Factors Affecting the Stability and Conformation of *Locusta migratoria* Apolipophorin III†

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Received October 26, 1993; Revised Manuscript Received January 10, 1994®

ABSTRACT: Apolipophorin III (apoLp-III) from the migratory locust, *Locusta migratoria*, represents the only full-length apolipoprotein whose three-dimensional structure has been solved. In the present study, spectroscopic methods have been employed to investigate the effects of deglycosylation (via endoglycosidase F treatment) and complexation with lipid on the stability and conformation of this protein. Addition of isolated lipid-free apoLp-III to sonicated vesicles of dimyristoylphosphatidylcholine (DMPC) resulted in the formation of relatively uniform disklike complexes with an average Stokes diameter of 13.5 nm. Flotation equilibrium experiments conducted in the analytical ultracentrifuge revealed a particle molecular mass of 588 500 Da. Chemical cross-linking and compositional analysis of apoLp-III·DMPC complexes indicated five apoLp-III molecules per disk and an overall DMPC:apoLp-III molar ratio of 122:1. Circular dichroism (CD) spectra of apoLp-III samples suggested a loss of α-helical structure upon deglycosylation, while complexation with DMPC did not significantly alter the helix content (estimated to be >75%). Fluorescence spectroscopy revealed that the apoLp-III tryptophan fluorescence emission maximum was blue-shifted from 347 to 332 and 321 nm upon deglycosylation and complexation with DMPC, respectively. In quenching experiments with native apoLp-III, tryptophan residues were shielded from the positively charged quencher, CsCl. Increased exposure to KI, CsCl, and acrylamide was observed upon deglycosylation, whereas complexation with DMPC yielded lower K_{sv} values for KI and acrylamide and an increased value for CsCl versus native lipid-free apoLp-III. In guanidine hydrochloride denaturation studies monitored by CD or fluorescence, native, lipid-free apoLp-III displayed a denaturation midpoint of 0.60 M, and $\Delta G_D^{H_2O} = 5.37$ kcal/mol was calculated. Deglycosylation decreased the stability of apoLp-III, while complexation with lipid stabilized its structure (increasing the denaturation midpoint substantially). The effect of solvent pH on the secondary structure stability of apoLp-III suggested that the protein is relatively resistant to pH-induced denaturation. This result is consistent with the concept that ion-pairing interactions between oppositely charged amino acid side chains may not play a significant role in stabilizing the lipid-free conformation of this protein. Near-UV CD spectra of apoLp-III revealed distinct peaks attributable to its two tryptophan and two phenylalanine residues. Deglycosylated apoLp-III possessed the same peaks, but these were reduced in magnitude. The spectra of apoLp-III·DMPC complexes also displayed extrema corresponding to tryptophan and phenylalanine residues, but these were reversed in sign and red-shifted. These data are consistent with a conformational change occurring upon the complexation of apoLp-III with lipid.

Apolipophorin III (apoLp-III[1]) from the migratory locust, *Locusta migratoria*, represents the only full-length apolipoprotein for which a three-dimensional structure has been determined. The structure was determined for this protein in the lipid-free state, wherein it adopts a stable globular conformation that is rich in α-helix (Breiter et al., 1991). apoLp-III is a glycoprotein of M_r 20 000, which contains 161 amino acids. The protein lacks methionine, tyrosine, and cysteine and contains two phenylalanine and two tryptophan residues. The five α-helices present in the protein are amphipathic and orient such that their hydrophobic faces are directed toward the center of a helix bundle. On the other hand, the hydrophilic faces of the amphipathic α-helices are exposed to the aqueous environment. Importantly, a similar elongated helix bundle structure has been reported for the N-terminal 22-kDa domain of human apolipoprotein E (Wilson et al., 1991).

apoLp-III normally exists in hemolymph as a lipid-free, water-soluble protein. During sustained flight, under the influence of peptide adipokinetic hormones (Beenakkers et al., 1985), apoLp-III associates with diacylglycerol-enriched low-density lipophorin (LDLp) particles [see Van der Horst (1990) for a review]. Association of apoLp-III with the surface of LDLp, which is dependent upon diacylglycerol enrichment of the particle, is postulated to stabilize its structure, permitting it to shuttle diacylglycerol from the fat body lipid storage depot to the flight muscle, where oxidation of LDLp-derived lipids serves to fuel sustained flight. Interestingly, LDLp

† This work was supported by grants from the Medical Research Council of Canada and NATO (RG 0786/88). M.W. and R.O.R. are the recipients of a Postdoctoral Fellowship and Medical Scholarship Award, respectively, from the Alberta Heritage Foundation for Medical Research.

