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Spectroscopic and Lipid Binding Studies on the Amino and Carboxyl Terminal Fragments of *Locusta migratoria* Apolipophorin III[†]

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ABSTRACT: The structural basis for the lipid binding capability of *Locusta migratoria* apolipophorin III (apoLp-III) was assessed by characterizing the amino and carboxyl terminal halves of the protein. The native molecule (~20 kDa) was deglycosylated with endoglycosidase F (molecular mass of deglycosylated species ~18 kDa) and cleaved with endoproteinase Arg-C to yield two fragments with molecular masses of ~9 kDa each. The two fragments were purified by reversed-phase HPLC and identified by mass spectrometry, amino acid analysis and N-terminal sequencing as the amino terminal (N9) and carboxyl terminal (C9) halves. Due to the apparent discrepancy of the protease digestion pattern obtained compared to that expected from the deduced amino sequence of apoLp-III cDNA, we carried out partial amino acid sequencing of the fragments and cDNA sequencing for the entire protein. Circular dichroism spectroscopy of the N9 and C9 peptides revealed that both exist in buffer in a random coil state. However, addition of trifluoroethanol, a helix-inducing agent, resulted in the formation of an α-helix, reflecting an innate propensity of the peptides to adopt a helical conformation. When cosonicated with dimyristoylphosphatidylcholine (DMPC) both peptides assumed an α-helical conformation, indicative of interaction with the phospholipid. In the presence of phospholipids, a 22 nm blue shift in Trp fluorescence emission was observed in the case of the C9 peptide, suggesting that the Trp residues are located in a more hydrophobic environment. Electron microscopy revealed that, compared to native apoLp-III, both peptides possessed a reduced ability to transform DMPC vesicles to disklike complexes. Further, both the N9 and C9 peptides were unable to interact with lipoprotein surfaces, as evidenced by their inability to prevent turbidity development due to aggregation of human low-density lipoprotein induced by phospholipase C. These results show that the isolated amino and carboxyl terminal fragments of the protein, while able to interact with lipids, cannot mimic the functional capacity of the intact protein. Thus, we conclude that, aside from specific amphipathic α-helices, structural elements of the intact protein contribute to physiologically relevant lipid binding abilities of apoLp-III.

Apolipophorin III (apoLp-III)[1] is an insect apolipoprotein which reversibly binds to diacylglycerol-enriched lipophorin particles. Release of adipokinetic hormone during insect flight (Beenakkers et al., 1985) leads to loading of diacylglycerol (DAG) from fat body onto preexisting hemolymph high-density lipophorin particles (HDLp). Diacylglycerol enrichment of HDLp results in increased surface hydrophobicity which induces association of several apoLp-III molecules ensuring stabilization of the lipid-loaded particle and, as a result, formation of low-density lipophorin (LDLp). At sites of energy utilization, such as flight muscles, LDLp-associated DAG is hydrolyzed and free fatty acids released, resulting in conversion of LDLp into HDLp and free apoLp-III, which can then be reused in another cycle of lipid loading. This loading−transport−shedding cycle has been termed the "DAG shuttle" and appears to enhance transport of DAG from fat body to flight muscles [see Ryan (1990), Van der Horst (1990), and Blacklock and Ryan (1994) for reviews].

At present, locust apoLp-III is the only apolipoprotein for which the entire three-dimensional structure has been determined in the lipid-free state. The five antiparallel α-helices are amphipathic, exposing their hydrophilic amino acid side chains to the exterior and the hydrophobic amino acid residues to the interior of the helix bundle (Breiter et al., 1991). Interestingly, a similar molecular architecture has been described for the LDL receptor binding domain of human apolipoprotein E (Wilson et al., 1991). Since hydrophobic amino acid side chains orient toward the center of the helix bundle, binding of apoLp-III to LDLp likely induces a conformational change of the protein, which enables interaction of the hydrophobic face of the amphipathic α-helices with LDLp−lipid. It has been postulated that apoLp-III interaction with lipid involves an opening of the helix bundle at putative hinge regions, thereby facilitating

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[1] Abbreviations: ApoLp-III, apolipophorin III; CD, circular dichroism, endo F, endoglycosidase F; Arg-C, endoproteinase Arg-C; PL-C, phospholipase C; DMPC, dimyristoylphosphatidylcholine.

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direct interaction of the exposed hydrophobic amino acid residues with lipid (Kawooya et al., 1986; Wells et al., 1987; Breiter et al., 1991). Making use of phospholipid−apoLp-III complexes, experimental evidence for such a conformational change upon lipid binding has been provided for apoLp-III from both *Manduca sexta* (Wientzek et al., 1994) and *Locusta migratoria* (Weers et al., 1994).

Despite knowledge of the three-dimensional structure of *L. migratoria* apoLp-III at 2.5 Å resolution, the mechanism for its conformational changes and the mode and sites of interaction with lipid surfaces remain undefined. Earlier work using *M. sexta* apoLp-III (Narayanaswami et al., 1994) indicated that the isolated carboxyl terminal helical segment is unable to interact with phospholipid bilayers or lipoprotein surfaces. The results suggested that the structural integrity of the terminal segment is maintained by domains within the protein which are required to initiate and stabilize the lipid binding interaction. In order to further understand the functional domains of apoLp-III, fragments of *L. migratoria* apoLp-III corresponding to the amino and carboxyl terminal halves (N9 and C9 peptides, respectively) were generated by proteolytic digestion using endoproteinase Arg-C (Arg-C), which cleaves specifically at the carboxyl side of arginine residues. Structural characterization of the fragments was carried out by circular dichroism and fluorescence spectroscopy, and functional aspects were determined by assessing their ability to bind to phospholipid bilayer vesicles and to lipoprotein surfaces. We also present the corrected primary sequence of *L. migratoria* apoLp-III as deduced from cDNA sequence and partial amino acid sequence determinations.

