Role of Phospholipids in the Protein Stability of an Insect Lipoprotein, Lipophorin from *Rhodnius prolixus*†

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ABSTRACT: Lipophorin (Lp) is the major lipoprotein in insect hemolymph. The structural organization proposed for Lp is basically the same as that suggested for vertebrate lipoproteins, consisting of a hydrophobic core containing neutral lipids, stabilized in the aqueous environment by surrounding polar moieties of protein and phospholipids at the particle surface. After complete removal of phospholipids from Lp by phospholipase A_2, the particle remains soluble [Gondim, K. C., Atella, G. C., Kawooya, J. K., & Masuda, H. (1992) *Arch. Insect Biochem. Physiol. 20*, 303−314]. However, studies on the roles of phospholipid on the structural stability of Lp are still lacking. In the present work, we have studied the structure and stability of dephospholipidated lipophorin (d-Lp). Trypsinolysis of d-Lp indicated no exposure of new cleavage sites on the protein when compared to Lp. However, an enhanced rate of proteolysis of the apoproteins (especially apolipophorin II) was observed in d-Lp. Circular dichroism analysis indicated that the secondary structure of Lp was not significantly affected by phospholipid removal. Furthermore, the exposure of tryptophan residues to the aqueous solvent in d-Lp was the same as in Lp, as indicated by intrinsic fluorescence emission spectra and fluorescence quenching experiments. Interestingly, d-Lp was more resistant to denaturation by guanidine hydrochloride than Lp. d-Lp was also found to be less sensitive than Lp to structural changes induced by hydrostatic pressure. Taken together, these results indicate that, although changes in its structural organization were subtle, dephospholipidated lipophorin may have additional protein−protein and/or protein−neutral lipid interactions that are responsible for the observed increase in stability. Therefore, phospholipids are not only not essential for Lp stability, but their presence in the particle seems to result in a less stable structure in the aqueous environment.

Lipid transport in the extracellular aqueous medium is limited by the hydrophobicity of these compounds. Lipid packing into lipoproteins creates a soluble particle, thus allowing transport of large amounts of lipids. The solubility of lipoprotein particles is thought to be accomplished in part by the localization of phospholipids (PL)[1] at the lipid−water interface (Lux *et al.*, 1972; Morrisett *et al.*, 1975; Edelstein *et al.*, 1979; Atkinson & Small, 1986; Gotto *et al.*, 1986), thus burying neutral lipids in the particle core.

Differently from mammalian plasma, where several lipoproteins can be found with distinct apoprotein and lipid compositions, lipophorin (Lp) is the single lipoprotein responsible for lipid transport in insect hemolymph. Lp is composed of two nonexchangeable apoproteins, apolipophorin I (apoLp-I) (240 kDa) and apolipophorin II (apoLp-II) (80 kDa), present in a 1:1 molar ratio (Chino *et al.*, 1969; Chino & Kitazawa, 1981; Soulages & Wells, 1994b; Blacklock & Ryan, 1994). In some species, an exchangeable apoprotein is present, apoLp-III (17 kDa), and its stoichiometry depends on the physiological state of the insect: particles with higher neutral lipid content (low-density lipophorins) have a higher apoLp-III content (van der Horst *et al.*, 1981; Kawooya *et al.*, 1984; Ryan & Law, 1984; Wells *et al.*, 1985; Chino & Yazawa, 1986). The high-density Lp (HDLp) of adult *Rhodnius prolixus* has 32% neutral lipids (mainly diacylglycerol) and 16% PL by weight (Gondim *et al.*, 1989a). This composition is similar to HDLp described in other insect species (Chino *et al.*, 1981; Ryan *et al.*, 1986; Rimoldi *et al.*, 1991). When compared to mammalian lipoproteins, a distinctive feature of Lp is that it acts as a reusable shuttle for carrying lipids between insect organs (Chino *et al.*, 1977; Beenakkers *et al.*, 1981; van der Horst *et al.*, 1981; Wheeler *et al.*, 1984; Downer & Chino, 1985; Kawooya & Law, 1988; Soulages & Wells, 1994b; Blacklock & Ryan, 1994). Insect Lp is multifunctional, transporting several classes of lipids including diacylglycerol (Chino *et al.*, 1969; van der Horst *et al.*, 1981), sterols (Chino & Gilbert, 1971), and hydrocarbons (Katase & Chino, 1982). Phospholipids are also transported by Lp, being loaded at the fat body or midgut and transferred to several tissues, such as ovaries or midgut (Thomas & Gilbert, 1967; Gondim *et al.*, 1989b; van Heusden *et al.*, 1991; Atella *et al.*, 1992, 1995; Machado *et al.*, 1996).

