Phospholipid Complexation and Association with Apolipoprotein C-II: Insights from Mass Spectrometry

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ABSTRACT The interactions between phospholipid molecules in suspensions have been studied by using mass spectrometry. Electrospray mass spectra of homogeneous preparations formed from three different phospholipid molecules demonstrate that under certain conditions interactions between 90 and 100 lipid molecules can be preserved. In the presence of apolipoprotein C-II, a phospholipid binding protein, a series of lipid molecules and the protein were observed in complexes. The specificity of binding was demonstrated by proteolysis; the resulting mass spectra reveal lipid-bound peptides that encompass the proposed lipid-binding domain. The mass spectra of heterogeneous suspensions and their complexes with apolipoprotein C-II demonstrate that the protein binds simultaneously to two different phospholipids. Moreover, when apolipoprotein C-II is added to lipid suspensions formed with local concentrations of the same lipid molecule, the protein is capable of remodeling the distribution to form one that is closer to a statistical arrangement. These observations demonstrate a capacity for apolipoprotein C-II to change the topology of the phospholipid surface. More generally, these results highlight the fact that mass spectrometry can be used to probe lipid interactions in both homogeneous and heterogeneous suspensions and demonstrate reorganization of the distribution of lipids upon surface binding of apolipoprotein C-II.

INTRODUCTION

Apolipoprotein C-II (apoC-II) is a soluble plasma protein with a mass of 8915 Da. It is one of a family of apolipoproteins involved in lipid transport and metabolism in plasma. In vivo it is found predominantly in chylomicrons and very low density lipoproteins (VLDLs) (Havel et al., 1973; Jackson and Holdsworth, 1986), where it binds reversibly to the polar phospholipid surface. It functions as an activator of lipoprotein lipase (LpL), the enzyme that hydrolyses triacylglycerols (Havel et al., 1973; Jackson and Holdsworth, 1986). The binding of apoC-II to the lipid surface is required to promote a high-affinity complex between apoC-II and lipoprotein lipase (Clarke and Holbrook, 1985). In vitro apoC-II is able to associate with a range of natural and synthetic lipid surfaces, with a change in secondary structure. Like other apolipoproteins, apoC-II associates with lipids via amphipathic helices (Segrest et al., 1992). In the absence of lipid, the protein shows very little secondary structure. In the presence of sodium dodecyl sulfate (SDS) micelles, an NMR structure of apoC-II has been determined (MacRaild et al., 2001). Three regions of α-helix were defined (Fig. 1), the largest comprising residues 16–36 and forming the SDS-binding domain (MacPhee et al., 1999). The hydrophobic face of this helix consists of two clusters of hydrophobic residues that form a convex face. The N-terminal 12 residues were found to be disordered and structurally heterogeneous. Since the chylomicrons are the largest lipoproteins in plasma, it has been proposed that the convex binding surface of apoC-II may explain its preference for these larger and therefore less curved structures (MacRaild et al., 2001).

For membrane proteins much progress has been made in developing sophisticated biophysical approaches (see for example Arora and Tamm, 2001, and references therein). Although electrospray mass spectrometry is increasingly being applied to define the stoichiometry of proteins in complex with other proteins, ligands, and cofactors (Last and Robinson, 1999), investigations involving protein and lipid interactions are limited. Typically, for electrospray mass spectrometry, procedures involving concentrated organic acid are required to extract protein from the membrane in a soluble form (le Coutre et al., 2000). These methods give rise to well resolved spectra of the protein but do not provide information about noncovalent lipid binding interactions. For transmembrane peptides direct analysis from lipid suspensions has been used to demonstrate the presence of residues that are protected from hydrogen exchange with aqueous solvents (Bouchard et al., 1999; Demmers et al., 2001, 2000). Of the few examples that have been reported that examine protein lipid interactions directly by mass spectrometry, the preferential binding and stability of single lipid molecules to a protein have been demonstrated (de Brouwer et al., 2002; Demmers et al., 2003). In this study we have used mass spectrometry to investigate the interactions between lipids in both heterogeneous and homogenous preparations. We also examine the tendency of the same lipid to associate to form homogeneous suspensions in the presence of other lipids and the effect on these associations when complexes are formed with apoC-II.

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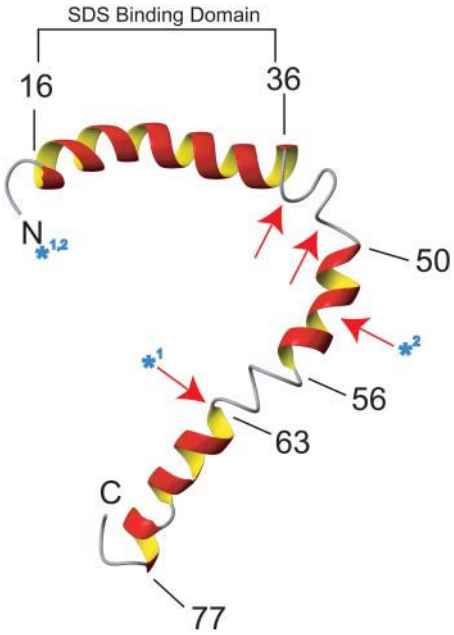

FIGURE 1 Structure of apoC-II derived from NMR experiments (MacRaild et al., 2001), produced using MolMol (Koradi et al., 1996). The residues at the N- and C-termini of each putative α-helix are marked. Regions at which chymotrypsin cleaves apoC-II in the absence of DMPC are indicated by red arrows. The convex SDS-binding surface is shown. Blue stars indicate the N- and C-termini of fragments 1 and 3, which are observed to bind DMPC.

MATERIALS AND METHODS

Apolipoprotein C-II

Apolipoprotein C-II was produced as described previously (Hatters et al., 2000). Briefly, it was expressed in *Escherichi coli* using the pET11a expression vector (Novagen, WI). Inclusion bodies were washed and resuspended in 5 ml of 5 M guanidine HCl, and 100 mM arginine, pH 12.0. The solution was sonicated for 1 min and centrifuged. The resulting solution was applied to a gel filtration column and the eluent applied to an anion-exchange column and eluted using a 0–500-mM NaCl gradient. The protein was dialysed against 10 mM ammonium bicarbonate, pH 8.0, at 4°C, lyophilized, and stored as a stock solution in 5 M guanidine HCl at −20°C.

Phospholipids

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (Avanti Polar Lipids, AL) have molecular weights of 678, 760, and 718, respectively. For a 1-mM solution of DMPC, 7 mg of the dry DMPC powder was suspended in aqueous buffer (10 ml ammonium acetate, 10 mM, pH 7.0). The resulting suspension was vortexed vigorously to homogeneity (turbid suspension), sonicated using a Soniprep sonicator probe (Integrated Services, NJ) for periods of 1 min with intervals of 1 min on ice, to prevent overheating. After ~15 min the suspensions became clear. Maximal clarity was achieved after ~45 min. For the mixtures of lipids two different preparations were examined. The first involves dissolution of 1:1 molar ratios of the dry phospholipid powders in $CHCl_3$, followed by solvent evaporation and resuspension in the aqueous buffer, according to standard protocols (New, 1990). For the second approach dry powders were mixed without prior dissolution in organic solvent and the same procedures as described above for homogeneous vesicle preparation were employed.

