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Hydrolysis of a Phospholipid in an Inert Lipid Matrix by Phospholipase A_2: A ^{13}C NMR Study†

Shastri P. Bhamidipati and James A. Hamilton*
Department of Biophysics, Boston University School of Medicine, Housman Medical Research Center, 80 East Concord Street, R111, Boston, Massachusetts 02118-2394

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ABSTRACT: A new approach to study phospholipase A_2 mediated hydrolysis of phospholipid vesicles, using ^{13}C NMR spectroscopy, is described. [^{13}C]Carbonyl-enriched dipalmitoylphosphatidylcholine (DPPC) incorporated into nonhydrolyzable ether-linked phospholipid bilayers was hydrolyzed by phospholipase A_2 (*Crotalus adamanteus*). The ^{13}C-labeled carboxyl/carbonyl peaks from the products [lyso-1-palmitoylphosphatidylcholine (LPPC) and palmitic acid (PA)] were well separated from the substrate carbonyl peaks. The progress of the reaction was monitored from decreases in the DPPC carbonyl peak intensities and increases in the product peak intensities. DPPC peak intensity changes showed that only the *sn*-2 ester bond of DPPC on the outer monolayer of the vesicle was hydrolyzed. Most, but not all, of the DPPC in the outer monolayer was hydrolyzed after 18–24 h. There was no movement of phospholipid from the inner to the outer monolayer over the long time periods (18–24 h) examined. On the basis of chemical shift measurements of the product carbonyl peaks, it was determined that, at all times during the hydrolysis reaction, the LPPC was present only in the outer monolayer of the bilayer and the PA was bound to the bilayer and was ~50% ionized at pH ~7.2. Bovine serum albumin extracted most of the LPPC and PA from the product vesicles, as revealed by chemical shift changes after addition of the protein. The capability of ^{13}C NMR spectroscopy to elucidate key structural features without the use of either shift reagents or separation procedures which may alter the reaction equilibrium makes it an attractive method to study this enzymatic process.

Phospholipase A_2 (PLA_2)[1] catalyzes the hydrolysis of the ester bond at the *sn*-2 position of a diacylphospholipid, releasing fatty acid (FA) and lyso-1-acylphospholipid (De Haas et al., 1968). Its activity is found intracellularly as well as in cell secretions; in particular, extracellular forms of the enzyme are found abundantly in mammalian pancreas and in the venom of snakes and bees (Volwerk & De Haas, 1982). While its functional role in facilitating fatty acid turnover is well-known (Van Deenen, 1965), its regulatory roles in eicosanoid synthesis (McKean et al., 1981; Imai et al., 1982) and detoxification of phospholipid peroxides (Van Kuijk et al., 1987) have been discovered more recently. Intracellular PLA_2 activity in vivo is presumably normally well regulated to provide small amounts of substrates such as arachidonic acid while leaving the bilayer structure of the membrane intact.

It is clear from analyses of the kinetics of PLA_2 action that PLA_2 activity exhibits strong dependence on aggregation state, phase, and structure of zwitterionic phospholipids (e.g., diacylphosphatidylcholine; Dennis, 1983). However, as pointed out recently (Lister et al., 1988), a detailed kinetic analysis of PLA_2 data requires knowledge of structural features such as changes in the substrate structure during hydrolysis and the location, distribution, and partitioning of the hydrolyzed products in the presence and absence of albumin. Previous studies of the structural organization of phospholipid bilayers subjected to PLA_2 treatment have examined extensive hydrolysis. The results of Kupferberg et al. (1980) suggested that the entire outer monolayer of the vesicle could be hydrolyzed and that the inner monolayer was inaccessible to the

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* Address correspondence to this author.

[1] Abbreviations: BSA, bovine serum albumin; DHPC, dihexadecylphosphatidylcholine ether lipid; DPPC, dipalmitoylphosphatidylcholine; FA, fatty acid(s); LPPC, lyso-1-palmitoylphosphatidylcholine; PA, palmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2.

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enzyme, while other investigators found more extensive hydrolysis of vesicles (Kensil & Dennis, 1979). A ^{31}P NMR study (Jones & Hunt, 1985) showed extensive hydrolysis of both outer and inner monolayer phospholipid without disruption of the bilayer structure. In another study Sundler et al. (1978) used chemical modification of phosphatidylethanolamine (PE) and found that PLA_2 action was limited to 80% of the modified PE in the outer leaflet of the vesicles. Results of the above studies showed variations in the extents of hydrolysis and accessibility of the inner monolayer phospholipid to hydrolysis as well as differences in localization of the hydrolyzed products (Kupferberg et al., 1980; Jones & Hunt, 1985).

