Determination of the Stability of the Noncovalent Phospholipid Transfer Protein−Lipid Complex by Electrospray Time-of-Flight Mass Spectrometry

A. P. M. de Brouwer,[‡] C. Versluis,[§] J. Westerman,[§] B. Roelofsen,[‡] A. J. R. Heck,[§] and K. W. A. Wirtz*,[‡]

Department of Biochemistry of Lipids, Institute of Biomembranes, Padualaan 8, 3584 CH Utrecht, The Netherlands, Department of Biomolecular Mass Spectrometry, Utrecht Institute of Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands

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ABSTRACT: Phosphatidylcholine transfer protein (PC-TP) containing different molecular species of PC and phosphatidylinositol transfer protein α (PI-TPα) containing either a PI, PC, or PG molecule were identified as intact complexes by nano-electrospray ionization time-of-flight mass spectrometry. The stability of these complexes in the gas phase was determined by elevating the cone voltage (cv) resulting in the appearance of the protein void of lipid. PC-TP containing a PC species carrying an *sn*-1 palmitoyl chain was less stable than PC-TP containing a PC species carrying an *sn*-1 stearoyl chain given that these complexes were dissociated for 50% at a cv of roughly 30 and 45 V, respectively. Different acyl chains on the *sn*-2 position did not lead to significant changes in stability of the complex. In the case of PI-TPα, the complexes containing PI and PG were dissociated for 50% at a cv of 100 V as compared to a cv of 40 V for the complex containing PC. We propose that this difference in stability is due to hydrogen bonds between the polar headgroup of PI and PG and the lipid-binding site of PI-TPα. This may explain why PI-TPα preferentially binds PI from a membrane interface.

Phospholipid transfer proteins are a group of water-soluble proteins, which are able to transfer phospholipids between membranes (*1*). Two well-characterized proteins in mammalian tissues are the phosphatidylcholine transfer protein (PC-TP)[1] specific for PC (*2, 3*) and the phosphatidylinositol transfer protein (PI-TP), which displays a marked preference for PI, but is also able to transport PC and PG (*4*−*7*). Although there is no sequence homology, the lipid-binding sites of these phospholipid transfer proteins show clear structural similarities (*8*−*10*). Phospholipids bound to transfer proteins reside in an enclosed hydrophobic cavity completely shielded from the medium (*8, 10*−*15*). The phospholipid polar headgroup interacts with charged amino acid residues inside this cavity, whereas the two acyl chains are accommodated in different hydrophobic pockets.

Until recently, water-soluble lipid−protein complexes could only be studied by mass spectrometry by analyzing the separate components. Normally the dissociation of these complexes results in the denaturation of the protein and a loss of information about the interaction with the lipid molecule. Electrospray mass spectrometry has been used to study noncovalent interactions between proteins and metals, ligands, peptides, oligonucleotides, DNA, and other proteins (*16*−*19*). Here we report the analysis of the noncovalent complexes of PI-TPα and PC-TP with phospholipids in the gas phase by nano-electrospray ionization time-of-flight mass spectrometry (ESI-TOF). Elevation of the voltage of the sample cone at the entrance of the mass spectrometer results in a collision activation and thus in a higher internal energy of the gas-phase ions. This higher energy leads to dissociation of the transfer protein−phospholipid complexes. In agreement with the ability of PI-TPα to preferentially extract PI from a membrane interface, the PI/PI-TPα complex was much more stable than the PC/PI-TPα complex.

EXPERIMENTAL PROCEDURES

Materials. PC-TP was isolated from bovine liver as described by Westerman et al. (*20*). Recombinant mouse PI-TPα was obtained as described by Bouma et al. (*21*). PD-10 columns were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). 10 and 100 kDa molecular mass cutoff filters were obtained from Amicon (Beverly, MA). Egg yolk PC, C16:0/C18:1-PC, C16:0/C20:4-PC, C18:0/C18:1-PC, C18:0/C20:4-PC, and bovine liver PI were purchased from Sigma (St. Louis, MO).