Apolipoprotein C-II with phospholipids

ApoC-II stock solution (29 mg/ml) was diluted to a final concentration of 20 μM in 10 mM ammonium acetate, pH 7.0, and 20-μl aliquots were applied to a Bio-spin 6 size-exclusion chromatography column (Bio-Rad Laboratories, CA). A quantity of 41.7 μl of the resulting apoC-II solution was added to 8.3 μl of the 1 mM phospholipid suspension to give a final protein/lipid concentration ratio of 1:10, in a volume of 50 μl.

For apoC-II with mixed lipid suspensions produced using the first approach, apoC-II stock solution (40 mg/ml) was diluted to a final concentration of 160 μM in 10 mM ammonium acetate, pH 7.0, and 20 μl was applied to a Bio-spin 6 size-exclusion chromatography column. A quantity of 5 μl of this solution was added to 5 μl of 10 mM 1:1 molar ratio lipid suspensions to a 1:63 final concentration ratio of protein to lipid. For apoC-II with mixed lipid suspensions produced using the second approach, 10 mM suspensions of the lipid mixtures were diluted 6.25 times to a concentration of 1.6 mM. A total of 5 μl of these solutions was mixed with an equal volume of apoC-II, 160 μM, to a final protein/lipid concentration ratio of 1:10.

Chymotrypsin digestion of apoC-II and apoC-II DMPC mixtures

A quantity of 2.5 μl of a 1-mg/ml solution of chymotrypsin (~40 μM) at pH 7.5 was added to 47.5 μl of either 20 μM apoC-II solution or apoC-II DMPC solution, such that the chymotrypsin/apoC-II ratio was 2:19 and 2:17, respectively. The mixtures were incubated on ice for a maximum of 3 h, and at specified time points 2-μl aliquots were removed for analysis.

Mass spectrometry

Nanoflow capillaries were prepared on a P97 Flaming/Brown micropipette puller (Sutter Instrument Company, Intracel, Hertfordshire, UK) using borosilicate glass capillaries of internal diameter 0.5 mm and external diameter 1.0 mm (Harvard Apparatus, Kent) and gold coated using an SEM coating system (Polaron, Energy Beam Sciences, MA). A quantity of 2-μl aliquots of sample were loaded into capillaries, and spectra were recorded on modified QToF2 (Sobott et al., 2002) and LCT mass spectrometers (Micromass UK, Manchester, UK). The capillary and cone voltages were maintained at 1800 V and 80 V, respectively. Pressure conditions were maintained in the LCT at 9×10^{-3} mbar in the analyser, 1.2×10^{0} mbar for the backing gas, and 3.2×10^{-7} mbar in the ToF, and 4×10^{-3} mbar in the ion guide, 2×10^{-5} mbar in the quadrupole analyser, and 5×10^{-7} mbar in the ToF for the QToF. The pressure in the analyser was raised to maintain a larger number of lipid/lipid and protein/lipid interactions. The highest analyser pressure used was 5×10^{-2} mbar in the QToF2 and 4×10^{0} mbar in the LCT.

Data analysis

Manipulation and analysis of mass spectra were performed with Masslynx v 3.1 (Micromass UK). The average mass of apoC-II was calculated from at least three charge states. All spectra were calibrated using 10 mM caesium iodide and the masses measured are centroided values. The estimated error in these measurements is calculated from the standard deviation of the charge states. All mass spectra have undergone minimal smoothing and no resolution enhancement, and are the average of 10–30 2-s acquisitions.

Charge state series are not a common feature of lipid and protein/lipid spectra. Rather, there are series of peaks with the same charge but increasing number of lipid molecules. It is not possible therefore to calculate the mass of these species using standard mass spectrometry software. To assign the peaks in the spectra, all possible combinations and charge states of lipid molecules in complex with one apoC-II molecule were calculated in Microsoft Excel. These m/z values were compared to those in the mass spectra. For the analysis of heterogeneous mixtures of phospholipids theoretical binomial distributions were calculated using Microsoft Excel.

RESULTS

Phospholipid suspensions dissociate in the mass spectrometer

The mass spectra obtained from homogeneous preparations of phospholipid vesicles consisting of DMPC, POPE, and POPC are shown in Fig. 2. POPC has the same phosphatidylcholine headgroup as DMPC, whereas POPE has a phosphatidylethanolamine headgroup. POPC and POPE have the same fatty acid tails, and are monounsaturated, whereas DMPC is fully saturated. A number of peaks are observed in the resulting mass spectra that correspond to different numbers of lipid molecules in complex. It is interesting to note that the peaks arise from association of lipid molecules rather than charging of a single species as is commonly observed in electrospray mass spectra of proteins. For example, for the 1+ charge state between one and six molecules of DMPC are observed, held together by noncovalent forces in the mass spectrometer. Within the same mass spectrum up to four charges are retained on the complex, giving rise to an assembly consisting of up to 25 DMPC molecules. The spectra of POPE and POPC give rise to a similar pattern of peaks, and in both spectra assemblies of up to 23 phospholipid molecules are observed. In all cases the monomeric lipid molecule is virtually absent, dimeric or trimeric species predominating in each case.

For protein complexes it is generally accepted that multiple low energy collisions within the flight path of the ion absorb excess translational energy and consequently reduce the internal energy of the ions (Rostom and Robinson, 1999). This process, often referred to as collisional cooling, has been shown to be effective in maintaining noncovalent interactions in large macromolecular complexes (Krutchinsky et al., 1998; Sobott et al., 2002). For lipid interactions collisional cooling gives rise to many more peaks at higher m/z values (Fig. 3) than those observed in Fig. 2. The increased number of peaks observed under these conditions is due to the reduction in the internal energies of the ions, which leads to an increase in the size of the assemblies that can be maintained. For example, for the 11+ charge state series (Fig. 3), complexes of up to 76 lipids are observed. In the 12+ charge state, the highest charge state that can be identified, the largest complex is assigned to 95 lipids with a mass of 64 410 Da.