This study focuses on monitoring the structural features during and following limited hydrolysis of small unilamellar vesicles by PLA_2. In order to limit the extent of hydrolysis and minimize the possibility of disruption of bilayer structure, we incorporated small amounts of hydrolyzable (ester-linked) PC into a nonhydrolyzable (ether-linked) PC which bears a close structural relationship to its ester analogue (Kim et al., 1987).

^{13}C NMR spectroscopy has seen little application to enzymatic processes despite its great potential for elucidating structural features, primarily because of the very low sensitivity of the method. Specific ^{13}C enrichment of the phospholipid was used to improve the detectability of the ^{13}C nucleus. The ester carbonyl carbon was selected for ^{13}C enrichment because it is the site of enzymatic action and its chemical shift is highly sensitive to both intrinsic (chemical bonding and structure) and extrinsic factors (hydrogen bonding and local environment). Furthermore, the ether-linked PC matrix decreases the rate of PLA_2 hydrolysis of the phospholipid component of the bilayer (Debose & Roberts, 1983) and thereby makes the reaction more amenable to ^{13}C NMR analysis.

Experimental Procedures

Materials. Dihexadecylphosphatidylcholine (DHPC) ether lipid was obtained from Serdary Research Laboratories, Ontario, Canada. Essentially fatty acid free bovine serum albumin (BSA), glycerophosphocholine cadmium chloride (crystalline), and lyso-1-palmitoyl-*sn*-glycero-3-phosphatidylcholine (LPPC) were obtained from Sigma Chemical Co., St. Louis, MO. Phospholipase A_2 (*Crotalus adamanteus*) was obtained from Sigma Chemical Co. as a lyophilized powder and used without further purification by dissolving in 0.05 M Tris buffer containing 0.22 M NaCl and 20 mM $CaCl_2$. [1-^{13}C]Palmitic acid (PA) with 90% enrichment was obtained from KOR Isotopes (Cambridge, MA), and [^{13}C]palmitic anhydride was prepared following the procedure of Selinger and Lapidot (1966). Dipalmitoylphosphatidylcholine (DPPC) with ^{13}C enrichment at the ester carbonyl of each chain was synthesized by condensation of glycerophosphocholine cadmium chloride with 1.6 mol equiv of [^{13}C]palmitic anhydride in the presence of dimethylaminopyridine in anhydrous chloroform (Gold Label, Aldrich Chemical Co., Milwaukee, WI) according to the procedure of Gupta et al. (1977). DPPC with ^{13}C enrichment at the *sn*-2 chain carbonyl position was synthesized by a modified method of Gupta et al. (1977), using LPPC and [^{13}C]palmitic anhydride. Both synthesized lipids were characterized by ^{13}C NMR in deuteriochloroform. All lipids were >95% pure by thin-layer chromatography and were used without further purification.

Vesicle Preparation. For preparation of ^{13}C-labeled DPPC/DHPC unilamellar vesicles, appropriate amounts of the lipids were co-dried from chloroform/mehanol (2:1 v/v) solution under nitrogen and then dried under vacuum overnight. The concentration of DPPC relative to total lipid was 10 or 25 mol %. The lipid film was hydrated in 1.6 mL of Tris buffer (0.05 M Tris-HCl and 1 mM EDTA, pH 7.4, containing either 0.22 M NaCl and 20 mM $CaCl_2$ or 0.10 M NaCl and 5 mM $CaCl_2$) and 0.2 mL of ^2H_2O. Vortex mixing of the sample was done at 50 °C, above the gel to liquid-crystalline phase transition of both the ether- and ester-linked phospholipids (Kim et al., 1987). These liposomes were sonicated on a Branson W-350 sonicator with a microtip in a pulsed mode (40% duty cycle) for 30 min at 48–55 °C (measured internally). The vesicles were subjected to a 10-min low-speed centrifugation in an oven at 50 °C to remove undispersed lipid and metallic particles from the sonicator tip.

