prior to NMR spectroscopy at 37 °C. DG = diacylglycerol; PL = phospholipid.

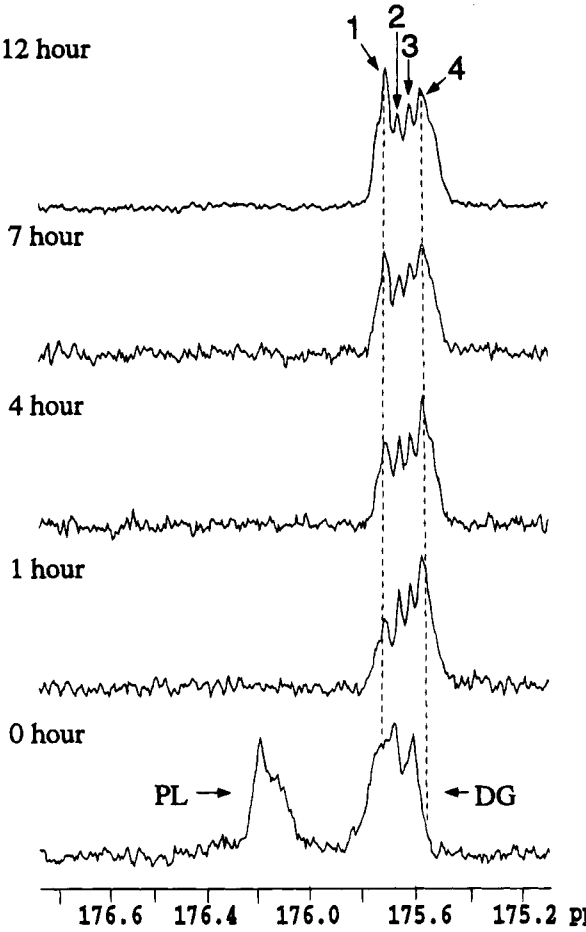

FIGURE 3: HDLp-W1 diacylglycerol carbonyl carbon spectra obtained following lipolysis by phsopholipase C. HDLp-W1 was treated with PL-C in the presence of apoLp-III as described in Materials and Methods. Spectra were recorded at 37 °C at the indicated times following phospholipolysis.

lipophorin subspecies HDLp-W2, HDLp-W1, and LDLp. HDLp-W2 and HDLp-W1 spectra reveal a significant resonance at 176.3 ppm corresponding to the phospholipid acyl chain carbonyls. In the DG acyl chain carbonyl region, two resonances, with chemical shifts of ~175.55 and 175.63 ppm at 37 °C, are observed. As was the case with ^{13}C-enriched HDLp, in HDLp-W2 (panel A) and HDLp-W1 (panel B), the two DG acyl chain carbonyl resonances were separated by 0.1 ppm and thus have been assigned as core localized 1,2- and 1,3-DG, respectively.

By contrast, the spectrum of LDLp (Figure 2, panel C, and Table 1) is more complex. The small phospholipid acyl chain carbonyl resonance compared to that for DG carbonyls is consistent with the known ratio of these lipids in LDLp (Ryan et al., 1986). Interestingly, DG acyl chain carbonyl resonances are split into four major peaks (numbered 1−4 in the Figure) with one shoulder peak. Two of these peaks (labeled as peaks 2 and 3 in Figure 2, panel C) have chemical shifts similar to that of HDLp DG resonances and were separated by 0.1 ppm. Therefore, these are assigned as 1,3-DG (peak 2) and 1,2-DG (peak 3) present in the particle core. The two additional major resonances present in panel C (peaks 1 and 4) were not observed in natural abundance or ^{13}C-enriched HDLp subspecies and displayed distinct chemical shifts separated by 0.2 ppm. It is noteworthy that, in all cases, $\Delta\delta$ between different pairs of resonances was independent of temperature (Table 1). Since it is generally accepted that some portion of LDLp DG resides at the particle surface where it interacts with protein and/or phospholipid (Wells et al., 1987; Wang et al., 1992), we postulated that these resonances may arise from 1,2-DG and 1,3-DG localized in the surface monolayer of LDLp.