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® Abstract published in *Advance ACS Abstracts*, March 1, 1994.

[1] Abbreviations: apoLp-III, apolipophorin III; DMPC, dimyristoylphosphatidylcholine; LDLp, low-density lipophorin; PAGE, polyacrylamide gel electrophoresis; TFE, trifluoroethanol; CD, circular dichroism.

0006-2960/94/0433-3617$04.50/0

particles are not internalized at the flight muscle (Van Antwerpen et al., 1988). Instead, diacylglcyerol is depleted from the lipoprotein through the action of a membrane-bound lipophorin lipase (Wheeler et al., 1984), which induces apoLp-III dissociation.

The dual existence of apoLp-III as a stable, lipid-free, water-soluble protein and as a lipid surface binding apolipoprotein implies conformational adaptability with respect to interaction with hydrophobic and hydrophilic environments. From monolayer balance studies at the air–water interface, it has been postulated that apoLp-III interacts with hydrophobic surfaces via one of its ends, whereupon it unfolds to occupy an increased surface area (Kawooya et al., 1986). A model depicting such a conformational change has been presented wherein the helix bundle opens via a "hinged" region between helices, thereby permitting contact between hydrophobic side chains and the lipid surface (Breiter et al., 1991). Recently, however, in studies of the interfacial behavior of *L. migratoria* apoLp-III at lipid–water interfaces, Demel et al. (1992) found that the molecular exclusion area of apoLp-III decreased upon interaction with lipid surfaces. Thus, at present, direct evidence that apoLp-III undergoes a conformational change upon interaction with lipid is lacking. In an effort to gain a better understanding of its conformational and stability properties, we have performed studies on native lipid-free apoLp-III, deglycosylated apoLp-III, and apoLp-III·phospholipid complexes.

MATERIALS AND METHODS

Isolation of apoLp-III. Apolipophorin III was isolated from the hemolymph of *Locusta migratoria* as described previously (Van der Horst et al., 1991), and the *b* isoform was used for all studies. Purified lipid-free apoLp-III was deglycosylated using endoglycosidase F (Boehringer) essentially as described by Hård et al. (1993).

Preparation of apoLp-III·DMPC Disks. Dimyristoylphosphatidylcholine (DMPC; obtained from Sigma Chemical Co.) vesicles were prepared by sonication according to Wientzek et al. (1993). apoLp-III·DMPC complexes were then prepared by mixing DMPC vesicles with apoLp-III at a DMPC:apoLp-III weight ratio of 2.5:1 (67:1 molar ratio) and incubating the sample for 18 h at 24 °C in buffer (0.2 M Tris (pH 7.2), 8.5% KBr, and 0.01% EDTA). Following incubation, the complexes were isolated by density gradient ultracentrifugation. The presence of complexes was confirmed by protein (bicinchoninic acid assay; Pierce) and enzyme-based phospholipid (Boehringer) assays, as well as native pore-limiting gradient PAGE and electron microscopy. Native PAGE analysis was on 4–20% acrylamide gradient slab gels run at a constant 150 V for 24 h (Ryan et al., 1985). Protein standards (Pharmacia) with the following Stokes diameters were used for calibration (Nichols et al., 1986): thyroglobulin, 17.0 nm; ferritin, 12.2 nm; catalase, 9.2 nm; lactate dehydrogenase, 8.2 nm; bovine serum albumin, 7.1 nm. Samples for electron microscopy were adsorbed to carbon-coated grids, rinsed three times with buffer (10 mM Tris, 10 mM NaCl, and 1.5 mM $MgCl_2$, pH 7.4), and negatively stained with 2% sodium phosphotungstate. Grids were photographed in a Philips EM420 operated at 100 kV. Chemical cross-linking of apoLp-III·DMPC complexes was conducted with dimethyl suberimidate (Pierce Chemical Co.) essentially as described by Davies and Stark (1970).