EXPERIMENTAL PROCEDURES

Protease Digestion and Reversed-Phase High-Performance Liquid Chromatography. ApoLp-III from *L. migratoria* was isolated as described, (Van der Horst et al., 1991). ApoLp-IIIa isoform was used in all experiments unless otherwise indicated. ApoLp-III was deglycosylated in 50 mM Tris-HCl, pH 8.5, containing 5 mM EDTA using endoglycosidase F/*N*-glycosidase F (Boehringer Mannheim GmbH, Mannheim, Germany; 0.005 units/50 μg of protein) during a 24 h incubation at 25 °C. After addition of endoproteinase Arg-C (Boehringer Mannheim; 0.2 units/50 μg of protein) the incubation was continued for 2 or 18 h at 37 °C. The reaction was stopped by the addition of sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample treatment buffer and subjected to tricine-based SDS−PAGE as described by Schägger and Von Jagow (1987). ApoLp-III fragments were separated by reversed-phase high-performance liquid chromatography (RP-HPLC; Bio-Rad, Munich, Germany), using an Aquapore RP-300 C9 column (Pierce, Rockford, IL). Gradient elution was performed with water and acetonitrile in the presence of 0.1% trifluoroacetic acid. The eluate was monitored for protein at 227 nm (Bio-Rad UV/visible Model 1706) and for tryptophan using a fluorescence monitor (Shimadzu, RF-530) with excitation and emission at 276 and 340 nm, respectively.

cDNA Cloning and Sequencing. Isolation of poly-$(A)^+$RNA from the fat bodies of adult locusts, synthesis of cDNA, and construction of a cDNA library in λgt11 were described previously by Van der Horst et al. (1993). ApoLp-III cDNA was amplified using synthetic oligonucleotide primers based on the locust apoLp-III cDNA sequence (Kanost et al., 1988) and subcloned in pBluescript vector (Stratagene, La Jolla, CA). The nucleotide sequence of several clones was determined from both DNA strands by the dideoxy chain termination method (Sanger et al., 1977). The experiment was performed a second time to confirm that the sequence did not include any errors caused by misincorporation of nucleotides during amplification.

Northern Blot Analysis. Total RNA was extracted from fat bodies of adult locusts by a modified method of Chomczynski and Sacchi (1987) using RNAzol B (Cinna/Biotecx, Houston, TX). A 10 μg sample of total RNA was treated with glyoxal/dimethyl sulfoxide, according to Thomas (1980) and probed with apoLp-III cDNA fragment by the random priming method using a random primed DNA labeling kit (Boehringer Mannheim).

Peptide−Lipoprotein Interactions. Phospholipase C- (PL-C-) induced aggregation of human low-density lipoproteins (LDL) (Suits et al., 1989) has been shown to be prevented by the presence of exchangeable apolipoproteins (Liu et al., 1993). Human LDL was isolated as described by Schumaker and Puppione (1986). The ability of apoLp-III, or fragments thereof, to associate with lipids was followed by this assay system consisting of 20 μg of human LDL protein in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 2 mM $CaCl_2$ at 37 °C in a final volume of 250 μL in a microtiter plate. Native apoLp-III was added as a positive control. The N9 and C9 peptides were added alone or in combination at the indicated levels and the reaction was started by the addition of 400 mU of PL-C. The absorbance was followed at 340 nm as a function of time in a plate reader (SLT Labinstruments).

Peptide−Diacylphospholipid Interactions. Native apoLp-III or the isolated peptides were added to a dried film of dimyristoylphosphatidylcholine (DMPC) in 150 mM NaCl, 1 mM EDTA, pH 8.0, at a lipid to peptide ratio of 2.5:1 (w/w) and sonicated in a bath sonicator at 24 °C for ~10 min until clear (Lund-Katz et al., 1990). The ability of the peptides to associate with multilamellar bilayer vesicles was determined by CD spectroscopy and electron microscopy.

Circular Dichroism and Fluorescence Spectroscopy. Circular dichroism (CD) spectra of the N- and C-terminal 9K fragments (N9 and C9 peptides, respectively), ~1.5 mg/mL, were obtained in 50 mM sodium phosphate buffer, pH 7.0, and in buffer containing 50% trifluoroethanol (TFE) on a Jasco J-720 spectropolarimeter at 25 °C (Jasco Inc., Easton, MD) as described earlier (Narayanaswami et al., 1994). The fluorescence emission of Trp was monitored at an excitation wavelength of 290 nm, and the emission was scanned from 300 to 400 nm using a Perkin-Elmer MPF-44B spectrofluorometer with the temperature maintained at 20 °C in a thermostated cell holder. The excitation and emission slit widths were 8 and 10 nm, respectively.

Other Analytical Methods. The lipid−peptide complexes were examined under a Philips EM420 electron microscope operated at 100 kV. The samples were adsorbed on a carbon-coated grid, washed with 10 mM Tris/10 mM NaCl/1.5 mM $MgCl_2$, pH 8.0, and negatively stained with 2% sodium phosphotungstate, pH 7.0. Molecular weight measurements were made using a VG Quattro electrospray mass spectrometer (Fisons Instruments, Manchester, UK). The electrospray potential was ~3.5 kV. N-Terminal sequencing of the peptides was performed using standard Edman chemistry on a Hewlett-Packard G1000A protein sequencer

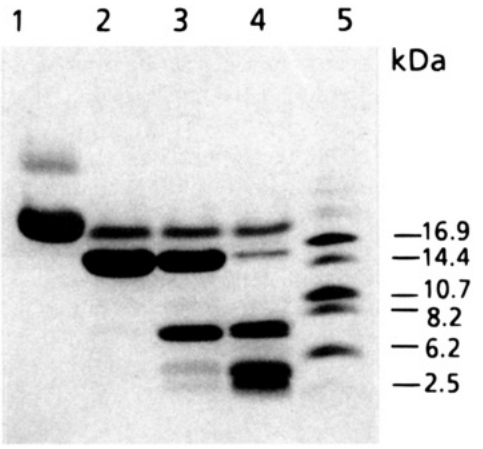

FIGURE 1: Tricine-based SDS−PAGE analysis of the endoprotease Arg-C digestion of deglycosylated apoLp-III: (lane 1) untreated native apoLp-III; (lane 2) deglycosylated apoLp-III; (lanes 3 and 4) deglycosylated apoLp-III treated with Arg-C for 2 h and 18 h, respectively; (lane 5) peptide molecular weight markers. Conditions for deglycosylation using endo F and proteolysis using Arg-C are as described under Experimental Procedures.

coupled to a HPLC (HP 1090LC) with a PTH-AA column (250 × 2.1 mm) with a 1090FPD detector set at 269 nm. Amino acid analysis of samples was carried out in a Beckman System 6300 amino acid analyzer, System Gold Version 6.01.