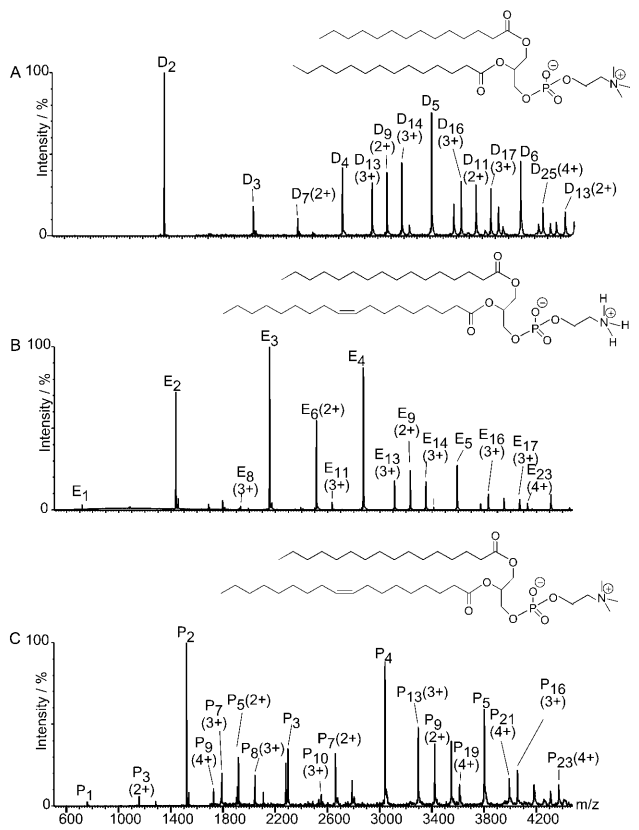

FIGURE 2 Electrospray mass spectra of homogeneous phospholipid suspensions formed from (*A*) DMPC (labeled *D*), (*B*) POPE (labeled *E*), and (*C*) POPC (labeled *P*). Peaks labeled with the number of phospholipid molecules are singly charged. For all other species the charge state of the ions is indicated in parentheses.

Apolipoprotein C-II phospholipid complexes

A typical positive ion nanoflow ESI mass spectrum of apoC-II introduced from aqueous solution is shown (Fig. 4 *A*). The charge states are well-resolved multiplets, the multiplicity resulting from sodium ions binding to the protein. There are also peaks that correspond to apoC-II with an additional mass of 131 Da. These peaks are due to incomplete processing of the protein and represent apoC-II with an N-terminal methionine. The measured masses of apoC-II, 8915.3 Da, and Met-apoC-II, 9046.3 Da, are within 0.1% of the

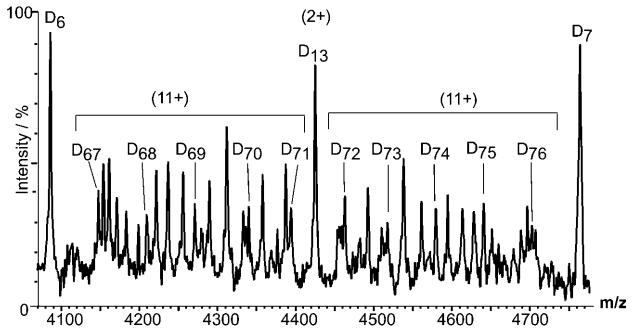

FIGURE 3 Electrospray mass spectrum of phospholipid suspensions formed from DMPC in the presence of collisional cooling of the ions. Under these mass spectrometry conditions associations of up to 76 lipid molecules are maintained. Only those peaks from the 11+ charge state series are labeled for clarity. It is interesting to note that when collisional cooling is employed all peaks except those corresponding to one and two lipids are 18 m/z units higher than their calculated m/z values. This difference remains the same over all of charge states, and is probably the result of binding of ammonium (NH_4^+) ions, present in the buffer, that contribute to the positive charging of these phospholipid complexes.

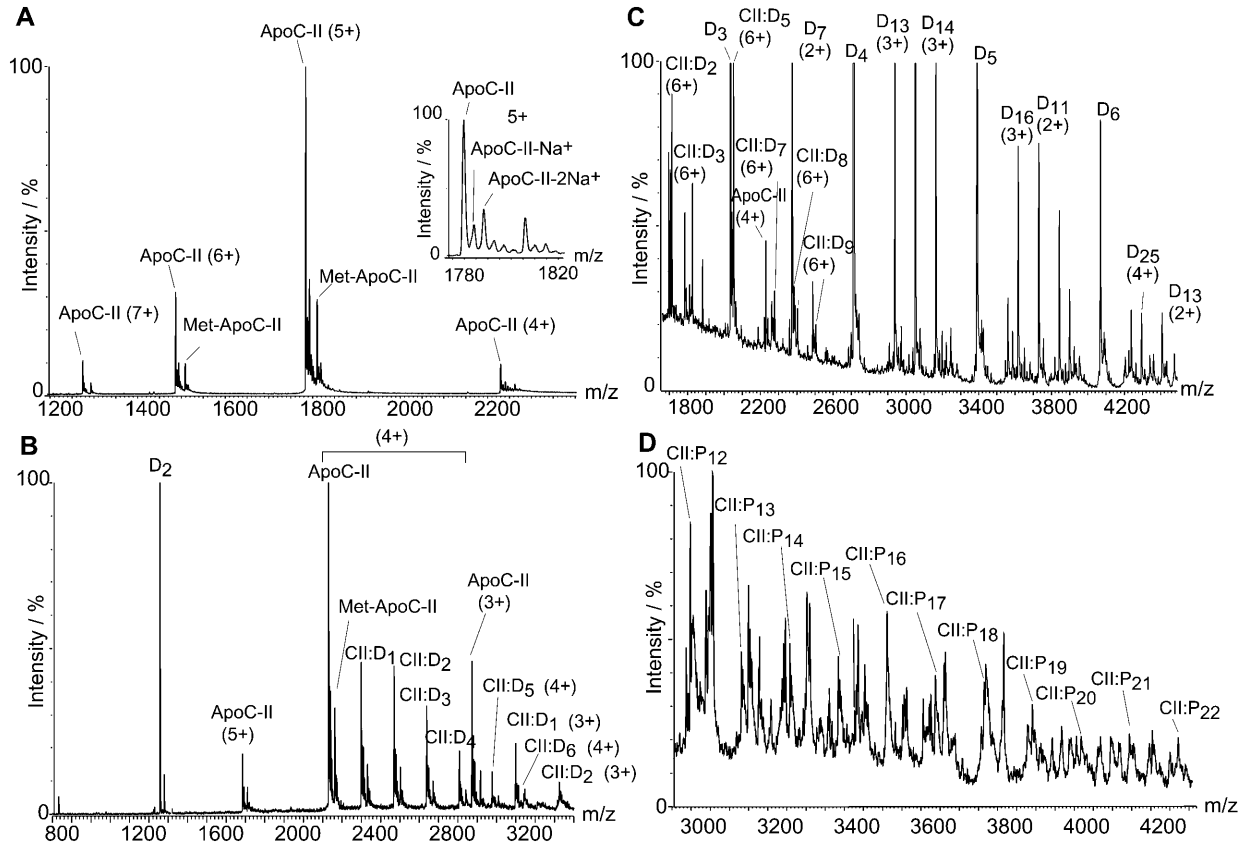

FIGURE 4 Electrospray mass spectra of apoC-II from aqueous ammonium acetate buffer (*A*) in the presence of DMPC vesicles (*B*) and in the presence of DMPC and POPC suspensions with collisional cooling (*C* and *D*).

calculated masses (8915.9 Da and 9046.9 Da, respectively). An interesting feature of the apoC-II spectrum is that the peak corresponding to the protein binding to two sodium ions is of higher intensity than that corresponding to the protein with one sodium ion. For nonspecific binding of sodium a statistical distribution of peaks would be anticipated. The results presented here therefore are consistent with the protein binding preferentially to two sodium ions.