Analytical Ultracentrifugation. A Beckman Model E analytical ultracentrifuge using the Rayleigh interference optical system was used for flotation equilibrium experiments, which were performed as described by Nelson et al. (1974). apoLp-III·DMPC complexes were dialyzed for 48 h against 100 mM Tris (pH 7.4) and 2.5 M KBr to equilibrate the complexes at a density of 1.21 g/mL. Runs were performed at 14 000 rpm at 20 °C in a double-sector, CFE sample cell equipped with sapphire windows for a minimum of 48 h before equilibrium photographs were taken. Sedimentation equilibrium experiments were performed in an analogous manner using native lipid-free apoLp-III (2.51 mg/mL) in 10 mM Tris (pH 7.4) and 100 mM KCl, at 20 000 rpm and 20 °C. Molecular mass calculations were carried out using an APL computer program. Partial specific volume values of 0.908 and 0.722 mL/g for apoLp-III·DMPC complexes (estimated from KBr density gradient ultracentrifugation) and lipid-free apoLp-III (calculated from compositional analysis), respectively, were employed. The ln Y versus r^2 data were fitted to a second-order polynomial equation using least-squares techniques, and the apparent weight-average molecular mass was calculated from the slope of the resulting plot.

Fluorescence Studies. Fluorescence experiments were performed on a Perkin-Elmer MPF-44B spectrofluorometer, with an attached, thermostated cell holder and a microprocessor-controlled differential corrected spectra unit (DCSU2). Temperature was controlled by a Lauda RMS water bath at 20 °C. The samples were placed in a semimicro 1-cm cell, with bandwidths of 6 nm used for excitation and 10 nm for the emission monochrometer. The samples were excited at 280 nm and emission was monitored at 328 nm to record either excitation or emission spectra with the blank mode set on the DCSU2.

For quenching studies, the samples were excited at 295 nm with emission monitored at 328 nm. Aliquots of quenchers (KI, CsCl, and acrylamide) were added, and the fluorescence was measured. An absorbancy correction was used for acrylamide of log $A/2$ at 295 nm and then dilution correction for all of the fluorescence readings at 328 nm. The OD for the quenchers was read on a Cary 3 spectrophotometer. The fluorescence data for each quencher were analyzed via the Stern–Volmer equation: $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F represent the fluorescence intensities at the emission maximum in the absence and presence of quencher, [Q]. The collisional quenching constant, K_{sv}, was obtained from the slope of a plot of F_0/F versus [Q]. Values for the average accessibility of the quencher to the two tryptophan fluorophores were obtained from modified Stern–Volmer plots, according to Lehrer (1971):

$$F_0/(F_0 - F) = 1/f_a K_q[Q] + 1/f_a$$

Here K_q is the Stern–Volmer quenching constant of the accessible fraction, and f_a is the fraction of the initial fluorescence that is accessible to quencher.

Circular Dichroism Experiments. Circular dichroism (CD) measurements were carried out on a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD) interfaced to an Epson Equity 386/25 computer controlled by Jasco software. The thermostated cell holder was maintained at 25 °C with a Lauda RMS circulating water bath (Lauda, Westbury, NY). The instrument was routinely calibrated with ammonium *d*-(+)-10-camphorsulfonate at 290.5 and 192 nm and with *d*-(−)-pantoyllactone at 219 nm. Each sample was scanned 10 times, and noise reduction was applied to remove the high frequency before calculating molar ellipticities. The voltage of the photomultiplier was kept below 500 V to prevent distortion of the CD spectrum. The cell used for the region below 250 nm was 0.02 cm (calibrated for path length). The protein concentration was between 0.5 and 1.0 mg/mL for far-UV