RESULTS

Endoprotease Digestion. In an effort to determine lipid binding domains in *L. migratoria* apoLp-III, proteolysis was employed to generate fragments which were then characterized in terms of structure and function. As only a few Arg residues have been reported to be present in apoLp-III (Arg 77, Arg 80, Arg 119) (Kanost et al., 1988), an enzyme that specifically cleaves at arginine residues, endoproteinase Arg-C (Arg-C), was tried. Arg-C cleaves at the carboxyl side of arginine residues and appears to have no preference for neighboring amino acid residues for optimal activity (Schenkein et al., 1977). Prolonged incubations of apoLp-III with Arg-C for 18 h at 37 °C or in the presence of urea or 0.1% SDS did not result in the production of demonstrable amounts of apoLp-III fragments. Examination of the amino acid sequence revealed the presence of a putative glycosylation site at Asn 83, suggesting that the glycosylated moiety at this site denies access to Arg-C. Indeed, deglycosylation of apoLp-III with endo-F followed by Arg-C treatment resulted in cleavage of apoLp-III into smaller fragments (Figure 1, lanes 1−4). Incubation for 2 h resulted in a major band of ~8 kDa (Figure 1, lane 3). The 2 h digest was subjected to RP-HPLC purification, and the eluate was simultaneously monitored for protein and tryptophan fluorescence. Two peptides were separated, one with an intrinsic tryptophan fluorescence emission and another that lacked fluorescence emission. Upon prolonged incubation (18 h), bands with lower molecular weights began to appear (Figure 1, lane 4).

Preliminary Identification of ApoLp-III Fragments. The RP-HPLC-purified peptides were subjected to electrospray ionization mass spectrometry, and the masses of the peptides lacking or containing intrinsic fluorescence were found to be 8863 and 8738 Da, respectively (Table 1). Due to the presence of tryptophan residues only in the C-terminal half

Table 1: Endoproteinase Arg-C Fragmentation Profile Expected in the Old and Corrected Amino Acid Sequence of apoLp-IIIa and Comparison with the Measured Molecular Masses of the Peptides Obtained by Electrospray Mass Spectrometry[a]

calcd masses[b] of Arg-C-treated apoLp-III fragments		measd masses of fragments[e]
old sequence[c]	corrected sequence[d]	
−2 to −2 (175)	−2 to −2 (175)	
78 to 80 (393)	−1 to 80 (8701)	
81 to 119 (4213)	81 to 162 (8732)	8738
120 to 161 (4374)	−2 to 80 (8856)	8863
78 to 119 (4587)	−1 to 162 (17414)	
−1 to 77 (8412)		
78 to 161 (8942)		
81 to 161 (8568)		
−2 to 77 (8568)		
−2 to 80 (8943)		
−1 to 80 (8787)		

[a] Endoproteinase Arg-C digestion was carried out at with deglycosylated apoLp-IIIa (residues −2 to 162) (0.2 unit/50 μg of protein) for 2 h at 37 °C. [b] The calculated masses were obtained from the amino acid sequence using the program MacProMass (Terry Lee, City of Hope, Duarte, CA). The size of the fragment is indicated by the residue numbers with the molecular weights in parentheses. [c] Primary deduced amino acid sequence of apoLp-III as reported by Kanost et al., (1988). [d] The corrected sequence as determined in this paper based on partial amino acid and complete cDNA sequencing. [e] Molecular weights of the peptides obtained as determined by electrospray ionization mass spectrometric analysis as described in Experimental Procedures.

of the protein, the latter peptide was identified as the C-terminal half and the former, lacking fluorescence, as the N-terminal half. The molecular weights of the fragments obtained did not, however, correspond to the expected values (Table 1) from the published locust apoLp-III sequence (Kanost et al., 1988). The peptide with positive Trp fluorescence gave rise to a peptide of ~6.5 kDa during extended incubation times. This was surprising since the C terminal peptide included an expected cleavage site at Arg 119 which would give rise to peptides of ~4 kDa in size. The peptide without intrinsic fluorescence was further degraded to yield peptides of lower molecular mass which could be accounted for only on the basis of nonspecific cleavage at lysine residue(s) (for example, K50) upon prolonged incubations (determinations done by amino acid and mass spectral analysis and N-terminal sequencing). Thus, the fragmentation pattern obtained did not correlate with the expected number and size of peptides calculated by assuming complete and/or partial digestion at Arg residues (see Table 1). This apparent discrepancy prompted us to perform amino acid and cDNA sequencing of *L. migratoria* apoLp-III. From the C-terminal fragment (8738 Da), the first 56 residues were determined by automated Edman degradation. There were several differences from the sequence published by Kanost et al. (1988).

cDNA Sequence Analysis. The nucleotide sequence of the apoLp-III cDNA and the deduced amino acid sequence are shown in Figure 2. The apoLp-III cDNA consisted of 614 bp and an open reading frame of 540 bp encoding the apoLp-III precursor of 180 amino acid residues. The site of cotranslational cleavage of the signal peptide was predicted by the method of Von Heijne (1986). Signal peptide cleavage occurs after Ala −3, giving rise to apoLp-IIIa, the longest isoform of apoLp-III, (Van der Horst et al., 1991) of 164 amino acids (see Figure 2). This is further processed in the hemolymph by the removal of the first two amino acids (Arg −2 and Pro −1) to apoLp-IIIb (1−162) (Weers