Preparation of Phospholipid Transfer Protein Complexes. PC and PI (1 μmol) were dried under nitrogen and dissolved in 40 μL of ethanol. Small unilamellar vesicles were prepared by injection of this solution in 10 mM Tris-HCl, pH 7.4, 10 mM β-mercaptoethanol (in the case of PC-TP) or in the same buffer containing 250 mM $MgCl_2$ (in the case of PI-TPα) (*22*). PC-TP (10 nmol) in 10 mM Tris-HCl, pH 7.4, 10 mM β-mercaptoethanol (1.5 mL) was incubated with the PC

* Address correspondence to this author at the Department of Biochemistry of Lipids, Institute of Biomembranes, Padualaan 8, 3584 CH Utrecht, The Netherlands. Tel: +31-30-2533443; Fax: +31-30-2533151; E-mail: k.w.a.wirtz@chem.uu.nl.

‡ Institute of Biomembranes.

§ Utrecht Institute of Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research.

[1] Abbreviations: cv, cone voltage; ESI-TOF, electrospray ionization time-of-flight mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; PC-TP, phosphatidylcholine transfer protein; PI-TPα, phosphatidylinositol transfer protein α; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid.

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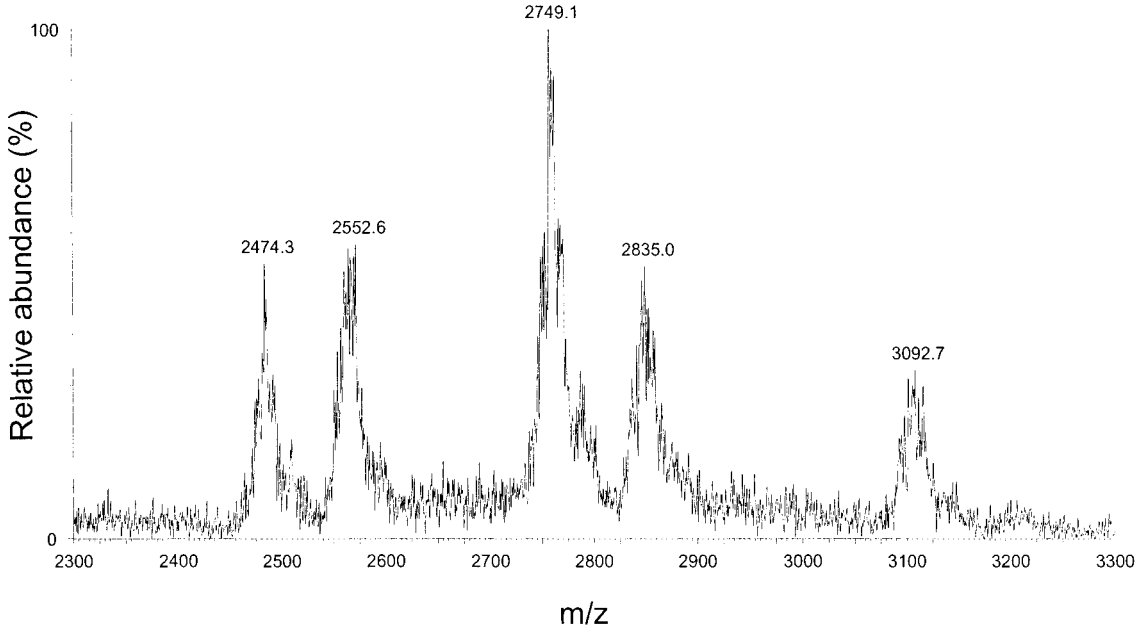