^{13}C NMR Resonances of DG Carbonyl Carbons Arising from Phospholipase C Treated HDLps. In experiments designed to verify the hypothesis that LDLp specific resonances (peaks 1 and 4) represent DG molecules in the surface monolayer, we conducted ^{13}C NMR measurements of the HDLp-W1 before and after conversion of its phospholipid moiety into DG by treatment with PL-C (Figure 3). We have previously shown that PL-C catalyzed conversion of lipoprotein phospholipid into DG results in particle instability, aggregation, and fusion, leading to sample turbidity (Liu et al., 1993; Singh et al., 1994). Importantly, however, it has also been demonstrated that inclusion of amphipathic exchangeable apolipoproteins, such as apoLp-III, prevents PL-C induced lipoprotein aggregation through formation of a stable binding interaction with lipolyzed particles (Liu et al., 1993; Singh et al., 1994). Since the phospholipid molecules are localized on the particle surface, PL-C activity should result in surface associated 1,2-DG. This expectation is consistent with the observed recruitment of exchangeable apolipoprotein as a function of PL-C mediated phospholipolysis. As discussed above, natural abundance ^{13}C NMR spectra of native HDLp-W1 display

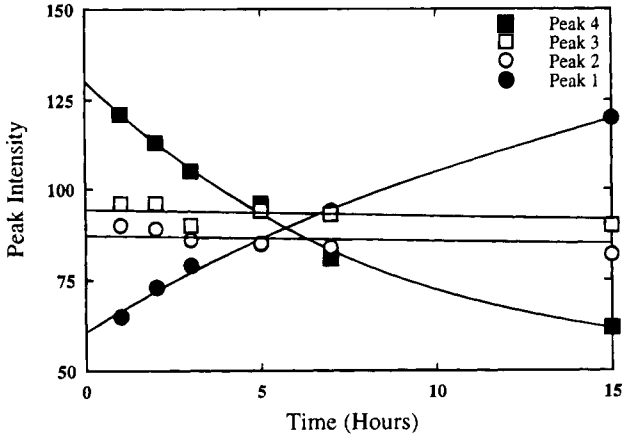

FIGURE 4: Relative peak height of HDLp-W1 DG carbonyl resonances following PL-C mediated hydrolysis of lipophorin phospholipid as a function of time at 37 °C. Phospholipase C treatment was performed as described in the legend to Figure 3, and NMR spectra were obtained as Materials and Methods. Peak heights determined at the indicated times correspond to those accumulated during lipolysis and numbers refer to peaks identified in Figure 3.

DG and phospholipid carbonyl resonances of nearly equal intensity (Figure 3), which reflects the relative amount of phospholipids and DG present in this subspecies (Prasad et al., 1986a). One hour after PL-C mediated conversion of lipophorin phospholipid into DG, the resonance attributed to phospholipid acyl chain carbonyl carbons was no longer present. In addition, the DG acyl chain carbonyl region of the spectrum contained a major new resonance (labeled peak 4; 175.55 ppm) 0.05 ppm upfield of the DG carbonyl resonances prior to lipolysis (Figure 3). Importantly, over the course of 12 h at 37 °C, the new upfield resonance generated by the action of PL-C on lipophorin phospholipid was reduced in intensity, concomitant with the appearance of a new downfield resonance (peak 1) at 175.74 ppm. Figure 4 shows changes in DG carbonyl resonance peak height for peaks 1−4 in Figure 3 as a function of time following PL-C treatment. The data show a direct correlation between the declining height of peak 4 and the increasing height of peak 1, while peaks 2 and 3 were unaffected. Thus, it is plausible that newly created surface 1,2-DG undergoes acyl chain migration but, apparently, does not appreciably equilibrate with core localized DG on the time scale of this experiment. Interestingly, the two new resonances observed in HDLp-W1 12 h after PL-C digestion are separated by 0.21 ppm and have chemical shifts nearly identical to corresponding resonances present in LDLp previously assigned as surface associated 1,2-DG and 1,3-DG.

To examine the effect of apoLp-III on the PL-C dependent appearance of new DG acyl chain carbonyl resonances in HDLp, the relatively DG-deficient lipophorin subspecies, HDLp-W2, was employed in phospholipolysis experiments in the absence of exogenous apoLp-III. This subspecies, which contains ~100 DG per particle, is relatively deficient in core lipid content causing the particle to adopt an unusual, asymmetric morphology (Ryan et al., 1992). PL-C treated HDLp-W2 is relatively resistant to aggregation and the accompanying sample turbidity development, presumably because DG generated at the expense of phospholipid can be effectively stabilized by apolipophorin I and apolipophorin II (Singh et al., 1994). As with HDLp-W1, PL-C treatment of HDLp-W2 resulted in the appearance of new DG carbonyl