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[1] Abbreviations: Lp, lipophorin; d-Lp, dephospholipidated lipophorin; PL, phospholipid(s); HDLp, high-density lipophorin; apoLp-I, apolipophorin I; apoLp-II, apolipophorin II; apoLp-III, apolipophorin III; HDL, high-density lipoprotein; HDL_3, high-density lipoprotein 3; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, 10 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4; GdnHCl, guanidine hydrochloride.

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The structure of Lp from various insects has been investigated in order to determine the localization of lipid and protein moieties in the particle. Diacylglycerol is located predominantly in the hydrophobic core (Soulages & Wells, 1994a). PL are known to be located at the surface of the particle (Katagiri, 1985; Katagiri *et al.*, 1987). Interestingly, after complete PL removal by treatment with phospholipase A_2, the Lp particle remains soluble in the aqueous medium, and the capacity for lipid transport is also retained in both *Rhodnius prolixus* and *Manduca sexta* (van Heusden *et al.*, 1991; Kawooya *et al.*, 1991; Gondim *et al.*, 1992; Atella *et al.*, 1995). However, it was not clear (from those studies) if phospholipids had a positive contribution to protein stabilization in the Lp particle, as expected from most lipoprotein studies. These observations, along with the fact that PL are also transportable lipids, point to the need for investigation of their contribution to Lp structure. In this work, the structural stability of dephospholipidated *Rhodnius prolixus* Lp was studied by several techniques, including limited proteolysis, fluorescence spectroscopy, circular dichroism, and structural perturbation by chaotropic agents and hydrostatic pressure. Comparison between the stabilities of Lp and d-Lp suggests that PL have a destabilizing role in the Lp particle.

MATERIALS AND METHODS

Insects. Adult mated female insects were taken from a *Rhodnius prolixus* colony, kept at 28 °C and 75% relative humidity, and fed on rabbit blood at 3-week intervals.

Lipophorin Purification. Three to five days after feeding, hemolymph was collected in the presence of phenylthiourea (3−13 mg/mL), 5 mM EDTA, 0.15 M NaCl, and 0.05 mg/mL soybean trypsin inhibitor (SBTI), leupeptin, and antipain, and 1 mM benzamidine. Hemolymph was centrifuged at room temperature for 5 min at 13000*g*, and lipophorin was purified from the supernatant as previously described (Gondim *et al.*, 1989a). The degree of purification was monitored by SDS−PAGE (Laemmli, 1970). Protein concentration was determined according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis. SDS−PAGE was carried out in 6−22.5% acrylamide gradient gels (Laemmli, 1970). Native PAGE was carried out in 3−10% acrylamide gradient gels (Davis, 1964). In both cases, proteins were eletrophoresed at 120 V at room temperature. Gels were stained with Coomassie Brilliant Blue G, according to Neuhoff *et al.* (1988). The molecular mass of polypeptides in SDS−PAGE was determined using the following proteins as standards: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and cytochrome *c* (12 kDa).

Treatment of Lipophorin with Phospholipase A_2. Lipophorin was completely depleted of phospholipids by incubation with phospholipase A_2 from *Crotalus durissus* (Boehringer Mannheim Biochemicals, Germany; 3 units of phospholipase/mg of Lp protein) as previously described (Gondim *et al.*, 1992). As determined by TLC analysis and measurements of ^{32}P-labeled phospholipids, all lysophospholipids and fatty acids were removed from Lp by this treatment (data not shown).

Trypsin Treatment. Purified Lp or d-Lp (1 μg/μL) were incubated with TPCK-treated trypsin (0.08 μg/μL) (Sigma) in 10 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4, at 37 °C. After different times of incubation, proteolysis was stopped with a 10-fold molar excess of SBTI (Sigma) over trypsin. Control samples were incubated in the absence of trypsin. Samples for SDS−PAGE were boiled for 5 min immediately after addition of sample loading buffer (Laemmli, 1970), to prevent the reactivation of trypsin that occurs in sample buffer.