FIGURE 1: Nano-ESI-TOF mass spectrum of the C18:0/C18:1-PC/PC-TP complex. The cone voltage is set at 50 V dissociating approximately half the complex. PC-TP void of PC is visible at *m*/*z* 2474 ($[M+10H^+]^{10+}$), 2749 ($[M+9H^+]^{9+}$), and 3092 ($[M+8H^+]^{8+}$). The complex can be found at *m*/*z* 2552 ($[M+10H^+]^{10+}$) and 2835 ($[M+9H^+]^{9+}$).

vesicles for 1.5 h at room temperature. PI-TPα (8 nmol) was incubated with PI vesicles under the same conditions in the presence of 250 mM $MgCl_2$. The proteins were separated from the vesicles using a 100 kDa cutoff filter. A high concentration of $MgCl_2$ was required so as to ensure an efficient separation of PI-TPα from the PI vesicles. $MgCl_2$ was removed from the run-through by using a PD-10 column.

Electrospray Ionization Time-of-Flight Analysis of Protein−*Lipid Complexes.* Prior to analysis, PC-TP and PI-TPα were concentrated to 2 mg/mL on 10 kDa cutoff filters and then diluted 10 times with 50 mM ammonium acetate, pH 7.4, to a final concentration of approximately 7 μM. Mass spectra were recorded using a quadrupole time-of-flight instrument (Micromass Ltd., Manchester, U.K.) operating in the positive ion mode equipped with a nano-electrospray source. The potential between the nano-electrospray needle and the sample cone was set at 1500 V, and the cv was varied between 20 and 120 V. Nano-electrospray needles were made from borosilicate glass capillaries with a P-97 puller (Sutter Instrument Co., Novato, CA, USA). Needles were gold-coated using an Edwards Scancoat Six sputter coater (Crawley, U.K.). The percentage of complex remaining at a fixed cv was calculated by the following formula: 100 × [(area of complex peaks combined)/(area of complex peaks combined + area of protein void of phospholipid peaks combined)].

Lipid Analysis by Fast Atom Bombardment Mass Spectrometry. Lipids were extracted from recombinant PI-TPα by the method of Bligh and Dyer (*23*). The lipid extracts were analyzed by fast atom bombardment and tandem mass spectrometry (FAB-MS) as described by Geijtenbeek et al. (*24*).

RESULTS

ESI-TOF Analysis of the Phosphatidylcholine Transfer Protein. Nano-electrospray ionization time-of-flight mass spectrometry was used to analyze the intact C18:0/C18:1-PC/PC-TP complex (Figure 1). At a cv of 50 V, free PC (*m*/*z* 788), three peaks representing the $[M+10H^+]^{10+}$ (*m*/*z* 2474), $[M+9H^+]^{9+}$ (*m*/*z* 2749), and $[M+8H^+]^{8+}$ (*m*/*z* 3093) of the protein void of PC, and two peaks representing the $[M+10H^+]^{10+}$ (*m*/*z* 2553) and $[M+9H^+]^{9+}$ (*m*/*z* 2835) of the PC/PC-TP complex were detected. The difference in average mass calculated from the three charged states of the PC/PC-TP complex (25 520 ± 4 Da) and the free protein (24 737 ± 5 Da) correlates with the mass of C18:0/C18:1-PC (788 Da). Close examination of the ion signals revealed several additional peaks. Hence, to facilitate the interpretation of the data, the charge-state spectrum was deconvoluted to a neutral mass spectrum using MaxEnt (Figure 2). The peaks representing the complex and the free protein were clearly separated. Each peak was composed of three major peaks, each of which again was composed of three minor peaks. The difference between each of the major peaks is about 76 Da, indicating that most likely β-mercaptoethanol has reacted with a cysteine of PC-TP (*25*). The presence of this reaction product also explains the mass of the first major peak (i.e., 24 737 Da), which differs approximately 76 Da from the calculated mass of PC-TP (i.e., 24 655 Da). The difference in mass (22 Da) between the minor peaks can be accounted for by the presence of sodium. As shown in Figure 2, the dissociation of the C18:0/C18:1-PC/PC-TP complex increases with the voltage applied to the cone. By increasing the voltage from 20 to 90 V, the dissociation of the complex increased from 15 to 100% (panels A−D).