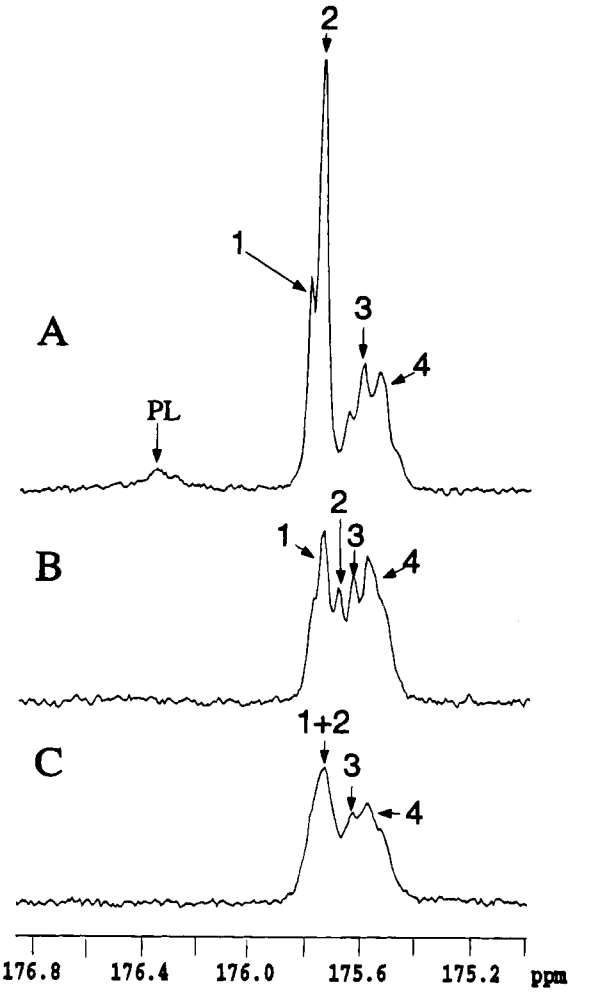

FIGURE 5: Natural abundance ^{13}C NMR spectra of the carbonyl carbon region of native and lipolyzed lipoproteins. (A) Native LDLp (48 mg of protein); (B) HDLp-W1 (180 mg of protein) plus apoLp-III (40 mg) after incubation with phospholipase C; (C) HDLp-W2 (200 mg of protein) after incubation with phospholipase C. Spectra were recorded at 37 °C. DG = diacylglycerol; PL = phospholipid.

Table 2: Carbonyl Chemical Shifts (ppm) of DG in Different Lipophorins at 37 °C before and after PLC Digestions[a]

	1,2-DG	1,3-DG	Δδ
before PLC digestion			
HDLp-W2 (core)	175.61	175.72	0.11
HDLp-W1 (core)	175.61	175.72	0.11
LDLp (core)	175.63	175.73	0.10
LDLp (surface)	175.54	175.77	0.23
after PLC digestion			
HDLp-W2 (core)	175.57	175.67	0.10
HDLp-W2 (surface)	175.50	175.69	0.19
HDLp-W1 (core)	175.60	175.68	0.08
HDLp-W1 (surface)	175.55	175.74	0.19

[a] The chemical shift values reported in this table is referenced by using DSS as the standard.

carbon resonances with a chemical shifts very similar to those appearing in PL-C treated HDLp-W1. Direct comparison of the resonance patterns observed in native LDLp and PL-C-treated HDLps (Figure 5) provides evidence that a detectable distinction exists between DG carbonyl carbon resonances alternately located at the surface and core of lipophorin particles.

Table 2 lists the DG carbonyl chemical shift values before and after PL-C digestion. In general, the chemical shift values of 1,2-DG and 1,3-DG resonances, in both the core

and surface, exist within a narrow range (175.50−175.77 ppm at 37 °C) which differs slightly among the various lipophorin particles examined. However, differences between 1,2-DG and 1,3-DG chemical shifts are consistent among these particles such that $\Delta\delta$ between positional isomers of DG present in the core is invariably 0.09 ± 0.02 ppm while $\Delta\delta = 0.21 \pm 0.02$ ppm for surface localized DG positional isomers.