Chemical Methods. All vesicles samples were analyzed by thin-layer chromatography (TLC) after sonication for lipid integrity. Total phospholipid was quantitated by the Bartlett method (Bartlett, 1959). In the case of samples treated with PLA_2, phospholipid (unreacted [^{13}C]DPPC and DHPC) and lysophospholipid (LPPC) were quantitated individually by the Bartlett method after separation by TLC. Fatty acid was also separated from PLA_2-treated samples by TLC and identified and quantitated by gas–liquid chromatography after methylation.

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were recorded at 50.3 MHz (4.7 T) on a Bruker WP-200 NMR spectrometer equipped with an Aspect 2000A data system and a Bruker B-VT-1000 variale-temperature unit. Broad-band proton decoupling (1.0 W) centered at 3.4 ppm downfield from tetramethylsilane was used. ^2H_2O was used as an internal lock and shim signal. The terminal methyl peak of the lipid fatty acyl chains at 14.10 ppm was used as a chemical shift reference (Hamilton et al., 1974); 1–5 μg of enzyme in the same buffer used for the substrate vesicles (see above) was added to each sample. The sample was maintained at 50 °C during addition of the enzyme and transferred without cooling to the NMR probe preequilibrated at 47 °C. Spectra were obtained as a function of time at 46–47 °C during the course of the enzymatic action. This temperature is above the peak transition temperature for the gel to liquid-crystalline transition of the constituent phospholipids; below this transition, the phospholipid [^{13}C]carbonyl peaks would be significantly broadened and unobservable on our NMR instrument. In a typical kinetic experiment, spectra were obtained every 0.5 h for the first 5 h and every 1 h for the next 18 h.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra of the vesicle samples were obtained at 81.0 MHz with broad-band proton decoupling on the same NMR instrument as described above. Chemical shifts of the phospholipid signals were measured with reference to external phosphoric acid. Spectra were accumulated with a 5000-Hz spectral width, 8192 time domain points, a 3.0-s pulse interval, and 300 scans.

Results and Discussion

Small unilamellar vesicles prepared by cosonication of 10 or 25 mol % DPPC with ^{13}C enrichment at both carbonyl carbons and 90 or 75 mol % DHPC ether lipid at ~50 °C were characterized by ^{13}C NMR spectroscopy. The carbonyl region of the substrate spectrum, as illustrated for a sample containing 10% DPPC (Figure 1A), contained peaks from the outer (P_o) and inner (P_i) monolayer carbonyl carbons of DPPC at 174.00 and 173.75 ppm, respectively (Schmidt et al., 1977). The ether-linked DHPC does not contribute to this region of the spectrum, allowing clear quantitation of the DPPC component by ^{13}C NMR. The resolution of the P_o and P_i peaks is somewhat lower than that usually seen for DPPC vesicles at this magnetic field (Hamilton, 1989), possibly because the