Fluorescence Measurements. Steady-state fluorescence measurements were performed on an ISS Inc. (Champaign, IL) GREG-200 spectrofluorometer. Lipophorin concentration ranged from 100 to 200 μg/mL. Excitation was at 280 nm, and emission was either scanned from 295 to 450 nm or fixed at 323 nm, as indicated under Results. Spectral areas and centers of mass (average emission wavelength) were calculated with software from ISS Inc., as follows:

$$\text{area} = \sum I(\lambda)$$

$$\text{center of mass} = \sum \lambda I(\lambda) / \sum I(\lambda)$$

where λ is the emission wavelength (nm) and $I(\lambda)$ is the fluorescence intensity at a given wavelength.

In unfolding experiments, lipophorin samples (100 μg/mL) were incubated with different GdnHCl concentrations ranging from 0.5 to 7 M, in 10 mM Tris-HCl, 0.15 M NaCl, pH 7.4. Fluorescence measurements were carried out after 12 h of incubation at 4 °C. Appropriate blanks containing different GdnHCl concentrations were prepared, and their spectra were subtracted from spectra of Lp in the presence of GdnHCl. Fluorescence anisotropy measurements were carried out as described above using Corning 7-54 and 7-61 filters in the excitation and emission, respectively.

In fluorescence quenching experiments, KI or acrylamide was added to the protein sample from freshly prepared concentrated (2 M) stock solutions. Before KI addition, the samples were diluted in 10 mM Tris-HCl buffer, 0.15 M NaCl, pH 7.4, containing 2 M KCl, in order to maintain constant ionic strength throughout the experiment. Fluorescence intensities were corrected for the dilution caused by addition of small volumes of quencher solution to the cuvette. Acrylamide and iodide quenching data were analyzed according to the Stern−Volmer equation (Lakowicz, 1983):

$$F_0/F = 1 + K_{SV}Q$$

where K_{SV} is the Stern−Volmer quenching constant, Q is the molar concentration of quencher, and F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively.

Fluorescence measurements under pressure were performed using a pressure bomb (ISS Inc.) similar to the one originally described by Paladini and Weber (1981), equipped with sapphire optical windows. Spectra were recorded after 30 min of equilibration at the desired pressure.

Circular Dichroism. Circular dichroism spectra were recorded on a Jasco J-720 spectropolarimeter, using PBS, pH 7.4, in a 1 mm cuvette. The spectra shown are averages of six spectra each, recorded at 50 nm/min at 0.5 nm steps. Spectra were corrected for protein concentration according to protein determination by the Lowry method (Lowry *et*