Interaction of PC Molecular Species with PC-TP. The stability of PC-TP containing C16:0/C18:1-PC, C16:0/C20:4-PC, C18:0/C18:1-PC, and C18:0/C20:4-PC was examined by ES-TOF analysis. The areas of the combined peaks were used to follow the dissociation of the complex as a function of cv. As shown in Figure 3, PC-TP containing PC species carrying a C16:0 fatty acid on the *sn*-1 position was less stable than PC-TP containing PC species carrying a C18:0 fatty acid. At a cv of 35 V, 50% of the C16:0/C18:1-PC/

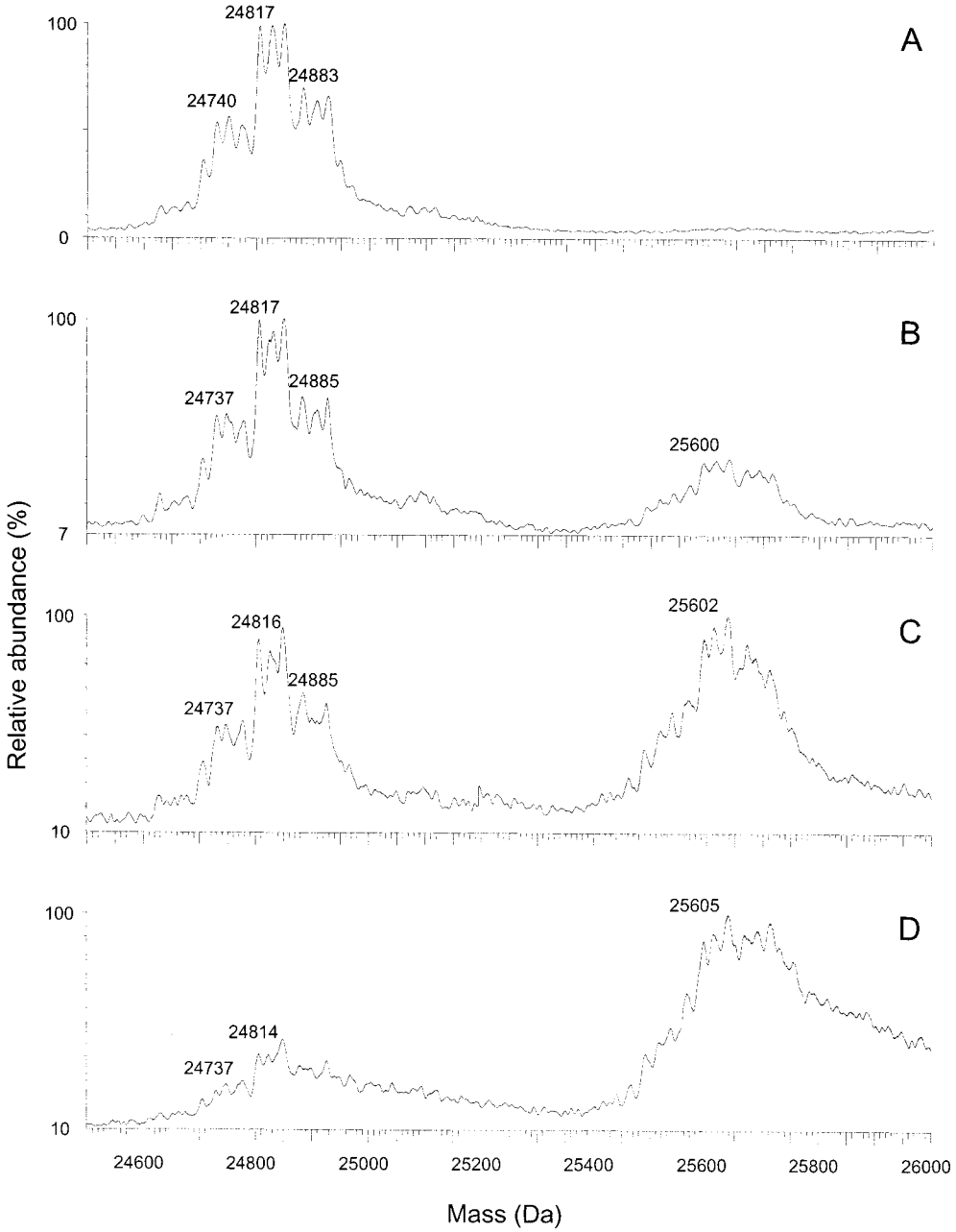

FIGURE 2: Transformed data from nano-ESI-TOF mass spectra of the C18:0/C18:1-PC/PC-TP complex. Shown is the dissociation of the PC/PC-TP complex at a cone voltage of 90 (A), 50 (B), 40 (C), and 20 V (D). The peak at 24 817 Da represents the protein void of PC and the peak at 25 602 Da the complex.

PC-TP complex was dissociated, while a cv of 45 V was required to dissociate half of the C18:0/C18:1-PC/PC-TP (Figure 3A). In the case of C16:0/C20:4-PC, the required cv was 25 V as compared to 40 V for C18:0/C20:4-PC (Figure 3B). Taking all cv's into consideration, the acyl chain on the *sn*-1 position (C16:0 or C18:0) has a significant effect on the stability of the complex; this does not seem to be the case for the acyl chain on the *sn*-2 position (C18:1 or C20:4).