ApoC-II was investigated in the presence of homogeneous suspensions of the phospholipids DMPC, POPE, and POPC. A typical spectrum recorded for a solution of apoC-II with DMPC is shown (Fig. 4 *B*). The spectra show some free protein as well as lipid/lipid and protein/lipid interactions. It is interesting to note that there is little change in the charge state distribution of the protein when in aqueous solution and when bound to lipid (highest intensity charge states 5+ and 4+, respectively). Since the phospholipid molecules used in this study have no net charge, this suggests an ion pair formation in the more compact lipid-bound protein. Also present within this mass spectrum are a number of peaks corresponding in mass to apoC-II complexed with DMPC molecules. It can be seen that from one to six lipid molecules remain bound to apoC-II in the 4+ charge state series under these mass spectrometry conditions.

Spectra were also recorded employing a higher degree of collisional cooling (Fig. 4 *C*). Under these conditions many more lipid/lipid interactions are maintained (up to 25 as opposed to only two under the conditions used to record the spectrum in Fig. 4 *B*). This spectrum also highlights an interesting feature of apoC-II in complex with DMPC. In this case the 6+ charge state series is predominant for the protein/lipid complex. Careful examination of this series reveals complexes with three, five, and seven lipid molecules bound to the protein. Interestingly the binding of four or six lipid molecules is not detected. This observation was found to be highly reproducible. In the 4+ charge state series, however, all species are represented (Fig. 4 *B*), the number of lipid molecules binding to the protein increasing consecutively. This is the same for all other charge states observed for this protein/lipid complex (data not shown). For apoC-II in the 6+ charge state, however, protein/DMPC complexes show preference for numbers of lipid molecules, a property not observed for other charge state series.

Phospholipid vesicles composed of POPE were examined in the presence of apoC-II using the same procedures as described in Materials and Methods. Mass spectra of POPE vesicles and apoC-II solutions show only the monomeric protein. No lipid molecules, whether in complex with the

protein, in complex with other lipid molecules, or in monomeric form, could be detected (data not shown). POPE has a small headgroup and an unsaturated fatty acid chain. The ratio of the cross sectional area of the headgroup to that of the tail dictates the orientation of lipid molecules within micelles. A small headgroup/tail area ratio results in inverted micelles in organic solvent. However, in aqueous solution the formation of inverted micelles is unfavorable since the hydrophobic hydrocarbon tails would be in contact with water. Consequently in aqueous ammonim acetate, POPE probably forms a hexagonal phase and precipitates. It is plausible therefore that apoC-II is not able to bind to POPE in this configuration, since POPE is not suspended in solution and is therefore not able to bind to apoC-II. Mass spectra of apoC-II with phospholipid vesicles formed from POPC, however, give rise to free protein, lipid complexes, and a large number of peaks that correspond to protein/lipid complexes. There are a number of differences between the apoC-II/DMPC and apoC-II/POPC mass spectra. For apoC-II/POPC complexes examination of the 4+, 5+, and 6+ charge states reveals peaks corresponding to apoC-II binding to consecutive numbers of lipid molecules. Consequently the binding of POPC to apoC-II does not show preference for binding discrete numbers of lipid molecules in any charge state series. In the 6+ charge state, in the presence of collisional cooling, up to 22 lipid molecules are seen binding to the protein (Fig. 4 *D*). The fact that this number is lower than that observed under comparable conditions for the lipid associations (up to 76, cf. Fig. 3) in the absence of the protein suggests some disruption of lipid associations by protein binding to the phospholipid surface.

As a control experiment to determine whether the interactions observed in the mass spectra represent those present in solution, or whether nonspecific complexes could be formed during the electrospray process, a mixture of the SH3 domain of bovine PI3 kinase and DMPC suspensions was examined under identical solution and mass spectrometry conditions as apoC-II and DMPC mixtures. SH3 is an 86-residue protein of 9638.6 Da, which does not bind to lipid molecules. The percentages of hydrophobic and hydrophilic residues in the two proteins are similar (30% and 62%, respectively for apoC-II; 27% and 55% for SH3). Their isoelectric points are also similar (the *pI* of apoC-II is 4.8; that of SH3 is 4.6). Mass spectra of SH3 with DMPC can be assigned to the free protein and complexes of lipid molecules, but there are no peaks that correspond to protein/lipid interactions. This demonstrates that proteins that do not bind to phospholipids in solution do not show binding to DMPC in mass spectra, under the same conditions where apoC-II exhibits significant binding. This suggests that the complexes observed between protein and lipid in the mass spectra are not simply governed by ion pair formation or aggregation but represent specific interactions of the lipid-binding protein with lipid molecules.

Binding of lipids to peptides derived from apoC-II

To investigate the degree of exposure of different regions of the protein in the absence and presence of lipid, proteolysis of apoC-II was carried out using chymotrypsin. Analysis of apoC-II in the absence of phospholipids reveals that a number of fragments are formed rapidly in the first 8 min of exposure to chymotrypsin (Table 1). After 50 min many of the peaks assigned to protein fragments have been further digested and the intact protein cannot be detected. This rapid proteolysis and large number of fragments is consistent with previous reports that apoC-II has little secondary structure in the absence of lipid or detergent (MacPhee et al., 1999). In complex with phospholipid vesicles formed from DMPC, the 5+ charge state of the protein remains clearly visible after 22 min of proteolysis and protein/lipid peaks are still observed after 40 min (Fig. 5). This indicates that the digestion is considerably slower than for the protein in the absence of lipids. The number of cleavage sites is also reduced, from four in the absence of lipid (Fig. 1) to two in the presence of lipid, namely, tyrosine residues 53 and 63. Two series of peaks in the mass spectra of the apoC-II/DMPC digestion do not correspond to intact protein, protein fragment, lipid, or protein/lipid peaks. These peaks result from association of DMPC molecules with proteolysis fragments F1 and F3 (sequences 1–63 and 1–53 of the apoC-II sequence, respectively; Fig. 1). These peaks correspond therefore to lipid binding to a region of the protein that includes the SDS-binding domain and the proposed lipid-binding domain. Such binding was not detected for other fragments derived from the protein. The protein fragments to which DMPC binds are considerably longer than the proposed lipid-binding helix, presumably because the lipid protects cleavable residues at the ends of the lipid-binding helix. Despite the fact that protein hydrolysis is considerably slower in the presence of lipid, digestion does still proceed. This observation is attributed to the fact that apoC-II is an exchangeable protein associating reversibly with the lipid surface, digestion being governed by its on-off rate from the lipid surface. The results presented here therefore demonstrate lipid binding to peptides generated from the proposed lipid-binding domain, exposure of the C-terminal domain to protease, and reversible interaction of the apoC-II with lipid suspensions.

