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Interaction of locust apolipophorin III with lipoproteins and phospholipid vesicles: effect of glycosylation

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Abstract Apolipophorin III (apoLp-III) from *Locusta migratoria* is an exchangeable apolipoprotein that binds reversibly to lipoprotein surfaces. The native protein is glycosylated at Asn-18 and Asn-85. Variable attachment of five distinct oligosaccharide moieties at the two glycosylation sites results in molecular weight heterogeneity, as seen by mass spectrometry. The main mass peak of 20,488 Da decreases to 17,583 Da after removal of carbohydrate, indicating that apoLp-III carbohydrate mass is ~14% by weight. Deglycosylated apoLp-III induced clearance of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol vesicles at a faster rate than glycosylated apoLp-III. However, in lipoprotein binding assays, in which apoLp-III interacts with surface-localized diacylglycerol, only minor differences in binding were observed. The fluorescence properties of 1-anilinonaphthalene-8-sulfonate were unaffected by the glycosylation state of apoLp-III, indicating that no changes in the relative amount of exposed hydrophobic surface occurred as a result of carbohydrate removal. We propose that glycosyl moieties affect the ability of apoLp-III to transform phospholipid bilayer vesicles into disc-like complexes by steric hindrance. This is due to the requirement that apoLp-III penetrate the bilayer substrate prior to conformational opening of the helix bundle. On the other hand, the glycosyl moieties do not affect lipoprotein binding interactions as it does not involve deep protein penetration into the lipid milieu. Rather, lipoprotein binding is based on oriented protein contact with the lipid surface followed by opening of the helix bundle, which allows formation of a stable interaction with surface exposed hydrophobic sites.—Weers, P. M. M., D. J. Van der Horst, and R. O. Ryan. **Interaction of locust apolipophorin III with lipoproteins and phospholipid vesicles: effect of glycosylation. *J. Lipid Res.* 2000.** 41: **416–423.**

Supplementary key words exchangeable apolipoproteins • lipid–protein interactions • lipophorin • apoLp-III • locust

Apolipophorin III is an exchangeable apolipoprotein found in several insect species and serves as a model for studies of the interaction of apolipoproteins with lipoprotein surfaces (1, 2). X-ray crystallography data of the protein in the lipid-free conformation showed that apoLp-III is organized as a bundle of five amphipathic α-helices (3) resembling the helix bundle architecture of the 22 kDa N-terminal domain of human apolipoprotein (apo) E (4). Hydrophobic amino acid side chains are oriented towards the protein core while hydrophilic side chains are fully exposed to the aqueous environment. Although high resolution structural data of the protein in the lipid-bound conformation are not available to date, several lines of evidence support the hypothesis that the protein bundle opens, exposing its interior upon lipid binding (3, 5–8). This repositioning of helices allows interaction of the hydrophobic faces of the amphipathic helices with the lipid environment. ApoLp-III molecules from more than a dozen different species have been identified, and those from the sphinx moth, *Manduca sexta*, and the locust, *Locusta migratoria*, have been extensively characterized. Two isoforms of locust apoLp-III have been identified, a full-length 164 residue apolipoprotein (a-isoform) which is secreted and subsequently converted into the b-isoform lacking two N-terminal residues (Arg-Pro) (9, 10). Both isoforms are able to bind to lipoprotein particles. ApoLp-III from *Manduca sexta* (166 residues) is a non-glycosylated protein, while its counterpart from *L. migratoria* contains two N-linked glycosylation sites. The carbohydrate structures have been elucidated and are complex in nature, containing fucose and covalently bound 2-aminoethylphosphonate (AEP) (11).

ApoLp-III fulfills a key role in diacylglycerol transport carried out by lipophorins, which are the main lipid-carrying vehicles in hemolymph. Despite significant differences in primary structure, apoLp-III from *L. migratoria* and *M.*

Abbreviations: apo, apolipoprotein; apoLp-III, apolipophorin III; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; LDL, low density lipoprotein; HDLp, high density lipophorin; LDLp, low density lipophorin; endoF, endoglycosidase F/N-glycosidase F; ANS, 1-anilinonaphthalene-8-sulfonate; PL-C, phospholipase C; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; G1cNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose; AEP, 2-aminoethylphosphonate.

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sexta are functionally indistinguishable (12). Recently, it was observed that recombinant apoLp-III lacking carbohydrates showed a much stronger interaction with phospholipid vesicles than insect-derived (native) apoLp-III (13). In the present study we used native *L. migratoria* apoLp-III to further investigate the role of glycosylation in apoLp-III–phospholipid vesicle interactions and lipoprotein binding. Special attention was focused on possible roles of carbohydrate in: *i*) shielding hydrophobic protein domains that are involved in lipid binding, *ii*) protein flexibility, and *iii*) steric effects. In addition, we compared the a- and b-isoforms of apoLp-III to examine whether deletion of the two N-terminal residues Arg-Pro has an effect on lipid and lipoprotein binding. The results of the present report show that glycosylation does not affect lipoprotein binding and support the view that interaction of apoLp-III with phospholipid vesicles and lipoprotein surfaces are two fundamentally different processes.