ESI-TOF Analysis of Recombinant Phosphatidylinositol Transfer Protein α. Analysis by ESI-TOF was carried out on recombinant PI-TPα as well. This protein contains PG as shown by Geijtenbeek et al. (*26*). The average mass of PG/PI-TPα is 31 764 ± 1 Da, which is almost identical to the calculated molecular mass of 31 762 Da (*26*). The difference between the complex and the empty protein is about 735 Da, which concurs with the average molecular mass of the PG molecular species bound (see below). PG species cannot be directly identified by positive mode ESI-TOF since PG is negatively charged. Analysis of the lipids extracted from recombinant PI-TPα, by negative ion FAB-MS, showed the presence of two major high-mass anions at *m*/*z* 733 (80%) and 759 (20%) (Figure 4D). By tandem mass spectrometry, the molecular ion at *m*/*z* 733 could be identified as C16:0/ΔC17:0-PG and the molecular ion at *m*/*z* 759 as C18:1/ΔC17:0-PG.

Interaction of Phospholipids with PI-TP. As shown in Figure 4C, the PG/PI-TPα complex (average mass of 32 500 Da) was nearly completely intact at a cv of 70 V. This was in striking contrast with the PC/PC-TP complex, which was dissociated for about 80% at this voltage (Figure 3). Increasing the voltage to 120 V showed that the dissociation between PG and PI-TPα, as apparent from the appearance