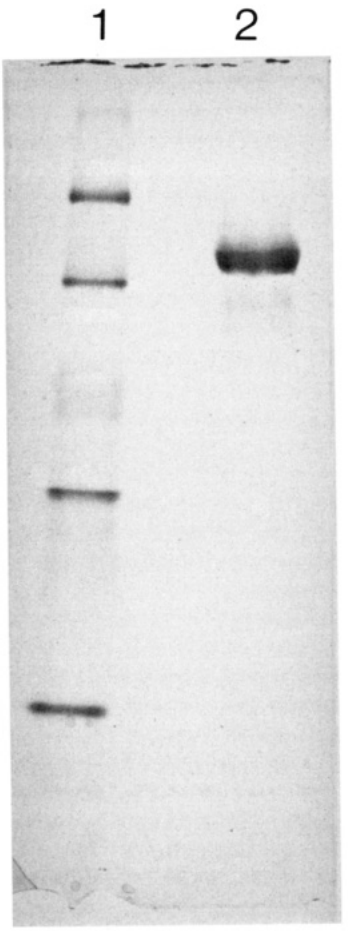

FIGURE 1: Native polyacrylamide gel electrophoresis of apoLp-III·DMPC complexes. A 4–20% acryalmide gradient slab gel was electrophoresed for 24 h at a constant 150 V and stained with Coomassie Blue. Lane 1, protein standards, from top to bottom: thyroglobulin, 17.0 nm; ferritin, 12.2 nm; catalase, 9.2 nm; lactate dehydrogenase, 8.2 nm; bovine serum albumin, 7.1 nm. Lane 2, apoLp-III·DMPC complexes.

spectra. Near-UV spectra were obtained in a 1.0-cm microcell with a protein concentration of 1 mg/mL. CD spectra were analyzed for secondary structure content using the Contin program (version 1.0) of Provencher and Glöckner (1981). Stock protein concentrations were established in the ultracentrifuge employing the Rayleigh interference optical system, assuming 41 fringes equal to 10 mg/mL (Babul & Stellwagen, 1969).

RESULTS

Characterization of apoLp-III·DMPC Complexes. Since apoLp-III represents one of three distinct apolipoprotein components of the hemolymph lipoprotein, lipophorin (Van der Horst, 1990), it is difficult to specifically evaluate its behavior in these complexes. Therefore, we employed purified apoLp-III complexed with the well-characterized phospholipid, DMPC, to create model lipid–protein complexes. When apoLp-III was added to sonicated vesicles of DMPC, a partial or complete clearing of sample turbidity was observed, indicating complex formation. Examination of the complexes by native pore-limiting gradient PAGE (Figure 1) revealed a single population of particles with an estimated Stokes diameter of 13.5 nm. Further characterization of the complexes by electron microscopy revealed disklike particles which, in some cases, stacked to form rouleaux (data not shown). To determine the molecular weight of the apoLp-III·DMPC complexes, flotation equilibrium experiments were conducted in the analytical ultracentrifuge. A plot of ln Y versus the square of the distance from the center of rotation yielded a linear relationship, confirming the presence of a single population of complexes. From the slope of this line, a calculated apparent molecular mass of 588 500 Da was obtained.

Compositional Analysis of apoLp-III·DMPC Disks. Compositional analysis of the apoLp-III·DMPC complexes was performed and yielded a final DMPC:apoLp-III molar ratio of 122:1. Chemical cross-linking experiments with dimethyl suberimidate revealed the presence of five apoLp-III molecules per disk (Figure 2). As expected, intermolecular cross-links were not detected with lipid-free apoLp-III, consistent with its existence as a monomeric protein. Together, the data indicate that complexation of apoLp-III with DMPC at this ratio gives rise to a relatively homogeneous population of disklike particles that are amenable to spectroscopic analysis.

Far-UV Circular Dichroism Studies. Circular dichroism spectroscopy was employed to assess the effect of deglycosylation or complexation with phospholipid on the secondary structure content of apoLp-III. The CD spectrum of native, lipid-free apoLp-III is consistent with its known high content of α-helix determined by X-ray crystallography (Figure 3). At apoLp-III concentrations of 0.1 mg/mL and below, a loss of negative ellipticity was observed. At concentrations above 0.1 mg/mL, no significant protein concentration effect on ellipticity was found. Since the apparent protein concentration-dependent induction of α-helix in this range may be indicative of self-association (Gwynne et al., 1975; Yokoyama et al., 1985), the molecular weight of apoLp-III was determined by sedimentation equilibrium experiments in the analytical ultracentrifuge. At a concentration of 2.5 mg/mL, a calculated molecular mass of 15 230 Da was obtained, indicating that apoLp-III is present as a monomer. Therefore, we conclude that the observed loss of negative ellipticity at low concentration may be due to adsorption to the cell wall or a surface denaturation phenomenon.