METHODS

Materials

Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids Inc., Alabaster, AL; phospholipase-C (PL-C), 1-anilinonaphthalene-8-sulfonate (ANS), and carbonic anhydrase were purchased from Sigma Chemical Co., St. Louis, MO. Endoglycosidase F/N-glycosidase F (Endo F) was from Boehringer Mannheim GmbH, Germany. Bicinchoninic acid protein assay and bovine serum albumin were from Pierce Chemical Co., Rockford, IL. Human low density lipoprotein (LDL) was isolated by sequential density ultracentrifugation (14). Insect-derived apoLp-III was isolated from *L. migratoria* as described previously (9). All apoLp-IIIs used in the present study were HPLC purified as described below.

Removal of carbohydrate

N-linked oligosaccharides were removed by treatment with endo F. ApoLp-III (10 mg) was dissolved in buffer (50 mm Tris-HCl, pH 8.5; 5 mm EDTA) and incubated with 1 U endo F at room temperature for 24 h. Deglycosylated apoLp-III was re-purified by reversed-phase HPLC (Beckman) equipped with a RXC-8 300 SB Zorbax column and eluted with a linear gradient of water–acetonitrile in 0.05% TFA at a flow rate of 1 ml/min. Molecular weight measurements of apoLp-III were obtained by electrospray ionization mass spectrometry using a VG Quattro electrospray mass spectrometer (Fisons Instruments, Manchester, UK).

Phospholipid vesicle clearance

Phospholipid vesicles were prepared by dissolving 5 mg DMPG or DMPC in chloroform–methanol 3:1 (v/v). After drying, pre-warmed buffer (10 mm Tris-HCl, pH 7.2, 150 mm NaCl, 0.5 mm EDTA) was added and vortexed. Subsequently, unilamellar vesicles were made by extrusion through a 200 nm membrane (Avanti Polar Lipids Inc). Light scattering was used to monitor apoLp-III-induced phospholipid vesicle clearance using a Perkin-Elmer spectrofluorometer (model LS 50B). Excitation and emission wavelengths were set at 600 nm (slit width 3 nm) and the temperature was maintained at 23°C (DMPG) or 23.9°C (DMPC) in a thermostated cuvet holder. ApoLp-III was dissolved in buffer and measured for protein concentration by the bicinchoninic acid protein assay. This protein solution was added to a 250 μg/ml vesicle dispersion at a final lipid:protein weight ratio of 12.5:1 (DMPG) or 1:1 (DMPC) and the decrease in light scattering was followed as a function of time.

Lipoprotein binding assay

The ability of apoLp-III to bind to spherical lipoprotein surfaces was examined using human LDL. The surface of LDL was modified by incubation with phospholipase C (PL-C) from *Bacillus cereus*, creating surface-localized diacylglycerol. As a result, LDL forms large aggregates (15). In contrast to untreated LDL, apoLp-III readily associates with PL-C-treated LDL, thereby providing protection against aggregation. In detail, LDL (50 μg protein) was treated with 160 mU PL-C in the presence or absence of apoLp-III in buffer (50 mm Tris-HCl, pH 7.5, 150 mm NaCl and 2 mm $CaCl_2$) at 37°C. To monitor formation of lipoprotein aggregates, the absorbance at 340 nm was measured at the times indicated. All measurements were done in triplicate with standard deviations shown if larger than the size of the symbol.

Competition binding assay

LDL (250 μg protein) was incubated with 0.88 U PL-C in the presence of 250 μg protein each of native apoLp-III and deglycosylated apoLp-III. The mixture was incubated for 40 min at 37°C and the reaction was stopped by the addition of KBr (2.8 m final concentration). The samples were subjected to KBr density gradient ultracentrifugation for 75 min at 65,000 rpm (4°C) in a Beckman VTi65.2 rotor to reisolate LDL. Top (LDL) and bottom (lipid-free apoLp-III) fractions were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gels were stained with Amido Black 10B.

Fluorescence spectroscopy

Binding of ANS to apoLp-III was monitored using a Perkin-Elmer spectrofluorometer (model LS 50B). Excitation was set at 395 nm with a slit width of 6 nm, and emission was monitored between 400 and 600 nm at 21°C (16). Spectra were obtained for ANS alone (250 μm in 50 mm phosphate buffer, pH 7.5), and in the presence of 5.6 μm apoLp-III. L32R/L34R/L95R mutant apoLp-III (17) was used as a positive control. Trp fluorescence spectra of apoLp-III were obtained by excitation at 280 nm and emission monitored between 290 and 450 nm using a slit width of 3.5 nm.

RESULTS

Deglycosylation and mass spectrometry

The mass of *L. migratoria* apoLp-IIIa was measured using electrospray ionization mass spectrometry, giving rise to a broad spectrum of masses ranging from 20,281 to 20,906 Da (**Fig. 1A**). The most abundant peak displayed a mass of 20,488 ± 7 Da. As shown in Fig. 1B, treatment of apoLp-IIIa with endo F resulted in the appearance of a single mass peak of 17,583 ± 2 Da, which closely resembles the expected mass based on the amino acid sequence (17,581 Da). Enzymatic removal of both oligosaccharide moieties reveals that carbohydrate mass represents ~14% of total glycoprotein mass. The carbohydrate structures of apoLp-III have been determined previously to be composed of five unique oligosaccharide chains (11). Knowing these precise carbohydrate sequences, we deduced that the main mass peak (20,488 Da) arises from an apoLp-III protein possessing two carbohydrate chains each containing three *N*-acetylglucosamine (GlcNAc), one fucose (Fuc), three mannose (Man), and two 2-aminoethylphos-