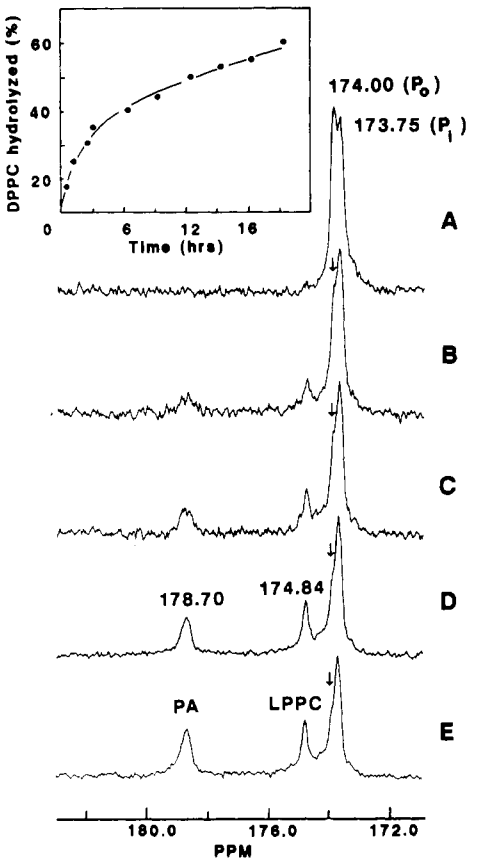

FIGURE 1: Carbonyl region from 50.3-MHz ^{13}C NMR spectra of 10 mol % *sn*-1 and *sn*-2 [^{13}C]carbonyl-labeled DPPC/90 mol % DHPC ether lipid vesicles hydrolyzed by PLA_2 (*Crotalus adamanteus*) at 46 °C as a function of time: (A) before enzyme was added (0.0 h); (B) 0.5 h, (C) 2.5 h, (D) 9.0 h, and (E) 18.0 h after the enzyme was added. P_o and P_i represent DPPC carbonyl signals from the outer and the inner monolayers, respectively. Outer monolayer P_o in (B) through (E) is referred to by an arrow. Spectra in (A), (B), and (C) correspond to 1000 accumulations with a pulse interval of 2.0 s and 2.0-Hz line broadening. Spectra in (D) and (E) were obtained under identical conditions except for the number of accumulations (2000). Numerical values above peaks are the chemical shifts in ppm. The inset shows the percentage of DPPC hydrolyzed as a function of time estimated from the loss in the total DPPC carbonyl signal intensity.

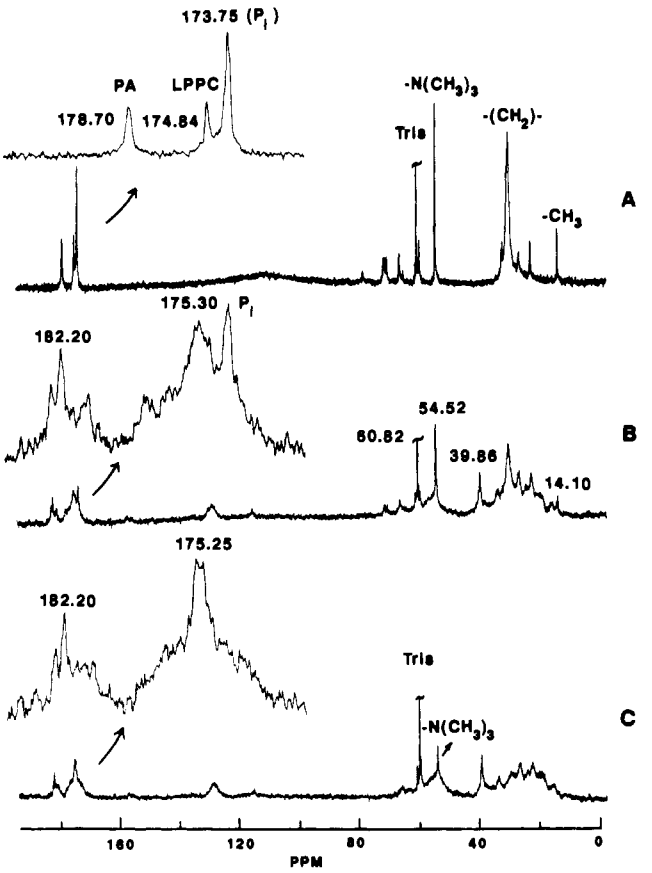

FIGURE 2: 50.3-MHz ^{13}C NMR spectra of (A) the substrate in Figure 1 after 24 h of hydrolysis by PLA_2, (B) after addition of BSA to sample shown in (A) at 46 °C, and (C) the same sample (with BSA) as in (B) at 30 °C. Spectrum shown in (A) was obtained under conditions identical with those mentioned in Figure 1, whereas spectra in (B) and (C) correspond to 4000 accumulations with a pulse interval of 2.0 s and 3.0-Hz line broadening. Chemical shifts (ppm) are given above selected peaks. Insets correspond to carbonyl regions.

ether-rich vesicles are somewhat larger than DPPC vesicles (DeBose & Roberts, 1983).

Following addition of the enzyme at 46 °C, two new peaks well separated from the PC carbonyl peaks were detected (Figure 1B). With time, these new peaks (178.70 and 174.84 ppm) increased in intensity (with no change in chemical shift values). The peak at 174.00 ppm from the outer monolayer DPPC carbonyl carbons gradually decreased in intensity with time, while the inner monolayer DPPC carbonyl signal at 173.75 ppm showed no intensity change (Figure 1B–E). The peak at 178.70 ppm was identified as unesterified fatty acid (PA), one of the products of hydrolysis, associated with the phospholipid bilayer (Bhamidipati & Hamilton, 1988a).[2] The PA chemical shift corresponds to ~50% ionization of the carboxyl group, the predicted ionization of long-chain fatty acids in PC vesicles at pH 7.4 (Hamilton & Cistola, 1986).[3] The presence of LPPC in equimolar, or nearly equimolar, ratio with respect to fatty acid does not alter the fatty acid ionization behavior (Bhamidipati & Hamilton, 1988a). The peak at 174.84 ppm was identified as LPPC, the other product of hydrolysis. The chemical shift value indicates the LPPC is present only in the outer monolayer of the bilayer. In model bilayer systems, LPPC on the inner leaflet appears at 174.60 ppm (Bhamidipati & Hamilton, 1988b).