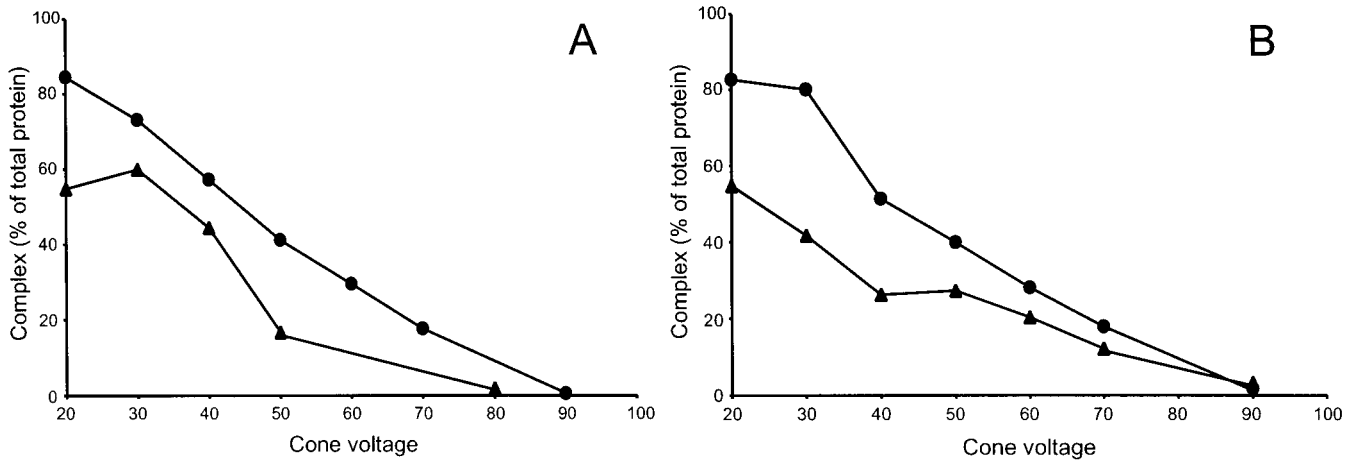

FIGURE 3: Dissociation of PC/PC-TP complexes caused by elevation of the cone voltage. Panel A: dissociation of C18:0/C18:1-PC/PC-TP (circles) and C16:0/C18:1-PC/PC-TP (triangles). Panel B: dissociation of C18:0/C20:4-PC/PC-TP (circles) and C16:0/20:4-PC/PC-TP (triangles). The data are representative for a series of measurements.