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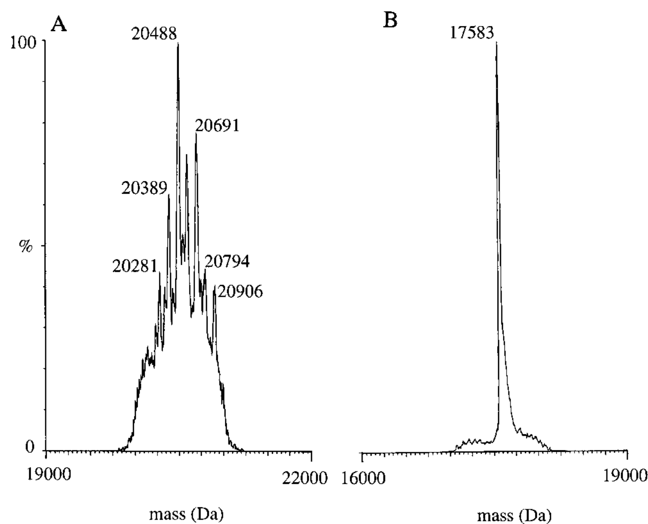

Fig. 1. Electrospray mass spectrometry of native apoLp-IIIa (panel A) and deglycosylated apoLp-IIIa (panel B). The numbers indicate the molecular weight of apoLp-III.

phonate (AEP) molecules (**Fig. 2A**), attached to Asn-18 and Asn-85 (Fig. 2B). The presence of an additional GlcNAc attached to the terminal Man on one of the oligosaccharide chains increases the mass of 20,691 Da, while two such chains results in a mass of 20,906 Da, the largest observed apoLp-III glycoprotein mass.

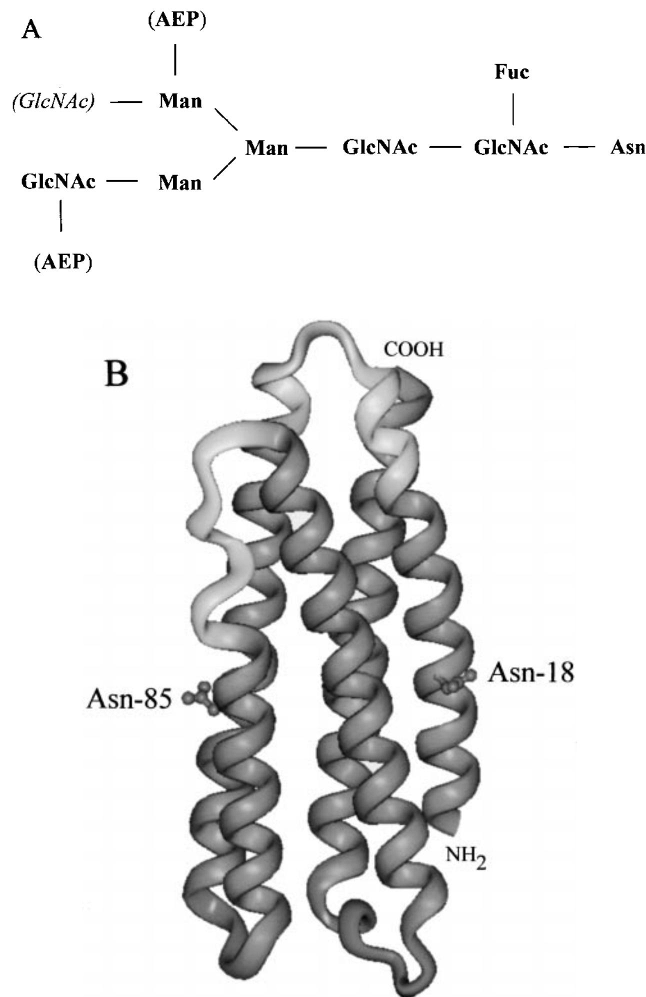

Fig. 2. The general structure of the oligosaccharide moiety is shown in panel A, as reported by Hård et al. (11). The units between brackets are optional and give rise to the heterogeneity observed by mass spectrometry. The main oligosaccharide moiety is shown in bold. Addition of GlcNAc (shown in italics) results in the largest observed oligosaccharide chain. Panel B: Ribbon diagram of *L. migratoria* apoLp-III according to Breiter et al. (3) depicting the location of the two glycosylation sites, Asn-18 and Asn-85.

Phospholipid vesicle clearance

When mixed with bilayer vesicles of DMPC, locust apoLp-III transforms the vesicles into disc-like particles with an average diameter of 14 nm, each disc containing five apoLp-III molecules (7). This process is conveniently monitored by light scattering as the turbid vesicle dispersion clears upon disruption and conversion to discoidal complexes. Using a lipid:protein ratio of 12.5:1, complete clearance of DMPG vesicles by apoLp-IIIa was achieved in about 600 s, while deglycosylated apoLp-IIIa cleared DMPG vesicles in about 120 s (**Fig. 3A**). The time required for 50% clearance ($t_{1/2}$) was 150 s for apoLp-IIIa and 13 s for deglycosylated apoLp-IIIa. Recombinant apoLp-III produced by *E. coli* cultures lacking covalently linked carbohydrate (18) behaved in a similar fashion as deglycosylated apoLp-IIIa. Comparison of the a- and b-isoforms demonstrated a somewhat faster clearance observed for apoLp-IIIa, which is possibly a result of the presence of Arg-1. Addition of apoLp-III to zwitterionic DMPC vesicles also resulted in clearance of the solution, although at a much slower rate than DMPG (Fig. 3B). Nevertheless, a similar trend was observed, with $t_{1/2}$ values of 3,380 s for apoLp-IIIa and 560 s for deglycosylated apoLp-IIIa, confirming previously reported results (13). Clearance rates of DMPC vesicles by the a- and b-isoforms were similar.