DISCUSSION

The present study was designed to determine the feasibility of utilizing ^{13}C NMR spectroscopy to differentiate between DG molecules present in the hydrophobic core of lipoprotein particles and those localized on the particle surface. The major insect lipoprotein, lipophorin, transports neutral lipid in the form of DG, and the DG content in different subspecies varies dramatically (Ryan, 1994). Furthermore, it has been shown that when the DG content of lipophorin is increased (through the action of adipokinetic hormone), there is a concomitant association of the amphipathic exchangeable apolipoprotein, apoLp-III. Studies investigating the process of apoLp-III association have suggested that partitioning of DG molecules from the core to the surface of lipophorin creates a binding site for apoLp-III (Wang et al., 1992). In support of this hypothesis, it has been demonstrated that lipid transfer particle mediated enrichment of human LDL with exogenous DG promotes association of apoLp-III with the particle surface (Singh et al., 1992). Also, treatment of LDL (Liu et al., 1993) or lipophorin (Singh et al., 1994) with PL-C to create surface DG, induces a stable association with apoLp-III. Finally, Soulages and Wells (1994) have shown that addition of exogenous short chain DG to lipophorin induces apoLp-III association and stabilization of the particle structure. Thus, it has been suggested that association of apoLp-III with the surface of lipophorin occurs as a function of DG partitioning to the particle surface.

^{13}C NMR spectra of HDLp particles (which do not contain apoLp-III and which are postulated to contain predominantly core localized DG) revealed two distinct DG acyl chain carbonyl resonances, separated by 0.1 ppm. This is similar to data reported by Soulages et al. (1994) except that, while we are able to distinguish two closely spaced resonances, these authors observed a single resonance using [^{13}C]palmitic or [^{13}C]oleic acid enriched lipophorins. This discrepancy may be reconciled if the lipophorin sample employed by these authors had not undergone significant acyl chain migration prior to NMR analysis. In any event, the narrow line widths of the DG carbonyl resonances in HDLp suggest these molecules are mobile within the particle. The assignment of the two peaks observed in HDLp samples in the present study as 1,2-DG and 1,3-DG is consistent with studies of these DG isomers in sonicated phospholipid vesicles. Hamilton et al. (1991a) showed that, unlike DG dissolved in organic solvents, the *sn*-1 and *sn*-2 acyl chain carbonyls of *sn*-1,2-DG in sonicated vesicles are not resolved, and, thus, this lipid gives rise to a single carbonyl resonance. 1,2-DG and 1,3-DG isomers present in sonicated phospholipid vesicles, however, can be resolved giving rise to distinct resonances separated by 0.15 ppm (Hamilton et al., 1991a,b). In HDLp subspecies, we observed similar resonances but these were generally separated by 0.09 ± 0.02 ppm. In organic solvents a trend exists with respect to the chemical shift difference, $\Delta\delta$, between resonances corresponding to the 1 position of *sn*-1,2-DG and the magnetically equivalent $^1/_3$ position of *sn*-1,3-DG wherein this value increases with increasing solvent polarity (Table 1). While $\Delta\delta$ is nonexistent in carbon tetrachloride, a 0.10 ppm chemical shift difference is observed between these carbonyls in chloroform. As the polarity of the solvent increased further, larger chemical shift differences are observed between these carbonyls (e.g., in 2:1 $CHCl_3/CH_3OH$ $\Delta\delta = 0.19$ ppm). Extrapolation of these data and comparison to the corresponding situation in aqueous systems (e.g., vesicles, lipophorin) suggests that the relative polarity of the DG carbonyl carbon environment may affect the chemical shift difference between 1,2-DG and 1,3-DG. Thus, it is reasonable to speculate that the decreased chemical shift difference between DG isomers in HDLp (0.09 ppm) versus vesicles (0.15 ppm) may reflect the sequestration of DG isomers in HDLp into the particle core.