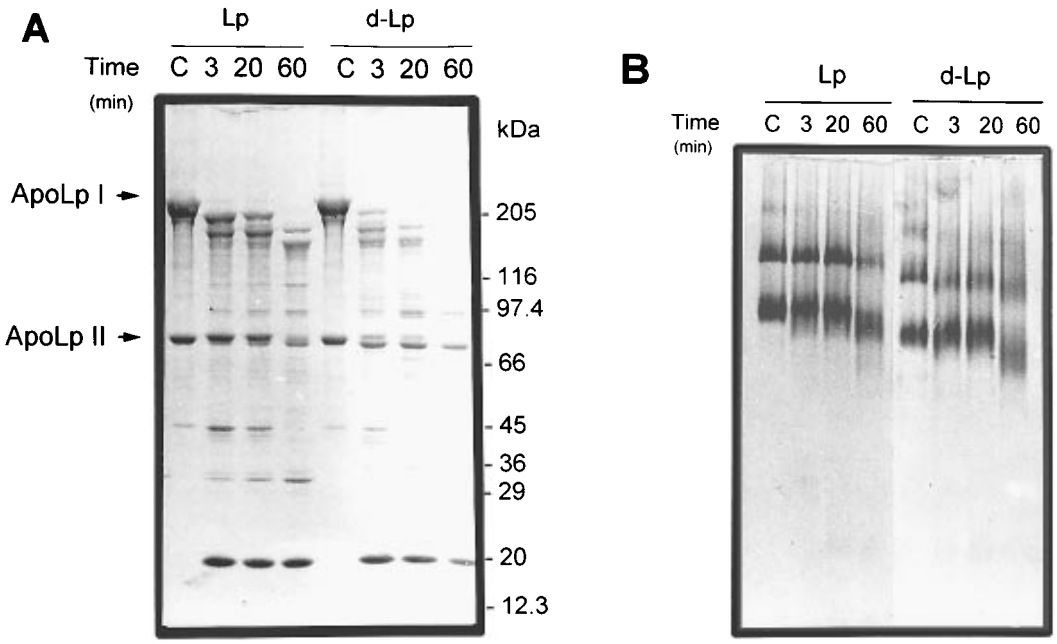

FIGURE 1: Effect of PL depletion on the proteolysis pattern of Lp. Lp was incubated with trypsin (13:1 Lp:trypsin ratio, w/w) in PBS at 37 °C. Reaction was stopped with a 10-fold molar excess of SBTI, after different incubation times as indicated in the figure. Panel A: SDS−PAGE (6−22.5% acrylamide); molecular mass standards and apolipoproteins are indicated by arrows. Panel B: native PAGE (3−10% acrylamide). Control samples (C) were incubated in the absence of trypsin.

al., 1951) and by the absorption at 280 nm. CD measurements were carried out at the Laboratory of Fluorescence Dynamics (University of Illinois at Urbana−Champaign).

RESULTS

Susceptibility of Lp to Proteolysis. In order to study the exposure of Lp polypeptide chains to the aqueous medium, the susceptibility of Lp to trypsin was analyzed. Lp was incubated with trypsin (13:1 Lp:trypsin ratio, w/w) for different times, and analyzed by SDS−PAGE (Figure 1A). In 3 min of proteolysis, a 45 kDa polypeptide was formed, and a corresponding decrease in the molecular mass of apoLp-I was observed, suggesting the existence of a highly susceptible cleavage site in apoLp-I. The 45 kDa peptide was degraded after 1 h incubation with trypsin (Figure 1A). Extensive cleavage of apoLp-II was only achieved at 1 h of proteolysis, suggesting that *R. prolixus* apoLp-II is less exposed to the aqueous medium than apoLp-I, similarly to what has been proposed for *Manduca sexta* and *Locusta migratoria* lipophorins (Shapiro *et al.*, 1984; Ryan *et al.*, 1992).

There was a significant increase in the rate of proteolysis of both apoproteins when dephospholipidated Lp (d-Lp) was subjected to trypsin treatment (Figure 1). This indicated that removal of PL facilitated access of trypsin to the protein. Comparison of the proteolysis patterns of Lp after 1 h incubation and d-Lp after 3 and 20 min showed that depletion of phospholipids led to an increase in the rate of proteolysis, especially of apoLp-II. Another interesting observation was the existence of a very susceptible cleavage site for trypsin proteolysis in Apo I of both Lp and d-Lp, producing a peptide of 45 kDa and one of 210 kDa (Figure 1). This was also observed when papain and chymotrypsin were used (data not shown).

The polypeptides formed by trypsin treatment of both Lp and d-Lp remained bound to the lipoprotein particle, as indicated by PAGE under native conditions (Figure 1B). More than one Lp band is observed, probably reflecting size and composition heterogeneity, similar to lipophorin from other insects (Soulages & Wells, 1994b). Even after 1 h of incubation with trypsin, only Lp and d-Lp bands were observed, with no detection of small molecular mass peptides. The slight change in electrophoretic migration produced at 1 h of proteolysis was possibly due to charge modification of the Lp particle. Depletion of PL also led to increased mobility of Lp in the nondenaturing gel (Figure 1B). This can be explained by changes in the overall charge as well as by a decrease in size of the Lp particle upon removal of PL. Indeed, when d-Lp and Lp were analyzed by means of pore-limiting native PAGE [according to Blanche *et al.* (1981)], a decrease in molecular mass compatible with total removal of PL was obtained for all d-Lp bands (data not shown).