Lipoprotein binding

Treatment of human LDL with PL-C results in the appearance of surface-localized diacylglycerol due to enzymatic removal of the phosphocholine headgroup of phosphatidylcholine. This is followed by a rapid aggregation of LDL and sample turbidity development. Inclusion of exchangeable apolipoproteins in the reaction mixture, however, effectively prevents aggregation through formation of a stable binding interaction with the lipolyzed lipoprotein surface (15). As shown in **Fig. 4**, 25 μg native or deglycosylated apoLp-III were equally effective in preventing PL-C-induced LDL aggregation. The apoLp-IIIb isoform was also fully active (data not shown). By contrast, bovine serum albumin and carbonic anhydrase, representing general examples of globular proteins, were completely ineffective. We also compared the concentration dependence of the ability of native and deglycosylated apoLp-III to prevent PL-C-induced aggregation (**Fig. 5**). Inclusion of limited amounts of apoLp-III (1–10 μg) resulted in partial protection over a 2-h time span, while 15 μg fully protected PL-C-treated LDL (50 μg pro-

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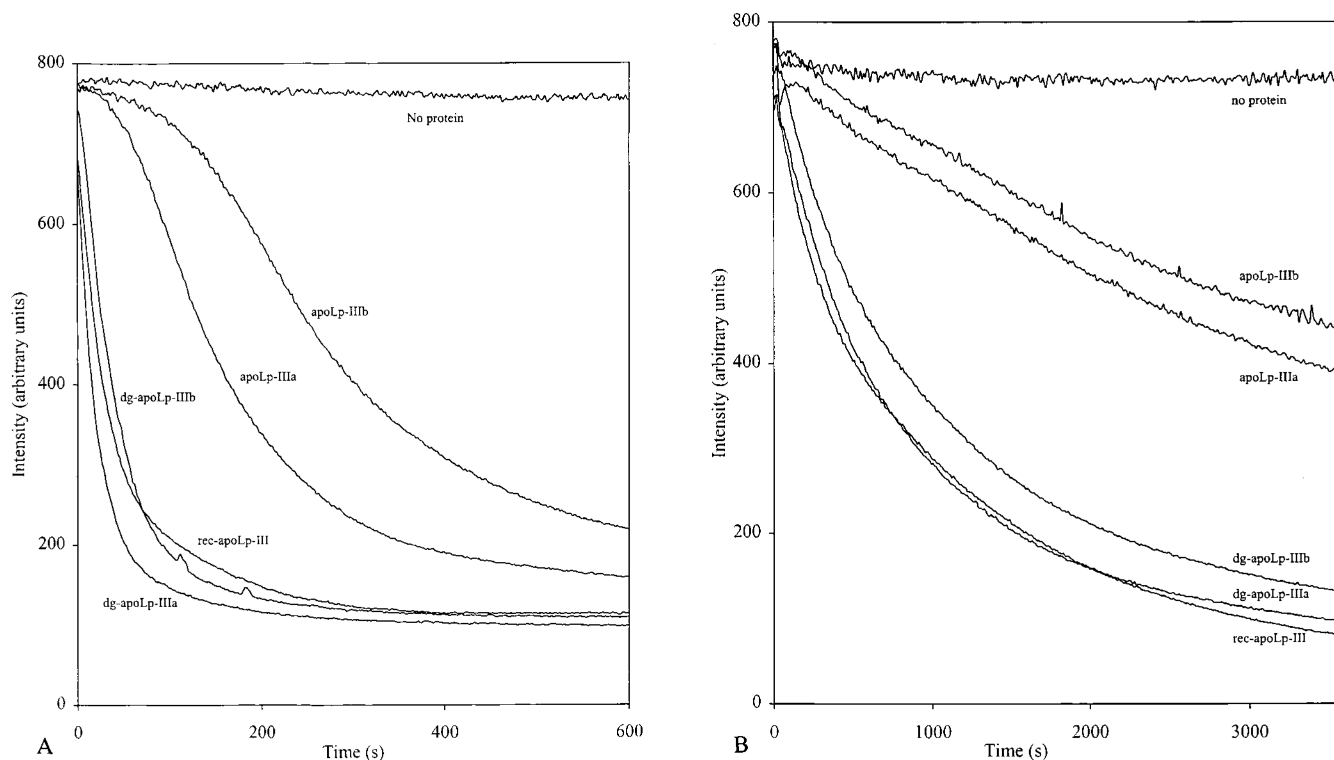

Fig. 3. The effect of apoLp-III on phospholipid vesicle clearance. Bilayer vesicles of DMPG (panel A) or DMPC (panel B) were incubated in the presence of different apoLp-III samples (native, deglycosylated (dg) and recombinant (rec)) at the lipid–gel to liquid–crystalline transition temperature at a lipid:protein ratio of 12.5:1 (DMPG) and 1:1 (DMPC), respectively. Sample light scattering intensity was followed as a function of time.

tein) from aggregation. A similar dose–response relationship was observed for deglycosylated apoLp-III. No differences were observed between the a- and b-isoforms (data not shown).