Interactions in heterogeneous lipid complexes

In a biological membrane a peripheral protein would interact with many types of lipid, including steroids and triacylglycerols, as well as different phospholipids. To mimic more closely this situation in vitro, heterogeneous lipid suspensions (1:1 molar ratio mixtures of DMPC and POPC, or POPC and POPE) were prepared using standard protocols (see Materials and Methods). The mass spectrum of DMPC/POPE mixtures reveals a distribution of peaks (Fig. 6 *A*),

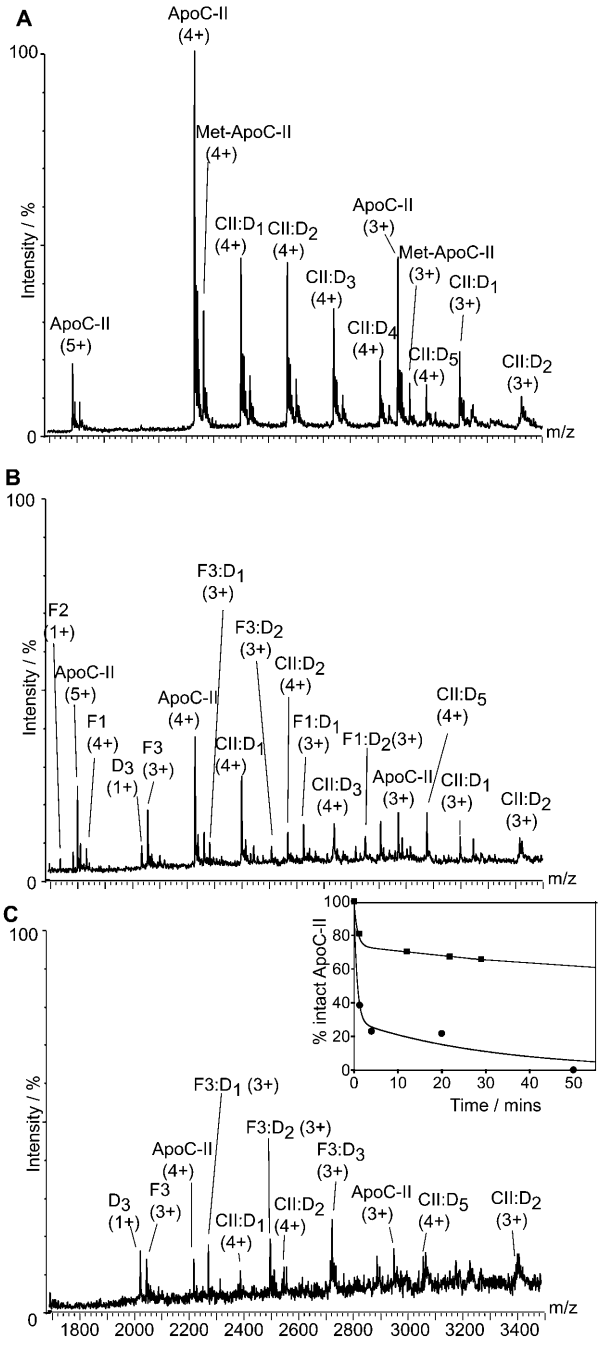

FIGURE 5 Chymotryptic digest of apoC-II in DMPC vesicles at $t = 0$ (*A*), after 12 min (*B*), and after 40 min (*C*). (*Inset*) The rate of digestion calculated from the intensity of the peaks in the mass spectra is plotted in the absence of vesicles (*circles*) and in the presence of DMPC vesicles (*squares*). Peptide fragments labeled F1 and F3 (see Fig. 1) bind up to two and three DMPC molecules, respectively.

which can be assigned to varying numbers of lipid molecules in relatively few charge states (in this case either the 1+ or 2+ charge states). These spectra also reveal the distribution of phospholipid molecules within each complex. If the interaction between the two different lipid molecules was random, a statistical, binomial distribution of lipids in each of the complexes would result; thus, for two lipid molecules in association, three peaks with relative intensities 1:2:1 would be anticipated. From Fig. 6 *A* it can be seen that for two molecules in complex the peak assigned to $(DMPC)_2$ is of greater intensity than those assigned to $DMPC_1POPE_1$ and $(POPE)_2$ (Table 2). As the number of lipid molecules in the complexes increases to five, however, the distribution approaches more closely that anticipated from statistical incorporation (Fig. 6 *B*; Table 2). This would suggest that POPE is found predominantly in larger complexes, and this may reflect its reduced solubility in aqueous suspensions. Similar observations were made for POPE/POPC interactions (Fig. 6 *C*). By contrast the distribution of peaks in the DMPC/POPC complexes is much closer to a statistical distribution even for bimolecular interactions. The observation presumably reflects their closely similar properties in aqueous suspensions.

When mixed with heterogeneous phospholipid preparations, apoC-II is exposed to two different phospholipid molecules. It should be possible therefore to study whether the protein is able to interact with different lipids simultaneously. To investigate the binding of apoC-II to these heterogeneous suspensions, complexes were prepared and spectra recorded under identical conditions to those described above. Interestingly it was not possible to observe protein/lipid binding interactions for the DMPC/POPC complexes. For DMPC/POPE, however, complexes of up to four POPE molecules binding to apoC-II are clearly identified in the 4+ and 5+ charge state series (Fig. 6 *D*). This is in contrast to the situation described above where preparations containing POPE alone showed no interaction with the protein. We conclude from these observations therefore that in the presence of DMPC, POPE does not precipitate, but rather is held in a configuration where its polar headgroup faces outwards toward the solvent, for surface binding of apoC-II.

We also investigated preparations of lipids formed by suspension in aqueous buffer but without prior dissolution in $CHCl_3$, since this was the method used to investigate interactions of apoC-II in homogeneous lipid suspensions. Interestingly the distribution of lipids within the complexes (Fig. 7) is different from those observed in Fig. 6. For the complex containing three lipid molecules, the distribution of lipids is found to be different to that predicted by random association (Table 2). Similarly, for a complex of seven lipid molecules associating randomly the highest intensity peaks should correspond to complexes containing either three POPE and four POPC, or vice versa. The most intense peaks are in fact those corresponding to seven molecules of either POPE or POPC (Table 2). This deviation from statistical behavior is consistent with a preference for the same phospholipid molecules to associate rather than to interact with molecules containing different headgroups or fatty acid side chains.