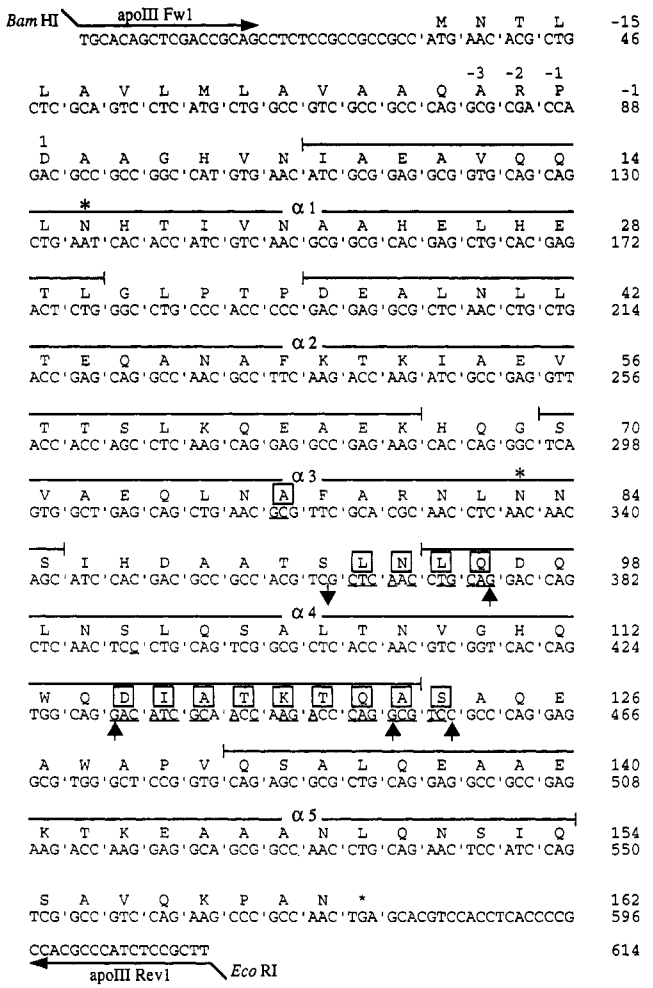

FIGURE 2: Nucleotide and deduced amino acid sequence of locust apoLp-III cDNA. Differences with the sequence reported by Kanost et al. (1988) are underlined at the nucleotide level and indicated with squares at the amino acid level; the positions of a single nucleotide deletion and the four nucleotide insertions are indicated with arrows. The forward and the reverse primers used are indicated. The putative signal peptide cleavage site is after Ala −3, thus resulting in a secreted apoLp-IIIa of 164 amino acid residues. The putative glycosylation sites are indicated by large asterisks and the stop codon by a small asterisk. The helical domains are indicated as α-1 to α-5 based on the consensus secondary structural analysis of three different criteria (distance matrix approach, the ϕ/ψ angles, hydrogen bonds) by the program VADAR (Wishart et al., 1994b), which uses the X-ray crystallographic coordinates (Breiter et al., 1991). The putative hinge domains are located between helices 2 and 3 and helices 4 and 5.

et al., 1993), the amino acid sequence of which starts with Asp 1 (Figure 2).

The nucleotide sequence obtained was different at several positions (including a single nucleotide deletion and four single nucleotide insertions) from the apoLp-III sequence reported by Kanost et al. (1988). The deduced amino acid sequence derived from the nucleotide sequence determined herein (Figure 2) was in full accordance with the amino acid sequence of the peptide fragments determined after Arg-C digestion of apoLp-III, (see below) as well as that recently reported by Smith et al. (1994) since submission of our work. To determine the length of the apoLp-III mRNA, RNA was extracted from locust fat body and prepared for Northern blot analysis. The apoLp-III cDNA probe showed hybridization with a single band of ~1050 nucleotides (results not shown).

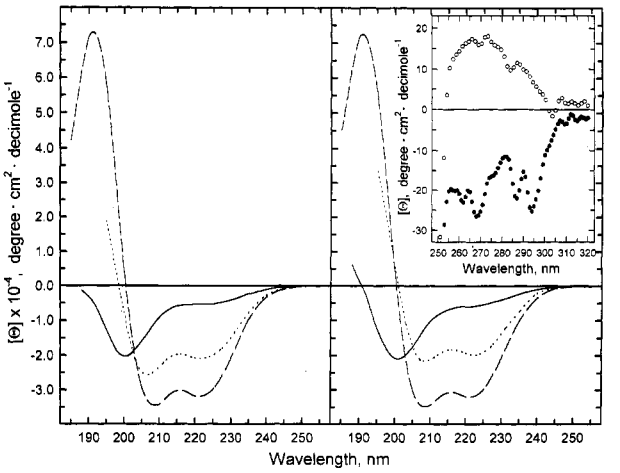

FIGURE 3: CD spectra of the N9 and C9 peptides recorded in buffer at a concentration of 1 mg/mL in 150 mM NaCl and 1 mM EDTA, pH 8.0 (straight line), in 50% TFE (dashed line), and in the presence of DMPC (dotted line) at a lipid/peptide ratio of 2.5:1 (w/w) in buffer: (panel A, left) N9 peptide; (panel B, right) C9 peptide. Inset shows near-UV CD spectrum of the C9 peptide in buffer (open circles) and in the presence of DMPC (2.5:1) (closed circles).