Competition assay

Native and deglycosylated apoLp-III migrate to different positions on SDS-PAGE, as a result of their ~3 kDa mass difference. This difference in electrophoretic mobil-

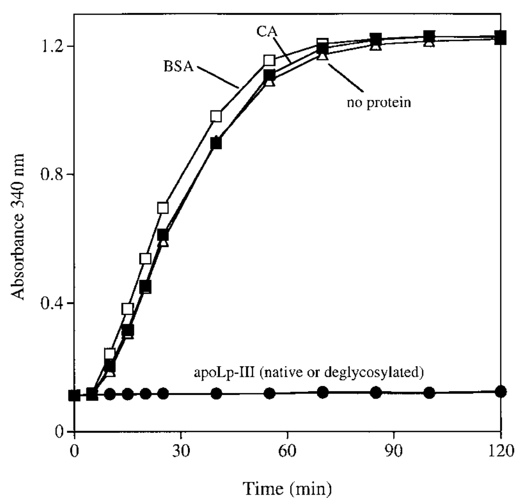

Fig. 4. Effect of apoLp-III on the turbidity of PL-C-treated LDL. Fifty μg LDL protein was treated with 160 mU PL-C and incubated in the presence of 25 μg native apoLp-IIIa (○), deglycosylated apoLp-III (●), bovine serum albumin (BSA, □) carbonic anhydrase (CA, ■) or no protein (△) at 37°C. Sample turbidity was monitored spectrophotometrically at 340 nm as a function of time.

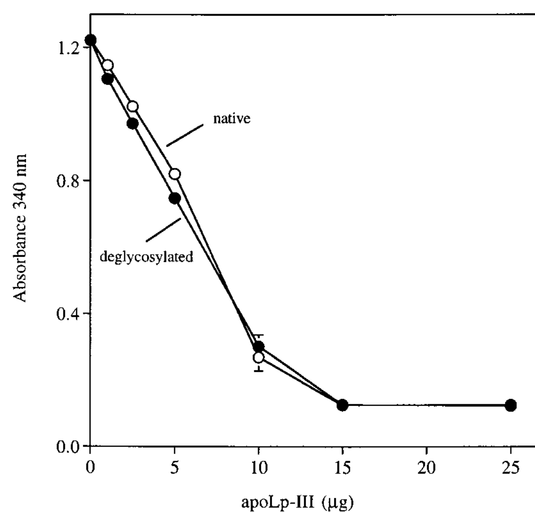

Fig. 5. The effect of apoLp-IIIa concentration of PL-C-induced LDL sample turbidity. LDL (50 μg protein) was incubated with 160 mU of PL-C and indicated amounts of apoLp-III. After 2 h incubation at 37°C, sample absorbance at 340 nm was measured. Native apoLp-III: (○); deglycosylated apoLp-III: (●).

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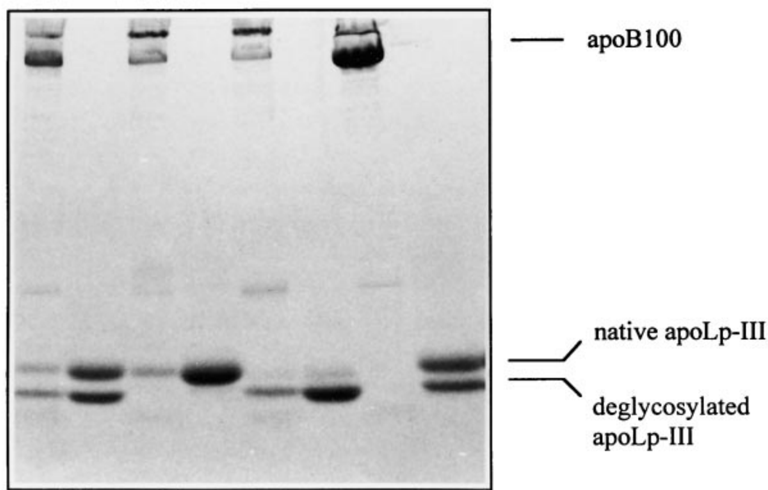

Fig. 6 Binding of native and deglycosylated apoLp-III to modified LDL. Native and/or deglycosylated apoLp-III (250 μg protein) were incubated with LDL (250 μg protein) in the presence or absence of PL-C. After incubation at 37°C for 40 min, the samples were subjected to KBr density gradient ultracentrifugation and the top (LDL containing) and bottom (lipoprotein-free) fractions were analyzed by SDS-PAGE (4–22% acrylamide gradient). Lanes 1, 3, 5, and 7 contain top fractions, lanes 2, 4, 6, and 8 contain bottom fractions. Lanes 1 and 2, incubation of LDL, PL-C and equal amounts of native and deglycosylated apoLp-III; lanes 3 and 4, incubation of LDL, PL-C and native apoLp-III; lanes 5 and 6, incubation of LDL, PL-C, and deglycosylated apoLp-III; lanes 7 and 8, incubation of LDL and equal amounts of native and deglycosylated apoLp-III in the absence of PL-C.

ity was used to assess the relative ability of native apoLp-III and deglycosylated apoLp-III to compete for binding sites created on the surface of LDL. Equal amounts of native apoLp-IIIa and deglycosylated apoLp-IIIa were included in incubations of LDL and PL-C. After the incubation, LDL-bound apoLp-III was separated from lipid-free apoLp-III by KBr density gradient ultracentrifugation. Top fractions (containing LDL-bound apoLp-III) and bottom fractions (lipid-free apoLp-III) were then analyzed by SDS-PAGE (**Fig. 6**). Based on staining intensity, the data showed minor differences in the amount of native (=glycosylated) and deglycosylated apoLp-IIIa recovered in the LDL fraction (Fig. 6, lane 1). Both native and deglycosylated apoLp-III alone were able to bind to LDL (lanes 3 and 5). In control incubations of LDL and apoLp-III in the absence of PL-C, apoLp-III binding to LDL was virtually absent with all apoLp-III recovered in the bottom fraction (lane 8).