The slow time course of the PLA_2-mediated hydrolysis yielded spectra with sufficient signal to noise ratios to give a good estimate of the reaction kinetics. The relative amount of DPPC hydrolyzed as a function of time was estimated from the decrease in absolute peak intensity of the total DPPC carbonyl signal. The observed time course of DPPC hydrolysis is shown in Figure 1, inset. At 18 h, the peak intensity decrease showed that 45% of the total DPPC was hydrolyzed. This amount is consistent with the result shown in Figure 1E, indicating that most, but not all, of the outer monolayer DPPC was hydrolyzed. The enzyme activity calculated from the initial four time points (Figure 1, inset) was 2.2 μmol min^{-1} mg^{-1}.

The complete ^{13}C NMR spectrum of the DPPC/DHPC vesicles after 24 h of hydrolysis is shown in Figure 2A. The

[2] In the absence of PC bilayers, aqueous PA (at the concentration present in hydrolyzed PC vesicles) precipitated from basic solution (pH 10) on addition of equimolar Ca^{2+} and remained insoluble at pH 7.0. There was no detectable ^{13}C spectrum. When Ca^{2+} was added to preformed egg PC vesicles containing 5 mol % PA in a 1:1 mole ratio with respect to PA, the PA carboxyl signal broadened slightly but did not shift. Thus, the PA signal seen in the hydrolysis experiments reflects PA in the bilayer, in accordance with the findings (Hauser et al., 1979) for various long-chain FA in egg PC multilayers which showed little interaction of bilayer-incorporated FA with Ca^{2+} at pH values near neutrality.

[3] The distribution of the fatty acid in the bilayer leaflets cannot be deduced from the ^{13}C chemical shift (Hamilton & Cistola, 1986).

overall appearance of the natural-abundance spectrum, representing mainly the DHPC ether lipid, is characteristic of small unilamellar vesicles (Levine et al., 1972; Hamilton & Small, 1981). (Note that the [^{13}C]carbonyl region verifies the bilayer structure of the DPPC component only.) The substrate spectrum did not change with time except for the carbonyl region (reflecting the hydrolysis of the minor component, DPPC). The inset (Figure 2A) showing the carbonyl region is similar to the 18-h spectrum (Figure 1E). To determine whether the fatty acid and/or lyso-PC could be extracted from the bilayer after hydrolysis, BSA was added to the reaction mixture in a 1:3 mole ratio with respect to total PA + LPPC. Transfer of PA (Bhamidipati & Hamilton, 1988a) and LPPC (Bhamidipati & Hamilton, 1988b) from bilayers to albumin can be detected directly in the reaction mixture, without separating the donor and acceptor, from changes in the carbonyl/carboxyl chemical shifts. After addition of BSA, the spectrum (Figure 2B) consisted of signals from both the protein and the lipid constituents; for example, the choline methyl signal at 54.54 ppm and the protein ϵ-Lys/β-Leu carbon signals at 39.86 ppm (Parks et al., 1983) were seen clearly. The aliphatic region contains overlapping protein and acyl chain peaks, as well as the terminal methyl signal of the acyl chain at 14.10 ppm. Inspection of the carbonyl region (Figure 2B, inset) shows that addition of BSA resulted in a set of new signals, the most prominent of which is at 182.20 ppm, characteristic of fatty acid now bound to BSA in different binding sites (Parks et al., 1983; Cistola et al., 1987a,b). A small peak at 178.30 ppm suggests that a minor proportion of PA may still be bound to the bilayer. In addition, the LPPC peak shifted from 174.84 to 175.30 ppm, indicating that LPPC was now bound to BSA (Bhamidipati & Hamilton, 1988b). Thus, most of the PA and LPPC were accessible to, and extracted by, BSA. The other remaining carbonyl peak, that of DPPC in the inner monolayer (173.84 ppm), reflects the bilayer integrity, while the broad envelope of resonances arises from the protein carboxyl/carbonyl carbons (Parks et al., 1983).