The relative percentages of secondary structure conformers were estimated using the method of Provencher and Glöckner (1981) (Table 1). When spectra of native apoLp-III were obtained in 50% trifluoroethanol (TFE), a known helix-inducing agent, there was an increase in α-helix content from 78% to 90%, indicating that the protein possesses the potential to increase its α-helix content. On the other hand, deglycosylation of native apoLp-III resulted in a decrease in α-helix content with a corresponding increase in other secondary structure conformers. As with native apoLp-III, however, spectra of deglycosylated apoLp-III obtained in 50% TFE revealed an induction of α-helix from 46% to 76%. Whereas native lipid-free apoLp-III was stable for extended periods, after 24 h the α-helix content of deglycosylated apoLp-III was reduced to 35%, and after 5 days it was only 8%. By contrast, Provencher–Glöckner analysis of CD spectra of apoLp-III·DMPC complexes revealed a high percentage of α-helical structure.

Near-UV Circular Dichroism. The near-UV CD spectrum of native apoLp-III in the absence of lipid reveals four peaks at 292, 286, 278, and 268 nm (Figure 3, inset). Between 250 and 270 nm evidence of the two phenylalanine residues can be seen. Peaks between 270 and 300 nm can be assigned to the two tryptophan residues in locust apoLp-III. Deglycosylated apoLp-III showed a similar pattern, although the peak intensities were lower, consistent with the observed loss of α-helix as detected by far-UV CD. Upon complexation with DMPC the spectrum is reversed in sign, becoming entirely