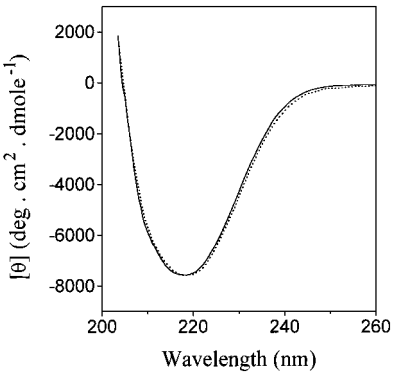

FIGURE 2: Circular dichroism spectra of Lp and d-Lp. Spectra of Lp (—) and d-Lp (•••) were measured in PBS, at a protein concentration of 0.34 mg/mL.

Circular Dichroism. Circular dichroism spectra of Lp and d-Lp are shown in Figure 2. The spectra for d-Lp and Lp were extremely similar, indicating preservation of the secondary structure of the protein after PL removal. This result was similar to that found by Katagiri (1985) for dephospholipidated lipophorin from *Locusta migratoria*.

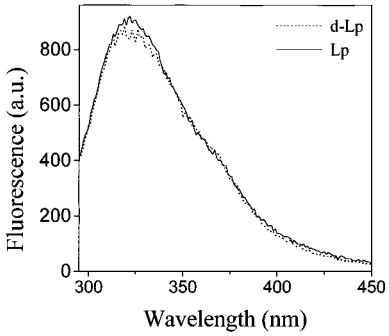

FIGURE 3: Intrinsic fluorescence emission spectra of Lp and d-Lp. Intrinsic fluorescence emission spectra of Lp (—) and d-Lp (•••) (100 μg/μL) were measured in 10 mM Tris-HCl, 0.15 M NaCl, pH 7.4, with excitation at 280 nm.

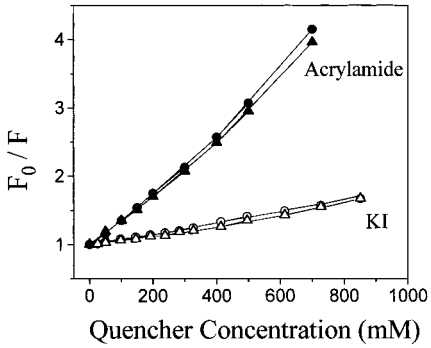

FIGURE 4: Lack of effect of PL depletion on the iodide and acrylamide quenching of Lp fluorescence. Stern−Volmer plots for iodide (open symbols) and acrylamide (closed symbols) quenching of the intrinsic fluorescence are presented for Lp (circles) and d-Lp (triangles). Fluorescence intensity was monitored at the emission peak (323 nm).

Solvent Exposure of Tryptophan Residues. In order to investigate possible conformational changes of Lp apoproteins upon PL removal, the exposure of tryptophan residues to the aqueous medium was assessed by fluorescence spectroscopy. As shown in Figure 3, the intrinsic fluorescence emission spectrum of Lp was not altered by removal of PL, exhibiting a maximum at 323 nm indicative of protected tryptophan environments in both Lp and d-Lp particles. The exposure of tryptophan residues to the aqueous medium was further investigated by fluorescence quenching measurements. PL depletion did not induce significant changes in the exposure of tryptophan residues to the medium, as indicated by identical Stern−Volmer quenching constants (K_{SV}) of 0.8 M^{-1} for iodide quenching of the fluorescence of Lp and d-Lp (Figure 4). Acrylamide Stern−Volmer plots also gave identical constants for Lp and d-Lp (K_{SV} = 4.2 M^{-1}; Figure 4).

Unfolding of Lp by Guanidine Hydrochloride. Lipophorin apoproteins dissociate and unfold in the presence of GdnHCl (Shapiro *et al.*, 1984; Kashiwazaki & Ikai, 1985; Kawooya *et al.*, 1989). The effect of this chaotropic agent on Lp and d-Lp was investigated by analyzing intrinsic fluorescence emission spectra at different GdnHCl concentrations (Figure 5). Changes in Lp spectra (Figure 5A) were more marked than in d-Lp (Figure 5B), especially at lower (2 M) concentrations of GdnHCl. Similar experiments to that shown in Figure 5 were also done with excitation at 295 nm, where only tryptophan fluorescence is excited, thus avoiding tyrosine emission (data not shown). These experiments showed that the peak at 310 nm, observed at high