Fluorescence studies

The decreased rate of vesicle clearance by native apoLp-III may be an indirect effect manifested by shielding of the hydrophobic regions involved in lipid binding by the carbohydrate moieties. The fluorescent probe ANS binds to exposed hydrophobic pockets of proteins, and such interactions are reflected by changes in ANS fluorescence wavelength emission maximum (λ_{max}) and intensity (16). Thus, ANS represents a suitable fluorescent probe to monitor changes in hydrophobic pockets in apoLp-III as a result of deglycosylation. As shown in **Fig. 7**, ANS in buffer displays a low fluorescence intensity with an emission maximum at 511 nm. Upon binding to native apoLp-III, ANS fluorescence emission intensity is highly increased and the λ_{max} is blue-shifted to ~475 nm. Similar fluorescence characteristics were observed for deglycosylated apoLp-III, indicating no major changes in exposure of ANS binding sites as a function of deglycosylation. A mutant apoLp-III, that displays altered lipoprotein binding, vesicle clearance and stability properties (17) was used as a positive control. This apoLp-III showed a much higher ANS fluorescence intensity compared to native apoLp-III or wild-type recombinant protein (data not shown), indicating that the mutation resulted in exposure of an increased number of ANS binding sites.

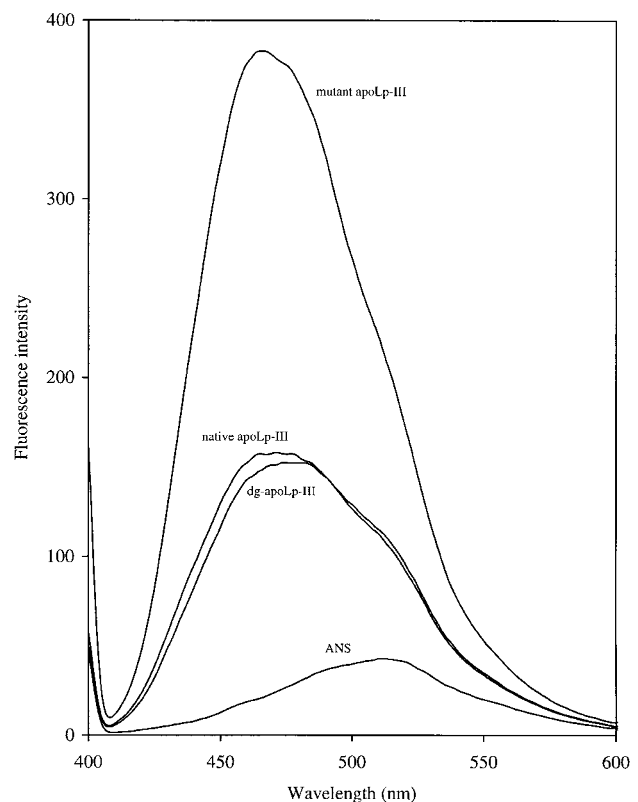

Fig. 7. Effect of apoLp-III glycosylation on the fluorescence properties of ANS. Spectra of ANS were obtained in buffer alone and in the presence of native apoLp-III, deglycosylated (dg) apoLp-III, or mutant apoLp-III. Samples were excited at 395 nm and emission spectra from 400 to 600 nm were collected (excitation and emission slit widths = 6 nm).

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To further study structural changes of apoLp-III as a result of deglycosylation, the fluorescence of the intrinsic fluorophores Trp-115 and Trp-130 was measured by excitation at 280 nm. An emission maximum of 332 nm was recorded for native apoLp-III, as well as for deglycosylated apoLp-III. Moreover, no differences in quantum yield were observed (data not shown).

DISCUSSION

ApoLp-III plays a key role in enhanced diacylglycerol transport during long-range flight in insects. Lipid-free apoLp-III and the major lipoprotein particle in hemolymph, high density lipophorin (HDLp) are abundant in adult hemolymph with concentrations up to 15 mg/ml (19). During diacylglycerol loading of HDLp, apoLp-III associates with the swelling lipoprotein particle, stabilizing the lipid-enriched structure yielding low density lipophorin (LDLp). Release of the diacylglycerol load results into dissociation of apoLp-III from the particle and recovery of HDLp. Both apoLp-III and HDLp can be reused for another cycle of lipid loading and delivery, a mechanism known as the lipid shuttle (20–22). Importantly, the dual existence of apoLp-III in hemolymph as a monomeric globular protein and LDLp apolipoprotein provides an attractive system for investigation of protein–lipid interactions.

L. migratoria apoLp-III contains 164 amino acid residues and is glycosylated on Asn-18 and Asn-85. Five unique carbohydrate chains have been reported and four of these contain AEP linked to Man or GlcNAc (11). Thus, in mass spectrometric analysis of apoLp-III, a heterogeneous population of mass peaks is observed. The major peak, with a mass of 20,488 ± 7 Da, results from an apoLp-III containing the structure depicted in Fig. 2A bound to both Asn-18 and Asn-85. Complete removal of carbohydrate was achieved by endo F treatment resulting in the appearance of a single mass at 17,583 ± 2 Da in agreement with the expected mass on the basis of its amino acid sequence (17,581 Da).