Confirmation of Fragment Identity. The fragmentation pattern of apoLp-III treated with Arg-C was consistent with that expected from the corrected locust apoLp-IIIa sequence. The calculated masses of the two peptides according to the corrected sequence are 8856 (residues −2 to 80) and 8732 Da (residues 81−162), which correspond very well with the measured masses of 8863 (without intrinsic fluorescence) and 8738 (with intrinsic fluorescence), respectively. These two peptides correspond to the N-terminal and C-terminal halves of the deglycosylated apoLp-III when cleaved at Arg 80 (Table 1, Figure 2) and will be referred to as the N9 and C9 peptides, respectively. Their identity was further confirmed by N-terminal sequencing (Arg −2 to Ile 8 in the N9 peptide and Asn 81 to Leu 135 in the C9 peptide) and amino acid analysis (data not shown). Apparently, cleavage at Arg −2 does not take place, as evidenced by N-terminal sequencing and mass spectrometry of the N9 peptide.

Structural Characterization of the N9 and C9 Peptides of ApoLp-III by Circular Dichroism Spectroscopy. CD spectra of the N9 and C9 peptides were carried out in buffer alone, as well as in the presence of 50% TFE or DMPC (Figure 3). While both peptides were present in a random coil state in buffer, each displayed the ability to adopt an α-helical structure when present in TFE, as evidenced by the appearance of the pronounced troughs at 222 and 207 nm. Further, when cosonicated with DMPC, both the peptides assumed an α-helical conformation although to different extents, comparable to that of native apoLp-III (Weers et al., 1994). The transition from random coil to helical conformation upon sonication of the two peptides with DMPC vesicles suggests an ability to interact with phospholipid bilayer surfaces. The near-UV CD spectrum of the C9 peptide (Figure 3B, inset) in the absence of lipids shows a profile similar to that of lipid-free native apoLp-III (Weers et al., 1994), although the extremes at 292, 286, 278, and 268 nm observed with the native protein were less discernible in the peptide due to its random coil state in buffer. Upon interaction with DMPC, however, the spectrum becomes more defined, with pronounced troughs that are reversed in sign, clearly indicating a more hydrophobic environment for the aromatic residues (Lux et al., 1972).

Fluorescence Measurements. Fluorescence emission of Trp was monitored for the C9 peptide to further assess the binding status of the peptide, with native apoLp-III as control. It has been shown that association of native apoLp-III with phospholipid bilayers resulted in a large blue shift in λ_{max} of emission (345−321 nm), reflecting a predominantly hydrophobic environment for Trp (Weers et al., 1994). Fluorescence emission of the C9 peptide containing the two Trp residues (Trp 113, Trp 128) was therefore monitored in the absence and presence of DMPC (data not shown). A blue shift in the λ_{max} of emission from 345 to 323 nm was observed, indicative of interaction with lipids and a largely hydrophobic environment for the Trp residues in the lipid-bound state of the C9 peptide.

Peptide−Phospholipid Bilayer Interaction. The ability of exchangeable amphipathic apolipoproteins to form disk-like complexes with lipid bilayers has been used as an indicator of functional phospholipid association. Native apoLp-III possesses the ability to transform multilamellar phospholipid vesicles to disk-like structures (Weers et al., 1994). The N9 and C9 peptides, however, failed to cause a similar transformation when they were added to preformed DMPC vesicles. Upon cosonication with DMPC in buffer, the peptides displayed a partial ability to alter the morphology of phospholipid vesicles (Figure 4, panels B and C). Under similar conditions the native protein elicited an efficient interaction, causing a complete transformation to disklike structures (panel A). Thus, although the spectroscopic data suggested a lipid binding interaction, it appears that, compared to intact apoLp-III, association of the N9 and C9 peptides with model lipid bilayers is incomplete.

Peptide−Lipoprotein Interaction. The ability of the C- and N-terminal halves of deglycosylated apoLp-III to associate with lipoprotein surfaces was assessed by an assay (Liu et al., 1993; Singh et al., 1994) which is based on the phospholipase C- (PL-C-) induced aggregation of human LDL (Suits et al., 1989). The ability of exchangeable apolipoproteins to bind to the phospholipid-depleted lipoprotein surface and prevent aggregation has been exploited as a facile and rapid assessment of the apolipoprotein function. In the absence of PL-C, LDL remains stable while addition of PL-C triggers onset of aggregation, as seen by the increase in turbidity as a function of reaction time (Figure 5). Native apoLp-III (50 μg) afforded protection by preventing the onset of turbidity to a large extent. On the other hand, the N9 and C9 peptides were unable to prevent turbidity development, when added in equimolar amounts, on an equal weight basis or when added in excess (data not shown). Further, when both C9 and N9 peptides (50 μg each) were added simultaneously in an effort to reconstitute the functional behavior of the whole protein, no additive or cooperative effect was observed. Deglycosylated apoLp-III (50 μg) on the other hand, afforded protection against turbidity, indicating that glycosylation has little role in lipid binding as reported previously (Demel et al., 1992).