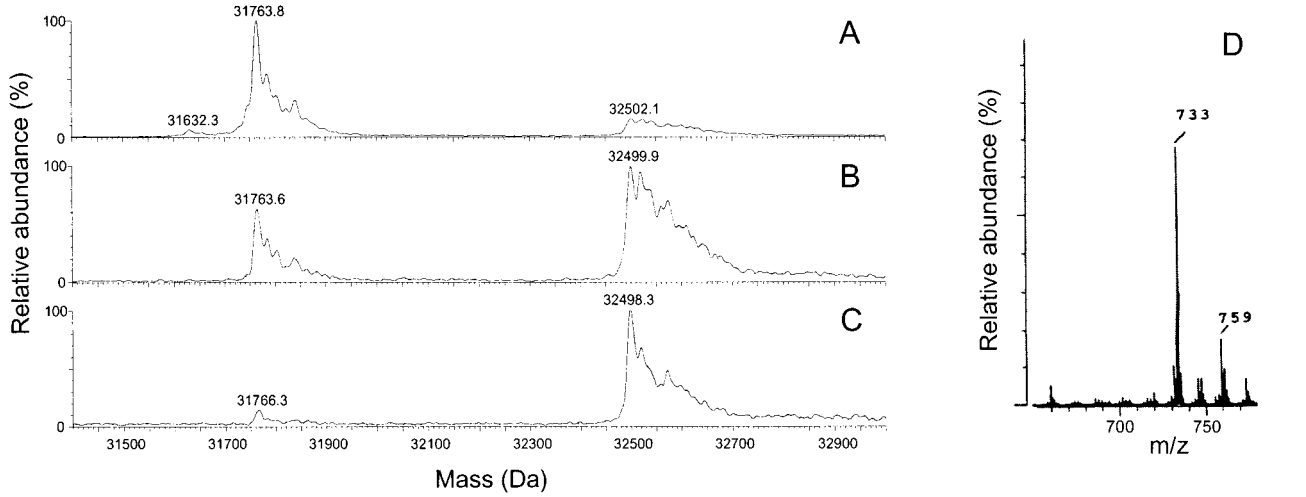

FIGURE 4: Transformed data from nano-ESI-TOF mass spectra of the PG/PI-TPα complex. Panels A−C: dissociation of the PG/PI-TPα complex at a cone voltage of 120 (A), 100 (B), and 70 V (C). The peak at 31 764 Da represents protein void of PG and the peak at 32 500 Da the complex. Panel D: negative-ion FAB mass spectrum of the Bligh and Dyer extract from recombinant PI-TPα. By tandem mass spectrometry using charge-remote dissociation, the peak at *m*/*z* 733 was identified as C16:0/ΔC17:0-PG and the peak at *m*/*z* 759 as C18:1/ΔC17:0-PG.

of a peak at a molecular mass of 31 763 Da, was not yet complete. At this high cv, an additional peak at 31 632 Da was observed (Figure 4A). The difference between PI-TPα void of PG and this peak is approximately 131 Da, most likely indicating the loss of the N-terminal Met residue (mass of 131 Da) or the C-terminal Asp residue (mass of 133 Da). The present measurements do not allow one to distinguish between these two residues, but do reveal that the noncovalent interaction between PG and PI-TPα in the gas phase is very strong and approaches that of a peptide bond. Since PI-TPα from mammalian cells carries either a PI or a PC molecule (*5*, *27*, *28*), we have analyzed these complexes as well. As shown in Figure 5, PI and PG dissociated from PI-TPα at a relatively high cv, whereas PC dissociated at a cv comparable to that observed for the PC/PC-TP complex. Cv's of 55, 110, and 115 V were required to dissociate half of the PC-, PG-, and PI/PI-TPα complexes, respectively. This difference shows that in the gas phase the negatively charged phospholipids interact much stronger with the lipid-binding site than the zwitterionic PC. Analysis of PI-TP complexes

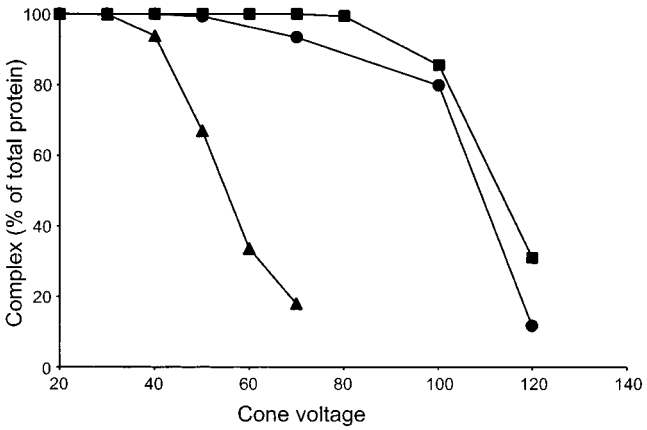

FIGURE 5: Dissociation of PC/PI-TPα (triangles), PG/PI-TPα (circles), and PI/PI-TPα (squares) complexes caused by elevation of the cone voltage.

containing either egg yolk PC, C16:0/C18:1-PC, or C18:0/C18:1-PC indicated that the dissociation was not affected by the fatty acid composition (data not shown).