The presence of ~14% (w/w) carbohydrate and its role in apoLp-III structure and/or function has been a subject of much speculation. ApoLp-III isolated from Lepidopteran species lack carbohydrate, while the Orthopteran species *Barytettix psolus* and *Melanoplus differentialis* contain high mannose structures (23). Glycosylation is not limited to Orthoptera as apoLp-III from *Derobrachus germanicus* (Coleoptera) is a glycoprotein as well (24). In contrast to locust apoLp-III, *M. sexta* apoLp-III is a non-glycosylated protein with a mass of 18,381 Da. Although these proteins have low sequence identity (29%), their molecular architecture is similar (3, 25). Furthermore, it has been shown that *M. sexta* apoLp-III can associate with *L. migratoria* LDLp. In in vitro assays it was determined that both natural and hybrid LDLp serve as substrates for flight muscle lipoprotein lipase (12). It was concluded that the carbohydrate moiety of *L. migratoria* apoLp-III is not required for recognition by the lipase. Recently, it was demonstrated that recombinant *L. migratoria* apoLp-III produced by *E. coli* (and thus lacking carbohydrate) showed enhanced activity in phospholipid vesicle clearance assays when compared to glycosylated (native) apoLp-III (13). However, other experiments suggest important distinctions exist between apolipoprotein-induced bilayer vesicle disruption and lipoprotein binding interactions (17). When Leu residues located in loops connecting helices were replaced by Arg, the hydrophobic character of the loop domains was altered. While these mutations caused an increase in the rate of phospholipid vesicle clearance, the ability of the mutant proteins to compete for a hydrophobic surface on PL-C-treated human LDL decreased, suggesting that fundamental differences exist between the two model assay systems. To further define the structure–function relationships in apoLp-III, we studied the effect of apoLp-III glycosylation in these assays. A summary of these data is presented in **Table 1**. Using negatively charged DMPG or zwitterionic DMPC vesicles, it was evident that enzymatic removal of the oligosaccharides resulted in a strong increase in the rate of vesicle clearance. However, removal of the carbohydrate chains had no effect on lipoprotein-binding, with both native and deglycosylated apoLp-III showing a similar ability to interact with PL-C-treated LDL. These data support the view that apoLp-III carbohydrate moieties do not affect the ability of this protein to bind to diacylglycerol-enriched lipoprotein surfaces.

ANS binding was used to determine whether the carbohydrate components of apoLp-III shield hydrophobic regions in the protein which function in lipid binding. ANS has been used to probe changes in the hydrophobic character of human (26) and chicken apoA-I (27). As we did not observe any changes in ANS fluorescence after deglycosylation, this indicated no increase in exposed hydrophobic surface as a result of carbohydrate removal. Therefore, we conclude that the carbohydrates do not shield such hydrophobic protein domains. Structural changes in the protein induced by deglycosylation might affect its flexibility and function. The observation that the protein Trp fluorescence emission maximum (332 nm) was not affected by re-

TABLE 1. Functional and structural comparison of native and deglycosylated apoLp-IIIa

	Native	Deglycosylated
DMPG clearance, $t_{1/2}$	150 s	13 s
DMPC clearance, $t_{1/2}$	3380 s	560 s
$[\text{ApoLp-III}]_{1/2}$ lipolyzed LDL[a]	6 μg	5.6 μg
$[\text{GdnHCl}]_{1/2}$[b]	0.60 m	0.58 m[d]
$\Delta G_D^{H_2O}$[c]	2.90 kcal/mol	2.68 kcal/mol[d]
Trp emission maximum	332 nm	332 nm
ANS emission maximum	475 nm	475 nm
Molecular mass	20,488 Da	17,583 Da

[a] $[\text{ApoLp-III}]_{1/2}$ lipolyzed LDL is the concentration of apoLp-III needed for 50% protection of PL-C-treated LDL (50 μg protein).

[b] $[\text{GdnHCl}]_{1/2}$ is the transition midpoint, the molar concentration of guanidine hydrochloride required to give a 50% decrease in ellipticity at 221 nm.

[c] $\Delta G_D^{H_2O}$ is the free energy of unfolding in the absence of guanidine hydrochloride.

[d] Data obtained from recombinant apoLp-III (18).

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moval of carbohydrate indicates no changes in the environment of Trp-115 and Trp-130. In addition, the midpoint of GdnHCl-induced denaturation of the protein was similar for recombinant apoLp-III and insect-derived protein (18), implying similar protein stability and flexibility. These results indicate that structural changes in apoLp-III are not responsible for the enhanced phospholipid clearance activity observed. Previous studies of apoLp-III interaction with lipid monolayers revealed a preference for diacylglycerol compared to phospholipids (28). This was not related to the presence of carbohydrate as its enzymatic removal had no effect on this preferential behavior. Also, deglycosylation did not affect the protein's interfacial behavior.