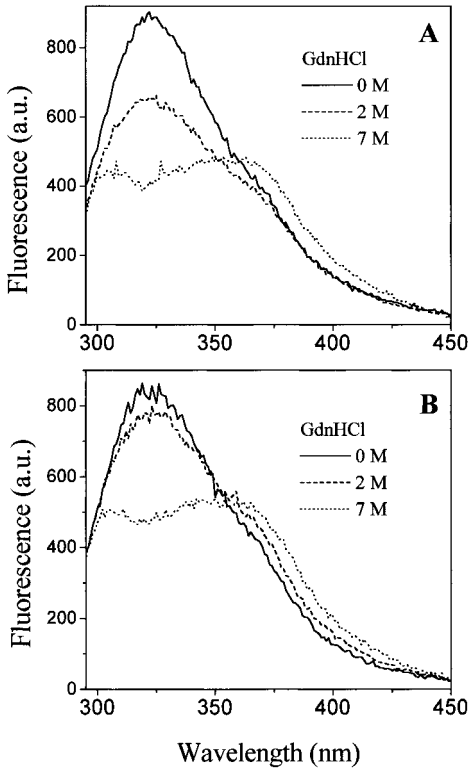

FIGURE 5: Effect of GdnHCl on the fluorescence emission spectra of Lp and d-Lp. Lp (panel A) and d-Lp (panel B) were incubated in the absence of GdnHCl (—), or in the presence of 2 M (---) or 7 M (•••) GdnHCl. Spectra were recorded after 12 h of incubation at 4 °C.

GdnHCl concentrations, disappeared when 295 nm excitation was used, indicating that this peak resulted from tyrosine emission, due to the increased distance between tryptophan and tyrosine residues in the unfolded protein. Increasing GdnHCl concentration promoted a red-shift in the Lp fluorescence spectral center of mass **(**Figure 6A**)** and a decrease in spectral area (Figure 6B). Removal of PL shifted the center of mass curve toward higher guanidine concentrations and conferred partial protection against the decrease in fluorescence spectral area, suggesting that perturbation of protein structure induced by GdnHCl was more marked in native Lp than in d-Lp particles.

Unfolding of Lp and d-Lp by GdnHCl was also followed by monitoring the decrease in intrinsic fluorescence anisotropy. For both Lp and d-Lp, similar decreases in anisotropy were observed up to 7 M GdHCl (data not shown). Given the short lifetime of tryptophan fluorescence (relative to the long rotational correlation time expected for the lipophorin particle), intrinsic anisotropy measurements will essentially convey information on the local mobility of the Trp residues. Thus, the similarity in the anisotropies measured for Lp and d-Lp suggests that removal of phospholipids does not significantly affect the average local mobility of the Trp residues of lipophorin.

Effects of Hydrostatic Pressure on Lp Structure. Hydrostatic pressure has been increasingly used in association with fluorescence spectroscopy to study protein interactions in several systems [for recent reviews, see Silva and Weber (1993), Mozhaev *et al.* (1994), and Gross and Jaenicke (1994)]. When Lp was submitted to 2.4 kbar of pressure, a 2.2 nm red-shift in the fluorescence emission was observed, indicating that the Lp particle is quite resistant to hydrostatic

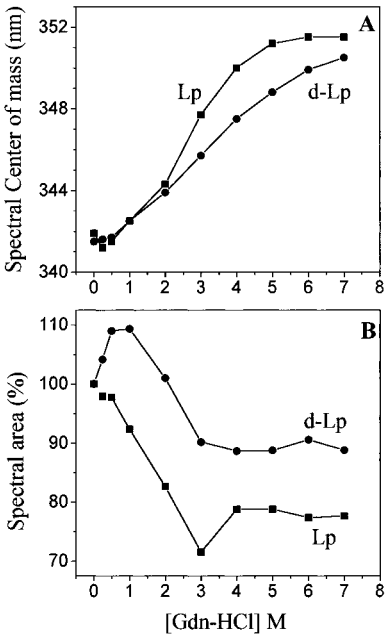

FIGURE 6: Fluorescence analysis of unfolding of Lp and d-Lp by GdnHCl. Lp (closed squares) and d-Lp (closed circles) were incubated at the indicated GdnHCl concentrations for 12 h, and intrinsic fluorescence spectra were recorded. Spectral center of mass (panel A) and spectral area (panel B) were calculated from fluorescence emission spectra, as described under Materials and Methods. The results shown are typical of four similar experiments.