DISCUSSION

Here we have demonstrated that PC-TP and PI-TPα complexed to phospholipid monomers can be detected by nano-electrospray ionization time-of-flight mass spectrometry. As far as we know, this is the first time that these intact complexes were analyzed by mass spectrometry. The gas-phase stability of these complexes was studied by adjusting the voltage applied to the cone. The intact protein−lipid complex could easily be distinguished from the protein void of phospholipids. Being a zwitterionic phospholipid, PC was also identified by ESI-TOF. However, since ESI-TOF was used in the positive ion mode to identify proteins in a protonated state, negatively charged phospholipids, such as PI and PG, could not be detected.The interaction of PC with PC-TP differed between PC species. C16:0/C18:1-PC and C16:0/C20:4-PC were released at a lower cv than PC species containing a C18:0 acyl chain on the *sn*-1 position. On the other hand, the release was barely affected by the fatty acid on the *sn*-2 position. This may indicate that the all-trans *sn*-1 acyl chain has a stronger interaction with the binding site than the *sn*-2 acyl chain containing cis-double bonds. Given that specific binding sites exist for the *sn*-1 and *sn*-2 acyl chains (*29*), it appears that the *sn*-1 saturated acyl chain dictates the stability of the complex more prominently than the *sn*-2 unsaturated acyl chain. Previously, by measuring the equilibration of PC between protein and membrane interface, PC-TP was found to more readily bind PC species carrying a C16:0 acyl chain than a C18:0 acyl chain (*30*). This difference in apparent affinity reflects the rates at which the PC monomer associates and dissociates from PC-TP and the membrane interface in the collision complex between PC-TP and the membrane. Thus, it appears that at the membrane interface PC-TP exchanges the C16:0 PC species more readily than the C18:0 PC species, yielding a higher apparent association constant. This is an agreement with our observations that in the gas phase the C16:0 PC species can leave the protein more easily than the C18:0 PC species.

In general, the phospholipid transfer protein complexes investigated in the present study do not dissociate in solution. One of the main reasons of this 'infinite' association is that the phospholipid bound to the protein is accommodated in a hydrophobic pocket shielded from the medium (*10*). Upon interaction with a membrane interface, the protein undergoes a conformational change such that the bound phospholipid can exchange for a molecule from the membrane (*31*). The rate at which this molecular exchange occurs is controlled by the association of the phospholipid molecule with the protein (hydrophobic/van der Waals for the acyl chains, polar/hydrogen bonding for the polar headgroup) and by the association of the phospholipid molecule with neighboring molecules in the membrane (hydrophobic/van der Waals). This implies that the actual binding constant of the phospholipid transfer protein complex cannot be determined under these molecular exchange conditions. Hence, the dissociation of these complexes in the gas phase as a function of the cone voltage gives information about interaction forces between lipid and protein (both van der Waals and polar and hydrogen bonding) that cannot be obtained by any other technique used to date. At this stage, this information is of a rather qualitative nature.

Apart from the acyl chains, the polar headgroup greatly determines the interaction of the phospholipid with the lipid-binding site both in solution and in the gas phase. This was clearly shown for PI-TPα, as the stability of the PG- and PI-containing complex was much higher than that of PC/PI-TPα. In earlier experiments, the phospholipid specificity of PI-TPα was determined by a phospholipid transfer assay (*4*, *6*, *32*, *33*) or by a competition binding assay (*28*, *34*). From these studies, it was gathered that PI-TPα binds PI about 16-fold better than PC or PG. However, ESI-TOF analysis indicates that the interaction between PI-TPα and PI or PG is about as strong, whereas the interaction with PC is much weaker. From recent X-ray data, it could be deduced that PI-TPα has separate binding sites for the phosphorylinositol and phosphorylcholine headgroup moieties (*31*). In contrast to the choline moiety, accommodation of the *myo*-inositol moiety in the binding site was stabilized by charged and polar amino acid residues (i.e., Thr59, Lys61, Glu86, Asn90) acting as potential hydrogen bonding partners to the hydroxyls. This may explain why the PI/PI-TPα and PG/PI-TPα complexes are much more stable than the PC/PI-TPα complex. In the gas phase, the interaction of PI and PG with PI-TPα is very strong and approaches that of a peptide bond.

In interpreting the above results, it should be noted that the dissociation of the complexes is carried out in the gas phase, whereas normally these complexes reside in an aqueous environment. We believe, however, that the information obtained is highly relevant, since phospholipids bound to the transfer proteins are present in a hydrophobic environment completely shielded from water (*8*, *10*, *15*). In summary, we have shown that intact water-soluble protein−lipid complexes can be studied by ESI-TOF. This may open also new perspectives for studying more complicated lipid−protein complexes, like serum lipoproteins.

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