The mass spectra of apoC-II in complex with DMPC/POPC and POPE/POPC prepared without prior dissolution

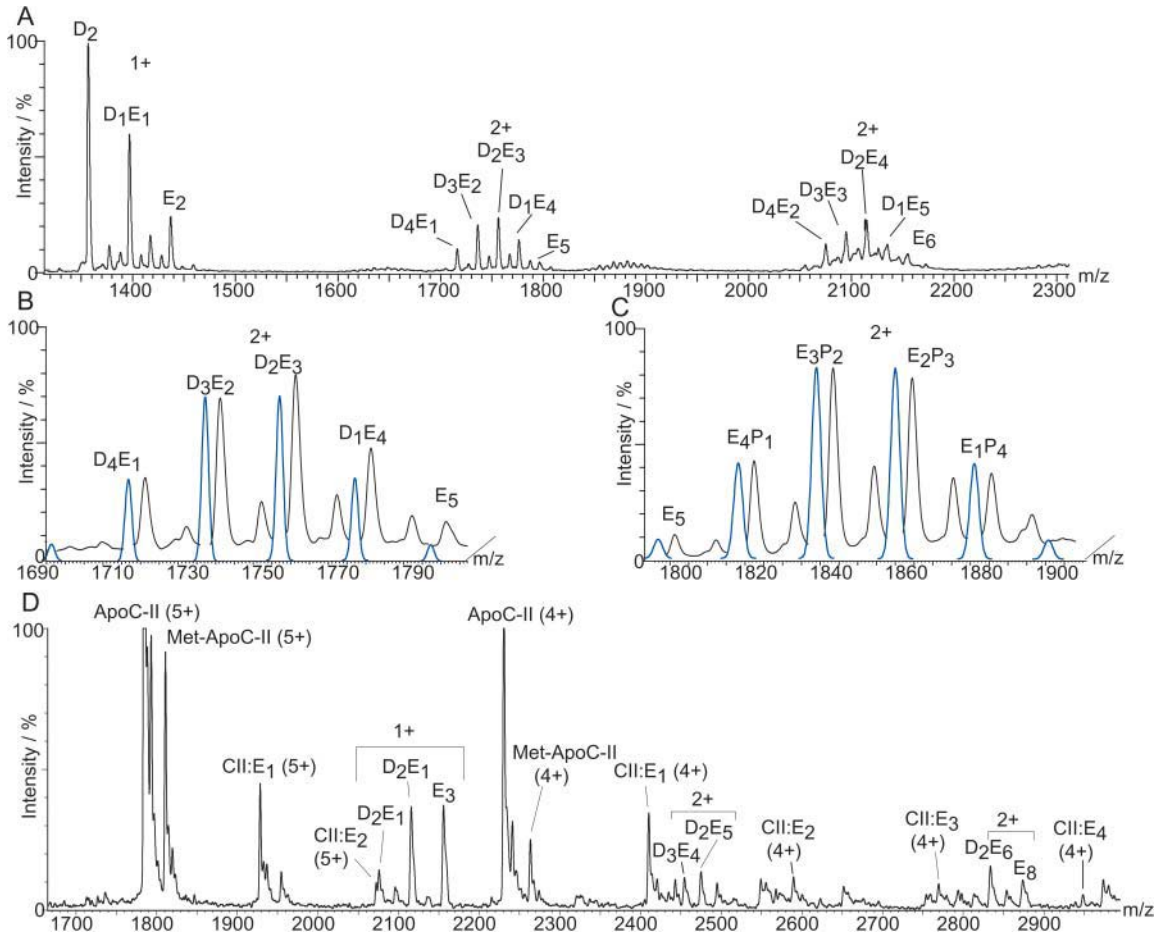

FIGURE 6 Electrospray mass spectra of complexes of heterogeneous lipid preparations formed from DMPC/POPE (*A* and *B*) and POPE/POPC (*C*) by dissolution of the dry powders in $CHCl_3$ and of complexes of apoC-II with heterogeneous lipid complexes formed from DMPC/POPE (*D*).

in $CHCl_3$ are shown in Fig. 8, *A* and *B*. As in spectra recorded for apoC-II with homogeneous lipid preparations, protein/lipid binding peaks are observed that correspond to the association of increasing numbers of lipids to a single protein molecule. In the presence of DMPC/POPC (Fig. 8 *A*) charge states 4+ and 5+ are present, with the latter having the highest intensity peaks for lipid binding to the protein. This is similar to the charge states observed in spectra of apoC-II with DMPC alone (cf. Fig. 4). Interestingly, despite the fact that apoC-II showed no interaction with homogeneous POPE molecules, for mixed phospholipid samples prepared either from $CHCl_3$ or by direct mixing (Figs. 6 and 8 *B*), interactions are clearly observed with POPE.

TABLE 1 ApoC-II fragments produced during proteolysis using chymotrypsin

Fragment number	Fragment residues	Mass/Da
1	1–63	7197
2	64–79	1736
3	1–53	6169
4	54–63	1046
5	38–48	1292
6	1–37	4234
7	38–53	1953
8	1–48	5508

It is also of interest to examine the distribution of lipid molecules in these complexes. Among the factors that determine this distribution are the tendency of identical lipids to interact and the affinity of the protein for the various lipid molecules. For apoC-II with DMPC and POPC, the peaks at m/z ~1400 can be assigned to the complexes $(DMPC)_2$, (DMPC/POPC), or $(POPC)_2$. For a statistical association of the lipid molecules in this bimolecular complex the ratio of intensities of the peaks would be 1:2:1. The experimentally determined ratio of intensities of the peaks is ~1:2.8:2 (Table 2). Similarly for the analogous complexes formed between POPE and POPC the ratio of intensities is ~1:2.4:2 (Table 2), indicating that the associations of the lipids are not random.

Close examination of the distribution of lipid molecules in the protein/lipid complexes reveals that for DMPC/POPC, apoC-II binding to one molecule of DMPC and one of POPC is more intense than peaks assigned to apoC-II binding to two molecules of the same lipid (Fig. 8 *D*). A similar situation is observed for apoC-II in complex with POPE/

TABLE 2 Distribution of peaks in lipid complexes formed under different conditions

Prep. of lipids	Number of lipid molecules	Statistical	Experimental DMPC/POPC	Experimental POPE/POPC	Experimental DMPC/POPE
1	2	1:2:1		1:4:8:7.5:3:0	5:3:1
	5	1:5:10:10:5:1			0:3:7:8:4:1
2	3	1:3:3:1		10:3.5:3:6	
	7	1:7:21:35:35:21:7:1		18:11:10:11:11:10:8:20	
3	2	1:2:1	1:3:2	2:3:1	
4	2	1:2:1	1:2.8:2	1:2.4:2	

Mixtures of lipids formed after dissolution in $CHCl_3$ (1), show a distribution closer to statistical as the size of the complex increases. From aqueous suspensions in the absence of apoC-II (2) the distribution is far from statistical. By contrast, in the presence of apoC-II the distribution is more similar to that predicted statistically, whether free in the spectrum (3) or bound to apoC-II (4).