By extension, it was hypothesized that LDLp particles, which contain up to 16 apoLp-III per particle, likely contain significant amounts of DG in the surface monolayer. Natural abundance ^{13}C NMR spectra of LDLp revealed resonances similar to those observed in HDLp subspecies. In addition, at least two additional resonances were observed that were unique to LDLp. These two resonances, which were separated by 0.23 ppm, were tentatively assigned as surface-associated 1,3- and 1,2-DG. To test this hypothesis, we subjected HDLp to phospholipolysis with PL-C to generate surface associated 1,2-DG. In the case of HDLp-W1, exogenous apoLp-III was added to the incubation to stabilize the lipolyzed particle. After the phospholipid acyl chain carbonyl resonances were depleted, a major new DG acyl chain carbonyl resonance (and a minor downfield resonance labeled peak 1) appeared upfield from other DG carbonyl resonances in HDLp-W1 (Figure 3, peak 4). This resonance displayed a chemical shift very similar to the unique upfield resonance present in LDLp, suggesting it corresponds to surface-associated DG (Table 2). This is consistent with an assignment of peak 4 as surface 1,2-DG since this is the expected product of PL-C activity. Over the course of 12 h following PL-C hydrolysis the intensity of peak 4 diminishes concomitant with a significant increase in the intenisty of the minor downfield resonance (peak 1 in Figure 3). This result is consistent with the occurrence of acyl chain migration, creating 1,3-DG at the expense of 1,2-DG (Hamilton et al., 1991a,b). An interesting aspect of these results pertains to equilibration of the putative surface and core DG pools. The present data suggest that these pools are distinct and do not readily mix. This interpretation is consistent with that derived from radiolabeling experiments reported by Kawooya et al. (1991). Similar results were obtained when the HDLp-W2 subspecies was subjected to phospholipolysis, although the individual peaks were less well resolved. Again, as the phospholipid resonance was depleted, new resonances, ascribed as surface-associated DG, appeared. Since apoLp-III was not present in the HDLp-W2 case, the appearance of distinct surface DG carbonyl resonances does not strictly require apoLp-III, and, thus, its chemical shift may be influenced by alternate interactions with solvent, phospholipid head groups, or apolipophorins I and II.

An important question related to the apparent distinction between surface and core DG molecules in lipophorin pertains to the reason for the observed chemical shifts for

surface associated DG relative to core localized DG. Studies of other carbonyl containing lipids, including phospholipids (Schmidt et al., 1977), triacylglcyerol (Hamilton & Small, 1981), and cholesteryl ester (Sears et al., 1976; Hamilton & Small, 1982), reveals that increasing solvent polarity induces a distinct downfield shift of carbonyl carbon resonances. A similar trend is observed for carbonyl resonances of pure DG in different organic solvents (Hamilton et al., 1991a) indicating solvent polarity (or DG carbonyl−solvent hydrogen-bonding capacity) exerts an influence on the chemical shift of DG carbonyls. Consistent with this concept, studies in aqueous systems have shown that the resonance arising from 1,2-DG in various systems (neat DG plus H_2O, DG in SDS micelles, and DG in phospholipid vesicles) follows a similar trend, wherein the carbonyl resonance shifts downfield as a function of their expected degree of hydration (Hamilton et al., 1991a). In the case of lipophorin particles, given the assignments presented, this overall trend is generally adhered to since all lipophorin DG carbonyl resonances fall within a narrow chemical shift range (175.5−175.8 ppm at 37 °C) that is intermediate between DG carbonyl resonances observed in CCl_4 (173.92 ppm) and aqueous SDS micelles (176.91 ppm; Hamilton et al., 1991a). Thus, rationalization of observed chemical shifts for resonances assigned as surface and core localized 1,2-DG and 1,3-DG must consider the fact that these chemcal shift differences are minor (range = 0.3 ppm) in the context of DG carbonyl chemical shifts in other systems (range = 3.0 ppm). The unique chemical shifts observed for a given differentially localized DG isomer within lipophorin, which show a minor nonconformity with the expected trend toward a DG carbonyl resonance to shift downfield as it partitions into the surface monolayer, may be explained by the relative complexity of the present system since, in addition to hydrogen-bonding potential, other factors, such as apolipoprotein or phospholipid interactions, may exert an influence.

In analyzing the present data, we noted a possible trend relating to the chemical shift difference between 1,2-DG and 1,3-DG as a function of their expected locations in either the core or surface of the particle. For example, in aqueous solvents, DG isomers present in the core of lipophorin are separated by 0.09 ± 0.02 ppm while these isomers in phospholipid vesicles are separated by ~0.15 ppm. In a similar manner the chemical shift difference between peaks corresponding to these isomers localized in the surface of lipophorin is 0.21 ± 0.02 ppm. Likewise, the chemical shift difference between resonances corresponding to 1,2-DG and 1,3-DG in organic solvents of differing relative polarity increases with increasing solvent polarity. Further studies of this and other systems will be required to verify the generality of this potential correlation.

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