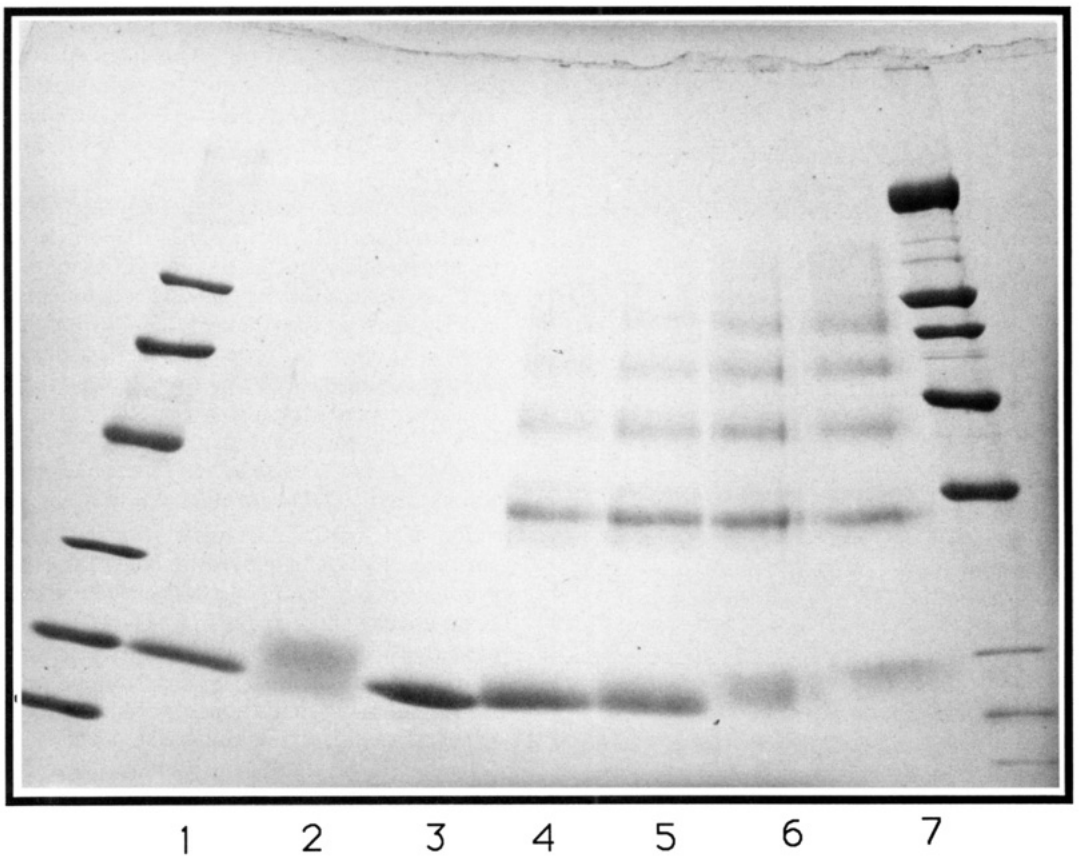

FIGURE 2: Chemical cross-linking of apoLp-III·DMPC disks. apoLp-III·DMPC complexes (50 μg of protein) were incubated with an increasing amount of dimethyl suberimidate (DMS) for 2 h at 22 °C. Following incubation, SDS sample treatment buffer was added, and the samples were heated to 100 °C, applied to a 4–20% acrylamide gradient SDS slab gel, and electrophoresed at 35 mA for 3.5 h: lane 1, apoLp-III control; lane 2, apoLp-III plus 2 mg/mL DMS; lane 3, control apoLp-III·DMPC disks; lane 4, apoLp-III·DMPC disks plus 0.2 mg/mL DMS; lane 5, apoLp-III·DMPC disks plus 0.4 mg/mL DMS; lane 6, apoLp-III·DMPC disks plus 1.0 mg/mL DMS; lane 7, apoLp-III·DMPC disks plus 2.0 mg/mL DMS; lanes at the far left and right correspond to molecular weight markers.

negative with troughs at 294, 287, 278, and 269 nm. In addition, the phenylalanine peaks are resolved to a greater extent than in the absence of lipid. The reversal of sign combined with the shift to longer wavelengths indicates that the tryptophan and phenylalanine residues contributing to the CD spectrum have assumed a new average conformation and are in a more nonpolar environment (Lux et al., 1972).

Fluorescence Studies of apoLp-III. In the lipid-free state, the apoLp-III tryptophan fluorescence emission maximum was 347 nm (excitation wavelength = 280 nm). Upon deglycosylation the emission maximum is blue-shifted to 332 nm. Complexation of native apoLp-III with DMPC also induced a blue shift to 321 nm. To obtain further information on the environment of the two tryptophan residues under these various conditions, fluorescence quenching experiments were performed. Stern–Volmer plots were linear for each of the three quenchers. K_{sv} values for KI yielded quenching constants ranging from 0.87 M^{-1} for apoLp-III·DMPC complexes to 3.87 M^{-1} for deglycosylated apoLp-III (Table 2). From the data obtained with KI, an accessibility value, f_a, of 0.6 was determined from the intercept of a modified Stern–Volmer plot, indicating that, on average, the two tryptophan residues in lipid-free apoLp-III are 60% accessible to this quencher (Table 2). Deglycosylation of apoLp-III results in an increase in quencher accessibility for KI from 0.6 to 0.75. This value increased to 0.92 upon storage following deglycosylation, suggesting deglycosylation-induced denaturation of the protein as a function of time. When CsCl was employed as quencher, native apoLp-III yielded a K_{sv} of 0.04 M^{-1}, suggesting that the tryptophan residues are shielded from this quencher. By contrast, the observed values for apoLp-III·DMPC complexes and deglycosylated apoLp-III were considerably larger. The low f_a values obtained for apoLp-III tryptophan fluorescence quenching by CsCl in the lipid-free state may be due to charge repulsion of the quencher by His_{111} in the case of Trp_{113} and by His_{67} in the case of Trp_{127}. This hypothesis was tested by performing quenching experiments at pH values in which the side chains of these histidine residues would be charged (pH 5.5) and uncharged (pH 7.8). Comparison of the f_a values obtained at these two pHs, however, did not reveal major differences, suggesting that some factor other than these histidine residues is responsible for the low K_{sv} and f_a values observed for apoLp-III when CsCl was employed as the quenching agent.