POPC (Fig. 8 *D*). In both cases therefore the peaks of highest intensity are assigned to protein in complex with two different lipid molecules simultaneously. The protein/lipid associations therefore follow a statistical distribution to a greater degree than when the protein is absent (Table 2). Interestingly the ratio of peaks assigned to the DMPC/POPC /protein complex is closely similar to those measured for interactions of lipid molecules alone within the same spectrum. By contrast for POPE/POPC the ratios are different, the peak assigned to protein binding to $(POPE)_2$ being of higher intensity than the analogous peak for binding to $(POPC)_2$, whereas for the free lipids within the same spectrum the opposite is true. Thus the distribution of lipid molecules differs when attached to the protein compared with when free within the same spectrum. For the heterogeneous phospholipid suspensions examined here, therefore, the distribution of lipid molecules is not necessarily the same whether in contact with the protein or with other phospholipids.

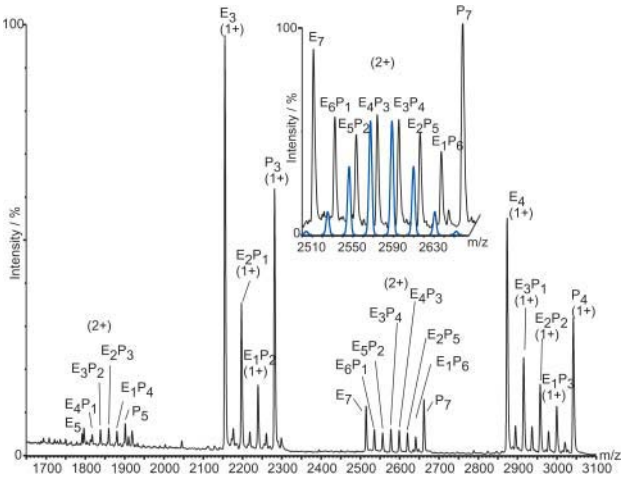

FIGURE 7 Electrospray mass spectrum of heterogeneous vesicles formed from equimolar ratios of POPE and POPC in aqueous suspensions. The distribution of lipids in the various complexes results from a tendency of the same lipid molecules to self-associate. (*Inset*) The distribution of species resulting from the formation of a complex of seven lipid molecules is compared with a theoretical binomial distribution assuming random association of lipid molecules in the bilayer. The intensity of the theoretical and experimental peaks is plotted against the *m*/*z* values of the peaks.

The second factor that is likely to play a part in the distribution of lipid molecules in heterogeneous suspensions is their propensity for binding to apoC-II. From Fig. 8 *C* it can be seen that the peak assigned to apoC-II/ $(POPC)_2$ is of higher intensity than the corresponding peak for DMPC. Similarly from Fig. 8 *D* it can be seen that apoC-II binding to two molecules of POPE is of greater intensity than that for binding to POPC. These results allow us to establish that apoC-II binds preferentially to POPE over POPC, and similarly that POPC binds preferentially over DMPC. Examination of the structures of these lipids allows us to conclude that lipids with an unsaturated side chain have a higher affinity for apoC-II than those with fully saturated hydrocarbon chains.

DISCUSSION

Human apolipoprotein C-II has been investigated in the presence of homogeneous and heterogeneous phospholipid preparations. The mass spectra of homogenous lipid suspensions demonstrate that complexes of large numbers of lipid molecules can be maintained within the mass spectrometer. For apoC-II in the presence of phospholipid suspensions the associations between protein and lipid can also be examined. This enabled us to probe the ability of the protein to bind to different phospholipid molecules. The results show that the number of lipid molecules that remain in association in the gas phase is significantly reduced when compared to spectra recorded for lipid suspensions in the absence of protein. Chymotryptic digestion of apoC-II in the presence and absence of phospholipid suspensions reveals the exposed regions of the protein. For heterogeneous lipid suspensions the results show a strong tendency for lipids of identical structure to form complexes. This property is significantly reduced in the presence of apoC-II, when a distribution closer to that predicted from random associaton of lipid molecules is observed. This result is in accord with the finding for homogeneous lipid suspensions where protein binding to the phospholipid suspensions disrupts the lipid packing forces, leading to significantly reduced numbers of interacting lipid molecules.

It is interesting to discuss whether the associations of apoC-II with phospholipid suspensions, as well as the lipid/