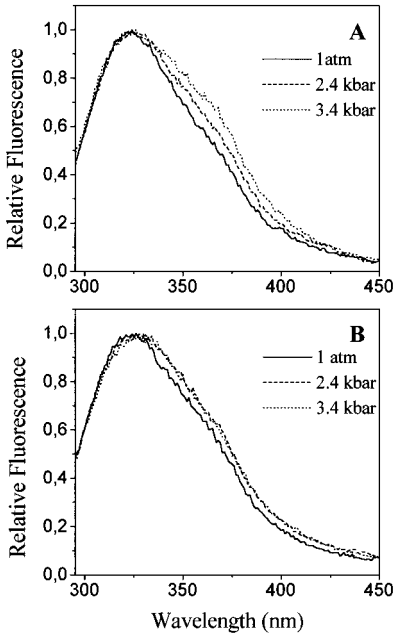

FIGURE 7: Effects of hydrostatic pressure on the fluorescence emission spectra of Lp and d-Lp. Lp (panel A) and d-Lp (panel B) fluorescence emission spectra were recorded at 1 atm (—), 2.4 kbar (---), or 3.4 kbar (···). Spectra are normalized for the maximum fluorescence intensity. The results shown are typical of three similar experiments.

pressure (Figure 7A). At 3.4 kbar, the red-shift in the emission increased to 3 nm (Figure 7A). For d-Lp, a red-shift of 1.8 nm was observed at 2.4 kbar (Figure 7B). Interestingly, increasing pressure to 3.4 kbar did not cause any further shift in the d-Lp spectrum, suggesting that PL-depleted particles are more resistant to high pressures than native Lp.

DISCUSSION

Mammalian high-density lipoproteins (HDL_2 and HDL_3) require PL to be reassembled *in vitro* from purified apoproteins (Hirz & Scanu, 1970). Other studies have also shown that isolated apoproteins from human HDL are stabilized by interaction with either PL or cholesterol (Tall *et al.*, 1975; Morrisett *et al.*, 1977; Atkinson & Small, 1986; Jonas, 1986; Jonas *et al.*, 1993). Kawooya *et al.* (1989) reported that lysophospholipids were necessary for reassembly of isolated apoLp-I. In contrast with these results indicating a stabilizing role of PL, mammalian HDL_3 has been shown to remain soluble even after complete removal of PL (Pattnaik *et al.*, 1976). In the present work, we show that PL are not only not essential for Lp stability, but rather that PL-containing particles were less stable than dephospholipidated particles toward denaturation by physical and chemical agents.

Analysis of intrinsic fluorescence emission and quenching experiments with Lp and d-Lp (Figures 3 and 4) showed no detectable change in the exposure of tryptophan residues to the aqueous solvent following PL removal. Furthermore, circular dichroism measurements indicated no significant change in the secondary structure of d-Lp relative to Lp (Figure 2). On the other hand, proteolysis data (Figure 1) indicated that removal of PL led to a higher susceptibility to trypsin, especially of apoLp-II, suggesting a close structural association between apoLp-II and PL. This suggests that dephospholipidation may bring about a conformational change that may be related to the enhanced stability of d-Lp.

Unfolding of *R. prolixus* Lp by GdnHCl promoted a 11 nm red-shift of the fluorescence emission, together with a 25% decrease in fluorescence intensity (Figure 6). Upon removal of PL, the effects of GdnHCl on both spectral area and center of mass were attenuated. GdnHCl promotes dissociation and denaturation of polypeptide chains by interacting with peptide bonds, with side chains of aromatic residues, and by interference with hydrophobic bonds (Robinson & Jencks, 1965; Aune & Tanford, 1969; Lee & Timasheff, 1974). Therefore, the higher stability of d-Lp in GdnHCl solutions may be explained by an increase in protein−protein and/or protein−neutral lipid interactions, which could decrease guanidine binding to the protein. Contrasting with our results, the soluble apolipoprotein A-I (the main apoprotein from mammalian HDL) (Reijngoud & Phillips, 1982) and *Locusta migratoria* apoLp-III (Weers *et al.*, 1994) were more resistant to denaturation by guanidine when associated with PL.