It should be possible to simplify the complex spectrum in Figure 2B containing contributions from phospholipids, BSA, PA, and LPPC by lowering the temperature below the phospholipid liquid-crystalline to gel transition temperature, which will result in severe broadening of all phospholipid (ether- and ester-linked) signals, except for the choline methyl group (Levine et al., 1972). If the peaks representing PA and LPPC bound to the phospholipid bilayer were to broaden along with the DPPC carbonyl peaks, then only the signals corresponding to BSA and the species bound to it would remain narrow. The spectrum at 30 °C (Figure 2C) consisted essentially of natural-abundance signals from BSA and carbonyl signals of PA and LPPC bound to BSA; of the phospholipid components, only the choline methyl was observed, as expected. Thus, the peak assignments for PA and LPPC bound to BSA were confirmed. Furthermore, the absence of the carbonyl signal at 173.80 ppm from unhydrolyzed inner monolayer DPPC shows that the DPPC has undergone a phase transition from liquid-crystalline to gel phase along with the DHPC ether lipid.

The above experiment using DPPC with ^{13}C enrichment at both ester carbonyl carbons allowed several of the salient features of PLA_2-mediated hydrolysis of DPPC to be followed by ^{13}C NMR. However, the spectral resolution of DPPC carbonyl signals from the outer and inner monolayers was compromised by the chemical shift inhomogeneity of *sn*-1 and *sn*-2 chain carbonyl carbon signals (Schmidt et al., 1977).

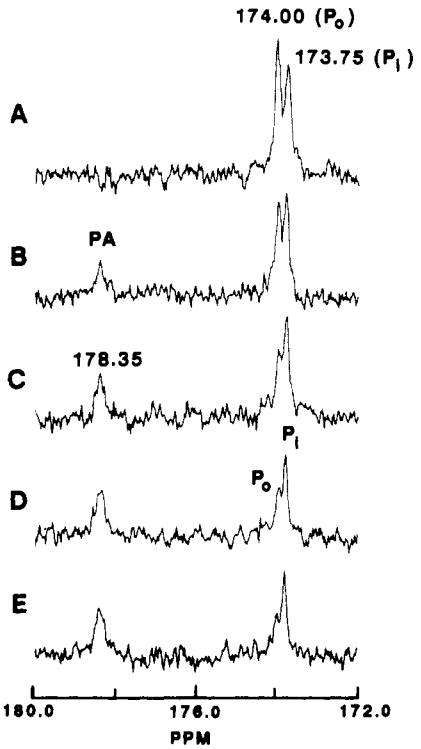

FIGURE 3: Carbonyl region of the 50.3-MHz ^{13}C NMR spectra of 10 mol % *sn*-2 [^{13}C]carbonyl-labeled DPPC/90 mol % DHPC ether lipid vesicles hydrolyzed by PLA_2 at 47 °C as a function of time: (A) before enzyme was added; (B) 1.0 h, (C) 3.5, (D) 13.0 h, and (E) 16.0 h after the enzyme was added. Each spectrum was recorded after 2000 accumulations, at a pulse interval of 2.0 s, and processed with 2.0-Hz line broadening. Chemical shifts (ppm) are indicated for each peak.

Hence, [^{13}C]DPPC labeled solely at the *sn*-2 position was incorporated into DHPC ether lipid vesicles at a level of 10 mol % DPPC, and PLA_2 hydrolysis at 47 °C was monitored by ^{13}C NMR in essentially the same fashion as above. The spectrum of the substrate (Figure 3A) shows DPPC carbonyl peaks from the outer (174.04 ppm) and inner (173.75 ppm) monolayers with improved spectral resolution because of the absence of overlapping signals from *sn*-1 chain carbonyls. The peak area ratio (P_o:P_i) of 1.5:1 is consistent with that expected for 300-Å vesicles (DeBose & Roberts, 1983). After addition of the enzyme, the peak at 174.04 ppm decreased in intensity while a new peak appeared at 178.30 ppm, corresponding to [^{13}C]PA (Figure 3B–E). In this experiment, where the outer and inner monolayer DPPC signals were seen more distinctly at all time points, it was clear that only the outer monolayer was hydrolyzed. The absence of a signal at 174.75 ppm showed that no ^{13}C-labeled LPPC was generated. Since the enzyme catalyzed the cleavage of the *sn*-2 ester bond alone, and the *sn*-1 position was not ^{13}C labeled, the unlabeled LPPC produced was not detected.