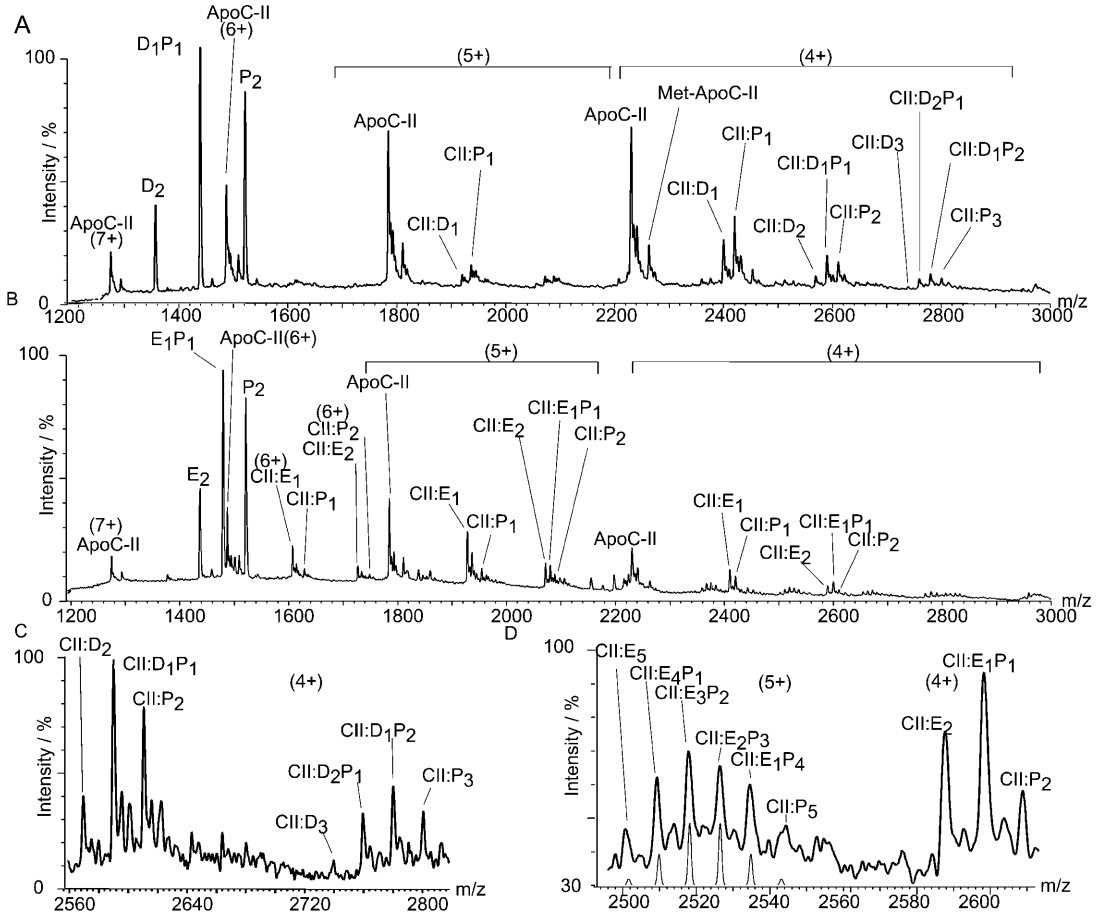

FIGURE 8 Electrospray mass spectra of complexes of apoC-II with heterogeneous lipid vesicles formed from DMPC/POPC (*A*) and POPE/POPC (*B*). The peaks assigned to protein and lipid molecules confirm that apoC-II is able to bind simultaneously to both lipids. Expansion of the peaks assigned to apoC-II bound to DMPC/POPC (*C*) and POPE/POPC (*D*) reveals that the distribution of lipid molecules in these complexes approaches a statistical distribution.

lipid associations themselves, reflect solution phase associations. For protein complexes, the overwhelming evidence of stoichiometry of interacting subunits identical to that defined by other techniques provides compelling evidence that noncovalent complexes that are observed in mass spectra under the appropriate conditions are those that exist in solution (Sobott et al., 2002). Several lines of evidence in this study support the proposal that the protein/lipid associations observed here can also be related to those occurring in solution. The first line of evidence comes from the fact that for lipid/lipid associations there is a tendency to form complexes containing the same lipid molecules. The fact that this preference is most likely a solution phase phenomenon that is clearly preserved in mass spectra supports the assertion that these solution phase interactions are maintained in the gas phase. The second line of evidence comes from proteolysis experiments with lipid binding to peptides generated from the proposed lipid binding domain and the absence of peaks corresponding to lipid molecules binding to the C-terminal region. Up to three lipid molecules are observed in complex with the peptide 1–63, whereas only two are observed interacting with the shorter fragment 1–53 (see Fig. 1 for locations of these residues). The third experimental evidence comes from the inability of a protein of closely similar properties to give rise to peaks in mass spectra that can be assigned to protein/lipid interactions. The final evidence in support of our assertion that these associations are derived from specific solution phase complexes comes from the interactions of the protein with mixed phospholipid suspensions. Here the favorable associations observed for the same lipid molecules are no longer maintained. The binding of the protein results in disruption of ordered suspensions such that the protein interacts with lipid molecules in such a way that the distribution approaches that predicted statistically. Such a distribution is also observed for lipid peaks not bound to the protein but present in the same solution as protein/lipid complexes. The deviations between the ratio of peaks in the lipid complexes and the protein/lipid complexes presumably arise from local and long range effects of protein binding to the lipid surface. The preferred association of identical lipid molecules in the absence of protein, lipid binding to peptides derived from the lipid binding domain, disruption of ordered suspensions in the presence of protein, and the failure of a protein with

similar characteristics to bind lipid molecules taken together provide compelling evidence that the associations observed in the mass spectra reflect, at least in part, the associations that are occurring in solution. The question now arises as to the extent these associations are preserved.

The approximate number of molecules in a DMPC bilayer vesicle of radius 100 nm has been calculated as ~568,000 molecules (Hanson, 2002). These interactions are held together principally by weak van der Waals forces. In this study the precise nature of the lipid complexes is unknown, but as the assemblies are transferred from solution to gas phase the internal energy of the ions increases such that disruption of these forces could occur, leaving only a fraction of the associations intact (50–100 lipids, or ~1.5%). For the complexes formed between protein and lipid up to 22 POPC molecules are observed in complex with apoC-II in the gas phase. The results from the chymotryptic digestion suggest however that up to three DMPC molecules are in direct contact with apoC-II. The interactions between protein and the 22 lipid molecules are presumably mediated through lipid/lipid as well as protein/lipid interactions, maintained by weak van der Waals forces. From the evidence presented here we propose therefore that the mass spectra recorded for phospholipid suspensions and their complexes with apoC-II represent a portion of the lipid assemblies rather than random associations through aggregation of individual lipid and protein molecules.

It is interesting to consider the findings of this study and their implications for the binding of apoC-II to lipid molecules. It is well established that the types of phospholipid present influence the bending rigidity of a phospholipid bilayer as well as the protein topology (Bogdanov et al., 2002), and that the distribution of lipids in a bilayer influences the folding stability of membrane proteins (Booth et al., 1997). The phosphoethanolamine headgroup decreases bilayer rigidity and increases curvature stress compared to the phosphocholine headgroup. POPE therefore increases curvature stress within a bilayer relative to POPC. The ability of apoC-II to bind POPE in mixed preparations is interesting, because in mass spectra of apoC-II/POPE alone, no peaks could be assigned to POPE or its complexes or to apoC-II/POPE. This is presumably because POPE and other unsaturated phosphatidylethanolamines have packing parameters greater than 1 (Janes, 1996), which results in an inverted cone shape and a tendency for formation of inverted micelles in organic solvent. In aqueous solvent POPE aggregates and precipitates, and apoC-II therefore cannot bind. Both DMPC and POPC have packing parameters between 0.5 and 1, a range of values known to drive the formation of bilayers. Although the lipid suspensions studied in our system are not homogeneous bilayers, these factors may still influence the packing of the phospholipids in these suspensions. The presence of POPC therefore induces POPE to bind to the protein, because in mixed systems it precipitates. When mixed lipid solutions are prepared using $CHCl_3$, POPE is the only lipid observed binding to the protein, and when $CHCl_3$ is not used, POPE binds to apoC-II to a greater degree than do POPC and DMPC, suggesting that POPE interacts favorably with apoC-II. This may reflect the preference of apoC-II to associate with POPE in vivo; although the protein is not associated with phospholipid suspensions in vivo, but rather with lipid monolayers on lipoproteins, it may bind to lipid surfaces with a similar degree of curvature stress to that in POPE suspensions. The preferential binding of apoC-II to POPE implies that, in the system studied, POPE binds as a well-defined cluster or microdomain. Microdomains in general can be functionally important structures in vivo, for example in the context of ''lipid rafts,'' localized regions of elevated cholesterol and glycosphingolipid content within cell membranes, known to be involved in cell signaling (Pike, 2003). Whether or not the microdomain formed by POPE is physiologically relevant, our results not only provide new insight into lipid binding interactions with apoC-II, but also serve to demonstrate the potential for mass spectrometry to compliment existing biophysical approaches.

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