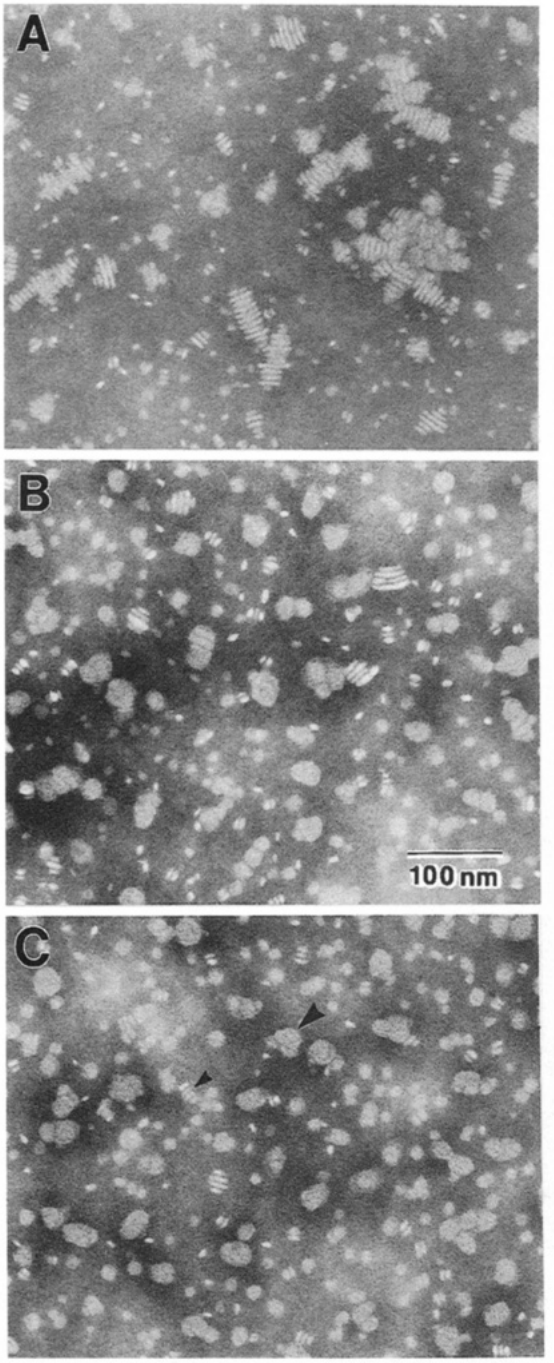

FIGURE 4: Effect of apoLp-III and the N9 and C9 peptides on the morphology of DMPC vesicles. The tranformation in the morphology of DMPC vesicles was observed under an electron microscope, in the presence of apopLp-III (panel A), the N9 peptide (panel B), and the C9 peptide (panel C), after adsorption of the samples on a carbon-coated grid and negative staining with 2% sodium phosphotungstate. The bigger arrow indicates a multilamellar vesicle and the smaller arrow indicates a stack of disks.

DISCUSSION

L. migratoria apoLp-III is an exchangeable apolipoprotein characterized by an up-and-down helical bundle motif as seen in the crystal structure which has been solved at 2.5 Å resolution (Breiter et al., 1991). The native protein is glycosylated and lacks Cys, Met, and Tyr residues. Two isoforms of *L. migratoria* apoLp-III have been identified, apoLp-IIIa (−2 to 162) and apoLp-IIIb (1−162) (Van der Horst et al., 1991), with an overall identical primary sequence except for the presence of two additional amino acid residues (Arg-Pro) at the N-terminus of apoLp-IIIa. Although the biochemical characteristics and biosynthetic pathways of apoLp-III are fairly well-characterized (Weers et al., 1993), the structural domains involved with respect to lipid binding remain unclear. In an attempt to map potential lipid binding domains and to address the question of the relevance of glycosylation and the structural elements involved in lipid

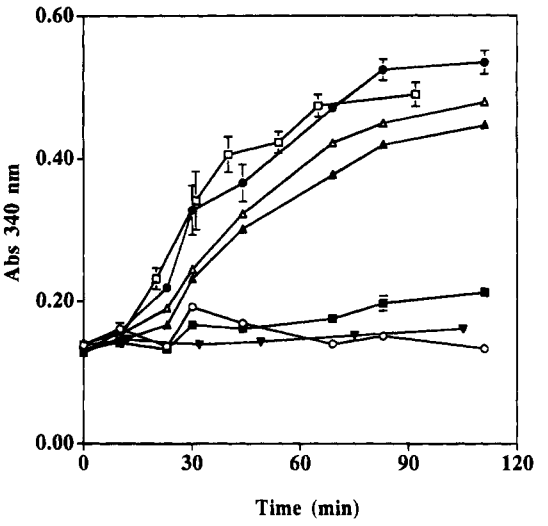

FIGURE 5: Effect of native apoLp-III and the N9 and C9 peptides on PL-C-induced aggregation of human LDL. The assay mixture contained human LDL (20 μg of protein) in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 2 mM $CaCl_2$, and 50 μg of the protein or peptide, at 37 °C in a final volume of 250 μL. The reaction was started by the addition of 400 mU of PL-C: open circle, control incubation without PL-C; closed circle, LDL plus PL-C in buffer; closed square, native apoLp-III; closed diamond, deglycosylated apoLp-III; open triangle, N9 peptide; closed triangle, C9 peptide; open square, 50 μg each of the N9 and C9 peptides. Turbidity was monitored at 340 nm. Controls with endo-F added to LDL and PL-C resulted in turbidity similar to that observed when LDL was treated with PL-C (data not shown). Values are mean ±SD (n = 3).

binding, apoLp-III was deglycosylated and treated with proteases to generate smaller fragments. Structural characterization of the peptides by CD and fluorescence spectroscopy and functional characterization by phospholipid binding studies were carried out and compared with the properties of the native protein reported earlier (Weers et al., 1994).

Arg-C was the protease of choice due to the low occurrence of Arg residues in native apoLp-III and the relatively higher specificity of this protease for Arg residues. There was, however, no concurrence between the sizes of the peptides obtained and the published primary sequence of *L. migratoria* apoLp-III (Kanost et al., 1988). This disparity, which could not be explained on the basis of nonspecific cleavage by Arg-C, prompted us to redetermine the amino acid and cDNA sequences of the protein. Based on the corrected sequence obtained, the fragmentation profile and sizes of peptides obtained by Arg-C digestion could be correlated directly. cDNA sequencing revealed differences in primary amino acid sequence, the most significant of which are the absence of Arg 77 and Arg 119 and Pro 95, Pro 118, and Pro 120 (Figure 2). The corrected sequence has not altered the overall sequence similarity of *L. migratoria* and *M. sexta* apoLp-III, which is still maintained at over 50%, as determined by SEQSEE (Wishart et al., (1994a,b).