To investigate the effects of higher concentrations of products (but below the amounts expected to cause bilayer disruption), hydrolysis was performed on a vesicle system consisting of 25 mol % DPPC (labeled at both *sn*-1 and *sn*-2 carbonyl carbons) and 75 mol % ether/DHPC. The product complex was examined by ^{13}C NMR with suppression of the nuclear Overhauser enhancement in order to quantitate the carbonyl peak intensities. In addition, the substrate vesicles and the product complex were characterized by ^{31}P NMR. Continuous monitoring of hydrolysis for 18 h by ^{13}C NMR showed results very similar to those for the 10 mol % DPPC/90 mol % DHPC systems (above). The reaction was stopped at

18 h by addition of sufficient EDTA to complex the Ca^{2+} in the reaction mixture. The ^{13}C spectrum of the product with nuclear Overhauser effects removed showed an intensity ratio of 1.08 LPPC/PA, close to the expected mole ratio of 1.0, and an intensity ratio for (LPPC + PA)/DPPC of 1.10, corresponding to 54% hydrolysis of the total DPPC. The extent of hydrolysis was slightly greater than that for the 10% DPPC sample but was slightly less than the total DPPC in the outer monolayer.[4] Both the substrate and the product complex showed ^{31}P NMR spectra characteristic of a bilayer (small unilamellar) structure consisting of two partially resolved resonances of unequal intensity at −0.96 and −1.15 ppm. Since the ^{31}P spectrum (unlike the [^{13}C]carbonyl spectrum) represents both DPPC and DHPC, the ^{31}P results directly verify preservation of the bilayer structure in this system (25% DPPC).

An important result of this study was that PLA_2 did not hydrolyze PC on the inner monolayer of the vesicle bilayer in the presence of small amounts of product, even after long periods of exposure of the product complex to enzyme. NMR provided a direct, rather than indirect (Kupferberg et al., 1980; Jain et al., 1986), method of addressing this issue, through observation of separate signals for the inner and outer leaflet PC. The total amount of lyso-PC generated in these model systems was less than that which disrupts the bilayer organization of liquid-crystalline DPPC (Van Echteld et al., 1981), and the presence of equimolar fatty acid with respect to lyso-PC would be expected to retard the bilayer-disrupting capability of lyso-PC (Jain et al., 1980). The NMR results demonstrated bilayer stability in our systems with respect to both phospholipid components, and this stability is undoubtedly a factor in the inaccessibility of enzyme to inner monolayer phospholipid. Moreover, these results show that PLA_2 products did not appreciably increase the slow ($t_{1/2}$ approximately days) transbilayer movement of PC in vesicle bilayers (Shaw et al., 1977). Higher concentrations of products may enhance PC flip-flop or alter the phospholipid bilayer organization to result in hydrolysis of PC on the inner monolayer, as seen by others (Kensil & Dennis, 1979; Jones & Hunt, 1985). Finally, the PLA_2 itself did not disrupt the bilayer or promote transbilayer movement, as expected from the low enzyme concentrations used (DeBose & Roberts, 1983).

In summary, the NMR results demonstrate that the PLA_2 (*Crotalus adamanteus*) cleaved the *sn*-2 ester linkage of DPPC located on the outer monolayer of the mixed phospholipid (ester/ether) bilayer. The bilayer structure was maintained throughout the course of hydrolysis, and the inner monolayer DPPC did not flip to the outer monolayer. The LPPC produced was present in the outer leaflet, did not flip to the inner leaflet, and was extracted by BSA. The PA produced was present primarily in the bilayer and was ~50% ionized at all points during hydrolysis. Most of the PA was extracted along with LPPC by albumin at pH 7.4. It was thus possible to deduce several key structural features of the PLA_2 reaction directly from the ^{13}C NMR spectrum of appropriately ^{13}C-enriched phospholipid. Procedures such as titrimetric measurement of released protons to follow the kinetics and to deduce fatty acid ionization (Kupferberg et al., 1981), sample fractionation to observe products bound to albumin (Bergmann et al., 1984), and the use of albumin (Bergmann et al., 1984) and shift reagents (Van den Besselaar et al., 1979) to determine the location of LPPC in the bilayer each provide only a part of the information obtained by ^{13}C NMR. While such procedures may be desirable in some cases (e.g., to determine very precisely the amount of product bound to albumin), they may also alter the reaction equilibrium or the product distribution equilibrium.