Application of high hydrostatic pressure often results in protein subunit dissociation and/or unfolding (Silva & Weber, 1993; Mozhaev *et al.*, 1994; Gross & Jaenicke, 1994). When pressures up to 3.4 kbar were applied to Lp, a red-shift of about 3 nm in the fluorescence emission was observed (Figure 7), significantly less than that observed with GdnHCl (Figure 6). This result indicates that complete unfolding or dissociation of Lp may not be achieved by application of pressure. Although there have not been studies on pressure effects on native lipid-associated proteins, detergent-solubilized erythrocyte membrane calcium-ATPase has been shown to undergo subunit dissociation under pressure, and in that case the fluorescence emission was also red-shifted by only a few nanometers (Coelho-Sampaio *et al.*, 1991). Interestingly, removal of PL from the Lp particle rendered it more resistant to pressure-induced unfolding (Figure 7). Although

it is not possible to state precisely which interactions are involved in the observed pressure-induced fluorescence red-shift, one possibility is that removal of PL may drive the Lp particle into a slightly more compact conformation, with stronger interactions between lipoprotein components (protein−protein or protein−neutral lipids). Pressure-induced conformational changes of Lp and d-Lp were completely reversible upon decompression (data not shown), suggesting that this approach may be useful for future studies in lipoprotein thermodynamics. To our knowledge, this is the first report to analyze conformational changes of whole lipoproteins under high hydrostatic pressure. Human soluble apolipoprotein A-I has been studied under pressure but in the absence of lipids (Mantulin & Pownall, 1985).

One possible explanation for the apparently conflicting results in the literature regarding the roles of PL in lipoprotein stability may be that reports that showed a stabilizing effect of PL were conducted with isolated apoproteins before association with PL. On the other hand, reports that showed PL as nonessential for particle solubility, or even a destabilizing effect of PL (present work), used entire lipoproteins before PL removal. In the case of insect Lp and mammalian HDL, there are substantial amounts of neutral lipids that may have a stabilizing role in whole lipoproteins. In the case of isolated apolipoproteins, in which neutral lipids are absent, PL could conceivably play a stabilizing role.

It is interesting to note that in mammalian HDL the major apoproteins are rich in surface-active amphiphilic helices (Jonas *et al.*, 1993) which are thought to be important in stabilizing the particle in aqueous medium. For *M. sexta* lipophorin, it is known that the α-helix content is about 35% (Ryan *et al.*, 1992). For *R. prolixus* lipophorin, analysis of the CD data (Figure 2) revealed an α-helix content of 23−27%, using the SELCON software (Sreerama & Woody, 1993). The amino acid sequence of *M. sexta* lipophorin has only very recently become available (Sundermeyer *et al.*, 1996). Thus, further studies will have to be carried out to establish whether the helices in Lp are of amphipathic nature, and whether they play any roles in interaction with phospholipids and/or particle stabilization.

Because phospholipids do not seem to play a role in lipoprotein stabilization, their roles should be further investigated. Previous studies have shown that PL may be transported by Lp (Gondim *et al.*, 1989b; van Heusden *et al.*, 1991; Atella *et al.*, 1992, 1995) in a way similar to that shown for neutral lipids (Chino *et al.*, 1969; van der Horst *et al.*, 1981). An interesting possibility is that PL might be important in lipoprotein stabilization during the assembly of lipoprotein particles, before neutral lipids are incorporated. After loading with neutral lipids, the hydrophobic core may play the stabilizing role initially performed by PL. Indeed, a newly secreted lipoprotein particle composed only by PL and apoproteins has been described both in insects (Prasad *et al.*, 1986; Soulages & Wells, 1994b) and in mammals (Atkinson & Small, 1986). Our data showing an increased susceptibility of d-Lp to proteases also suggest another possible function of PL, namely, to protect Lp against proteolysis, thus enhancing its half-life in the hemolymph.

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