Initially, the relevance of glycosylation to the lipid binding capabilities of apoLp-III was investigated. Earlier work on the structural stability of *L. migratoria* apoLp-III indicated that upon deglycosylation the α-helical content of the protein decreased from 78% to ~46% (Weers et al., 1994). However, upon interaction with lipids, the α-helical content increased to levels comparable to that observed with native apoLp-III, suggesting that glycosylation is probably not involved directly in lipid binding. This observation was confirmed by lipoprotein binding assays capable of detecting association of exchangeable apolipoproteins with the monolayer surface of human LDL. Deglycosylated apoLp-III prevented the PL-C-induced onset of turbidity to the same extent as the native protein suggesting that glycosylation in apoLp-III is not necessary for the initiation or stabilization of lipid binding. This is not unlikely since the functionally homologous *M. sexta* apoLp-III is not glycosylated (Kawooya et al., 1984). A role in maintaining the structural integrity of lipid-free apoLp-III has been attributed to glycosylation of apoLp-III at Asn residues (Weers et al., 1994). The physiological relevance of the unique oligosaccharide moieties (Hård et al., 1993) containing (2-aminoethyl)phosphonated mannose/*N*-acetylglucosamine in the carbohydrate network is unknown.

Deglycosylation of the native protein is a crucial step to enable access of Arg-C to Arg 80. The proximal location of Asn 83 (one of the two glycosylation sites) to Arg 80 conceivably shields the substrate from the protease (Hård et al., 1993). Upon deglycosylation, Arg-C cleavage was rendered facile, yielding digestion products corresponding to the N- and C-terminal halves (9 kDa each) of the whole apoLp-III. Recently, it has been shown with *M. sexta* apoLp-III that a 36 residue carboxyl terminal fragment, which corresponds to the entire fifth helix of *L. migratoria* apoLp-III, is unstructured when present in buffer and in the presence of phospholipid bilayers and is incapable of interaction with lipoprotein surfaces (Narayanaswami et al., 1994). This peptide has the propensity to form an amphipathic α-helix as predicted by the program SEQSEE (Wishart et al., 1994a) and as evidenced by CD spectroscopy in the presence of TFE or detergent micelles. It was suggested that helix−helix interactions might be required to maintain the helical integrity of the C-terminal segment to enable it to elicit phospholipid binding interactions. In the present study, the *L. migratoria* C9 peptide used encompasses helices 4 and 5 with a loop and a part of helix 3, while the N9 peptide includes helices 1 and 2 connected by a short loop (β-turn) and a part of the amino terminal end of helix 3 (Figure 6). Both the N9 and C9 peptides remain unstructured in buffer but adopt an α-helical conformation in the presence of TFE or upon interaction with DMPC bilayers. The transition from random coil to α-helical structure in the N9 and C9 peptides in the presence of phospholipid bilayers is a preliminary indication of their association with lipids. This was further supported by the blue shift in Trp fluorescence emission in the DMPC−C9 peptide complex. However, electron microscopic observations showed that both the peptides have only a partial ability to form disks compared to intact apoLp-III. Furthermore, lipoprotein binding assays indicated a clear inability of these peptides to interact stably with phospholipid monolayer surfaces. Thus, it is possible that additional structural elements are required for the N9 and C9 peptides to confer an ability to initiate and maintain an interaction with phospholipid mono- and bilayers.

In order to explain the mode of interaction of apoLp-III with lipophorin particles in hemolymph, a model was proposed where the protein opens at putative hinge domains (Breiter et al., 1991) and the exposed hydrophobic surface of the helical bundle interacts with diacylglycerol moieties in the expanding lipophorin particle (Wang et al., 1992). A similar conformational change possibly takes place during