[4] Our results for both 10% and 25% DPPC in DHPC vesicles showed less than complete hydrolysis of the DPPC in the outer monolayer, even with long incubation times. Using mixed PE/PC vesicles containing modified PE at levels of ~10% and 20% with respect to total phospholipid, Sundler et al. (1978) found that 20% of the modified PE in the outer monolayer was not hydrolyzed by phospholipase A_2 (*Naja naja*). However, different hydrolytic conditions, including a much shorter observation time in the latter case, may not make these results quantitatively comparable.

Acknowledgments

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Registry No. PLA_2, 9001-84-7; DPPC, 2644-64-6.

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Characterization of Glycosphingolipid Mixtures with Up to Ten Sugars by Gas Chromatography and Gas Chromatography–Mass Spectrometry as Permethylated Oligosaccharides and Ceramides Released by Ceramide Glycanase†

Gunnar C. Hansson,*,‡ Yu-Teh Li,§ and Hasse Karlsson‡

Department of Medical Biochemistry, University of Gothenburg, S-400 33 Gothenburg, Sweden, and Department of Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana 70112

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ABSTRACT: A novel, effective method for structural characterization of glycosphingolipids has been devised. It employs ceramide glycanase to release intact oligosaccharides followed by analysis using high-mass gas chromatography–mass spectrometry. The oligosaccharides and ceramides released by the glycanase were permethylated and analyzed. The capillary gas chromatography gave excellent resolution and separated, for example, two isomeric 10-sugar oligosaccharides with a molecular mass of 2150 daltons differing only by a Gal1-3GlcNAc and a Gal1-4GlcNAc linkage. The oligosaccharides released from sialic acid containing glycosphingolipids (gangliosides) were also analyzed for monosialo compounds. This analytical approach is simple, is quick, and can readily allow quantitation of individual glycosphingolipids.

Glycosphingolipids (GSLs)[1] are a very heterogeneous group of substances that are localized in the outer leaflet of the plasma membrane. In this leaflet they constitute the major type of lipids in the brush border membrane of epithelial cells (Simons & van Meer, 1988; Hansson and Simons, unpublished results). No unifying function of the glycan part of the GSLs has yet been described, but they can specify several of the blood group antigens (e.g., ABO, Lewis, P, and Ii), as well as being expressed in a tumor-associated way (Hakomori, 1984). Also, many bacteria and bacterial toxins can use the carbohydrate moiety of GSLs for specific adhesion to their target cells (Mirelman, 1986).

The GSLs vary among animal species, individuals, and strains as well as among the different organs and cells within one species (Hansson, 1988). Today more than 200 different GSLs have been described. With this great variety of GSLs very high demands are put on the analytical methods for their characterization. Gas chromatography (GC) has been adapted for the analysis of oligosaccharides having up to 10 sugar residues. The high resolution of capillary GC together with the structural information obtained by mass spectrometry (MS) is shown to be very useful in the characterization of GSLs after their cleavage with the recently discovered ceramide glycanase (Li et al., 1986; Ito & Yamagata, 1986). The approach taken allows a quick and simple characterization of the oligosaccharide portion, the ceramide portion, and the nondegraded monoglycosylceramides.

Materials and Methods

Materials. The following GSLs were used in this study: total neutral GSL from white rat small intestine, both epithelial cells and nonepithelial residue (Breimer et al., 1982a); total neutral GSL from mouse small intestine (Breimer et al., 1981a); lactosylceramide, fucosyllactosylceramide, globotriaosylceramide, isoglobotriaosylceramide, globotetraosyl-

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* Address correspondence to this author at the Department of Medical Biochemistry, University of Gothenburg, P.O. Box 33031, S-400 33 Gothenburg, Sweden.
‡ University of Gothenburg.
§ Tulane University School of Medicine.

[1] Abbreviations: GSL, glycosphingolipid; GC, gas chromatography; MS, mass spectrometry; Hex, hexose; HexNAc, *N*-acetylhexosamine.

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