ApoLp-IIIa and -b differ by the presence of an N-terminal Arg-Pro extension on apoLp-IIIa. This prompted us to investigate whether functional differences existing between the two isoforms could be detected. Whereas the rate of DMPG clearance was faster for apoLp-IIIa versus apoLp-IIIb, DMPC clearance was similar for both. This may be explained by the increase in net positive charge in apoLp-IIIa owing to the presence of Arg-1. This may promote a stronger electrostatic attraction towards the negatively charged DMPG. Lipoprotein binding showed no difference between the two isoforms, supporting the view that the extreme N-terminus of apoLp-III is not involved in recognition or initiation of lipoprotein hydrophobic defects (17, 29).

The opposing effects of apoLp-III glycosylation observed in vesicle clearance and lipoprotein binding assays must be explained by different working mechanisms. It is conceivable that the carbohydrate structures, located roughly in the center of the long axis of the globular helix bundle on helices 1 (Asn-18) and 3 (Asn-85) (Fig. 2), impede penetration of the phospholipid bilayer, a required step in apoLp-III-induced transformation of phospholipid bilayer vesicles into disc-like complexes (**Fig. 8A**). This is due to repulsion of the bulky, polar and hydrated oligosaccharide moieties from the non-hydrated phospholipid bilayer interior. By contrast, during interaction with

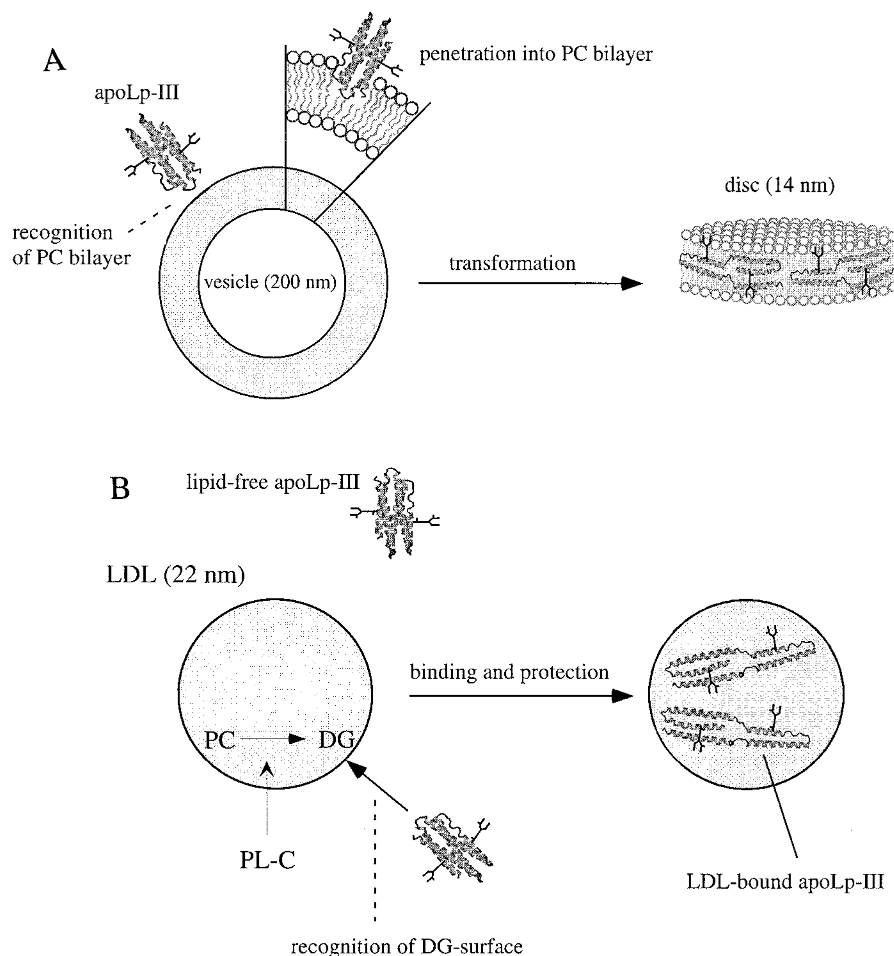

Fig. 8. Schematic representation of the interaction of apoLp-III with phospholipid vesicles (A) and lipoproteins (B). When encountering a bilayer vesicle of phosphatidylcholine (PC), apoLp-III penetrates the bilayer and disrupts it, inducing transformation into much smaller disc-like structures, a process that is impeded by apoLp-III oligosaccharide chains. In the case of lipoprotein binding, the appearance of diacylglycerol (DG) on the surface of spherical LDL results in recognition and binding of apoLp-III to the modified LDL particle surface, covering the otherwise exposed hydrophobic surface. Both processes are postulated to be accompanied by a major conformational change of the apolipoprotein. Figure is not drawn to scale. Stoichiometry of apoLp-III binding to LDL should not be inferred from inclusion of two apoLp-III molecules in the diagram.

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lipoproteins, apoLp-III is attracted to exposed hydrophobic regions on the surface of these spherical particles (Fig. 8B). Interaction is thought to occur via loops located at one end of apoLp-III and culminates in helix bundle opening and exchange of helix–helix interactions in the bundle for helix–lipid interactions on the lipoprotein surface (17, 29). Penetration of apoLp-III is not required, and binding is superficial, without reorganization of phospholipid molecules (30). The lipid-bound conformation of apoLp-III is thought to be similar in the two systems, adopting an open conformation where hydrophobic faces of the amphipathic helices interact with lipids.

It remains unclear why the protein is glycosylated in vivo. It cannot be excluded that the protein is involved in other processes which might benefit from the presence of carbohydrates, and recently new roles of apoLp-III in programmed cell death (31) and immune reactions (32, 33) have been reported.

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