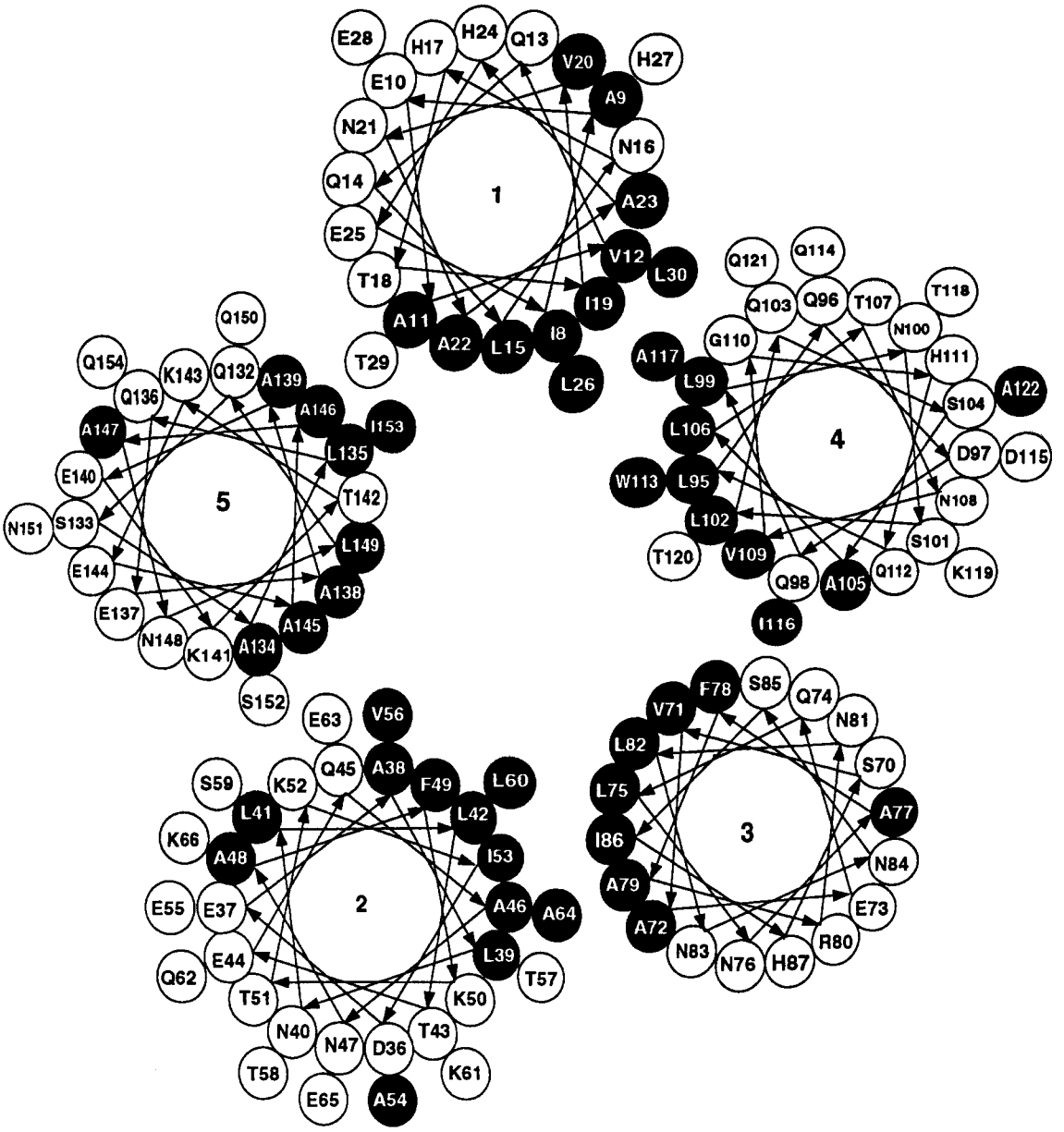

FIGURE 6: Helical wheel representation of apoLp-III in the lipid-free helix bundle state. The location of the helices are based on the analysis by the program VADAR (Wishart et al., 1994b), which uses the X-ray crystallographic coordinates (Breiter et al., 1991) (also see Figure 2). In helix 3, residues I86 and H87 have been included to maintain the continuity of the hydrophobic and solvent-exposed surfaces, respectively, even though VADAR excludes these two residues in the helical segment. The hydrophobic residues are shown in black and orient toward the interior of the helix bundle, and the other polar and nonpolar residues are shown in white. The helices are juxtaposed according to their orientation in the crystal structure (Breiter et al., 1991).

association of the native molecule with phospholipid bilayers, resulting in interaction of the hydrophobic surface with the methylene groups of the fatty acyl chains of the phospholipid bilayer. It is possible that the interaction of the two peptides with the phospholipid monolayer is weak and unstable in comparison with that of the native protein.

A helical wheel analysis of the five helices of locust apoLp-III was carried out to further characterize their helical properties (Table 2, and Figure 6). The mean hydrophobic moment and the hydrophobicity per residue of the nonpolar face were calculated for the individual helices in the two peptides. All the helices are amphipathic and have high to moderately high hydrophobicities and hydrophobic moments. The polar faces on these helices subtend angles of $>180°$ perpendicular to the helical axis. Further, helices 2 and 5 have the properties of a class A helix (Segrest et al., 1990, 1992, 1994) characterized by the presence of distinct polar and hydrophobic faces with an interface having a cluster of positively charged residues and the center of the polar face bearing negatively charged residues. The remaining helices do not have a distinct profile of charge clustering and therefore appear to fall under the category of class G*. In human apolipoproteins, Segrest et al. (1992, 1994) have shown the presence of amphipathic helical domains that do not conform to the characteristics of the class A motif. These have been classified as class G* with a random radial distribution of charged residues, or class Y where the charged residues are distributed in a "Y" motif (Segrest et al., 1992, 1994). Furthermore, helical segments with class G* characteristics appear to be nonlipid binding (Segrest et al., 1992). In the N9 and C9 peptides characterized here, it is possible that the presence of a single class A helix in each of the

Table 2: Hydrophobic Moment and Hydrophobicity Values of the Helices in *L. migratoria* ApoLp-III

helix	mean hydrophobic moment per residue[a]	mean hydrophobicity per residue of nonpolar face[b]	helix type[c]
1 (I8−L30)	0.487	0.878	G*
2 (D36−K66)	0.365	0.809	A
3 (S70−S85)	0.518	0.762	G*
4 (L95−A122)	0.373	0.694	G*
5 (Q132−Q154)	0.388	0.637	A

[a] Mean hydrophobic moment per residue was calculated using the normalized consensus hydrophobicity scale of Eisenberg et al., (1984). [b] Mean hydrophobicity of the nonpolar face per residue was calculated using the normalized consensus hydrophobicity scale of Eisenberg et al., (1984). [c] Classification of type of helix was done based on Segrest et al. (1990, 1992), and residues involved in helical conformation were obtained from the crystal structure (Breiter et al., 1991).

peptides (helix 2 and helix 5, respectively) confers a partial ability to interact with phospholipid bilayers.

The cleavage of apoLp-III to generate the N9 and C9 peptides appears to result in disruption of the helix−helix and hydrophobic interactions present in the intact protein which induces a loss of secondary structure and lipid binding capabilities. A large contiguous hydrophobic surface such as that present in the open form of the native protein is probably an absolute requirement for stable lipoprotein association. Such a surface is not recreated when the molecule is truncated into two halves. In contrast to the behavior of apoLp-III peptides, thrombolytic fragments of human apo E (22 kDa N-terminal and 10 kDa C-terminal peptides) retained their respective structural and functional characteristics (Aggerbeck et al., 1988; Gianturco et al., 1983). The 22 kDa N-terminal fragment encompassing the receptor binding domain and possessing some lipid binding capability has been crystallized, and the three-dimensional structure (Wilson et al., 1991) presents a striking similarity with that of the intact locust apoLp-III. Further, the N9 and C9 peptides are comparable in length to some human apolipoproteins such as apo C-I (57 residues), apo C-II (78 residues), and monomeric apo A-II (77 residues) [see Sparrow and Gotto (1980)]. These apolipoproteins (and some of their protease-digested fragments and synthetic peptides) have the capability to bind to lipoprotein particles in the plasma such as HDL, VLDL, and chylomicrons.

It is not clear what factors play a role in the initiation and stabilization of interaction of apolipoproteins with lipid surfaces. Both ionic (Zhang et al., 1993) and hydrophobic (Breiter et al., 1991) interactions have been suggested to play a role in initiating the binding events. Further studies are in progress to understand the mechanisms underlying the binding of apoLp-III to lipid surfaces and the conformational changes that take place in the native protein upon lipid interaction.

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