In the lipid-free native state, apoLp-III tryptophan residues were quenched by acrylamide with a K_{sv} = 1.72 M^{-1}. This value increases dramatically upon deglycosylation of apoLp-III and is reflective of near-complete exposure of the apoLp-III tryptophan residues to this quencher. The degree to which this result is due to deglycosylation-induced conformational changes versus denaturation of the protein cannot be assessed at present. When native apoLp-III is complexed with DMPC, however, the K_{sv} value decreased to 0.76 M^{-1}, indicating that the two tryptophan residues now reside in an environment that is shielded from the quencher, compared to the lipid-free state. With KI as the quenching agent, K_{sv} values for lipid-associated apoLp-III were decreased compared to lipid-free apoLp-III while the corresponding f_a was increased. In the case of CsCl, complexation of apoLp-III resulted in only small changes in K_{sv} and f_a versus the lipid-free protein.

Denaturation Studies. Upon the addition of increasing amounts of guanidine hydrochloride to a solution of lipid-free

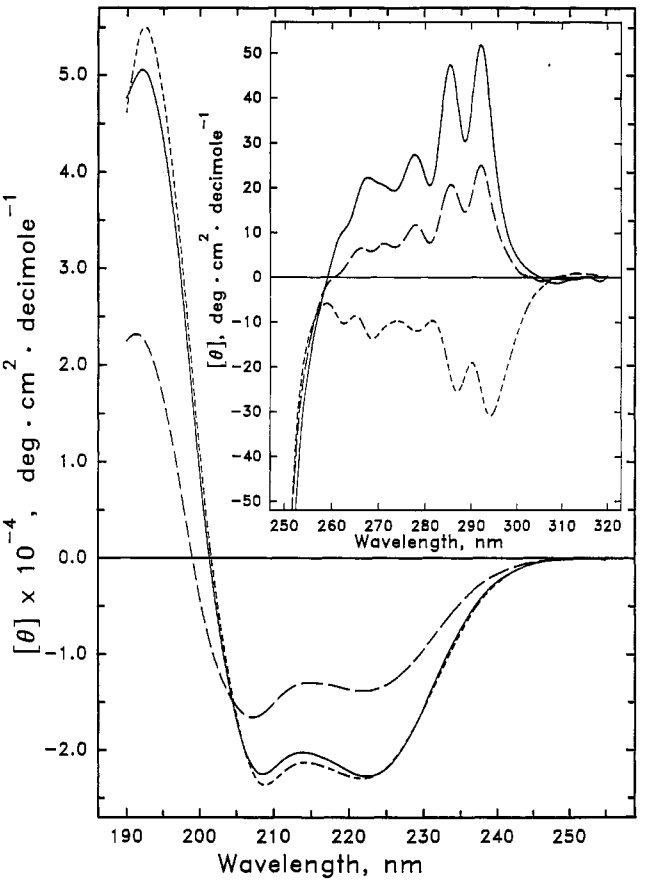

FIGURE 3: Far-UV and aromatic (inset) circular dichroism spectra of apoLp-III: native lipid-free apoLp-III (—); deglycosylated apoLp-III (– –); apoLp-III·DMPC complexes (- - -). Spectra were recorded in 50 mM sodium phosphate (pH 7.0) and 100 mM KCl.

Table 1: Provencher–Glöckner Analysis of Secondary Structure Conformers of apoLp-III

	% conformer			
sample	α-helix	β-sheet	β-turn	remainder
native apoLp-III	78	13	2	7
deglycosylated apoLp-III	46	23	11	20
apoLp-III·DMPC complexes	76	24	0	0

Table 2: Tryptophan Fluorescence Quenching of apoLp-III

	KI[a]		CsCl		acrylamide	
	K_{sv} (M^{-1})[b]	f_a[c]	K_{sv} (M^{-1})	f_a	K_{sv} (M^{-1})	f_a
apoLp-III	1.58	0.60	0.04	0.14	1.72	0.82
deglycosylated apoLp-III	3.87	0.75	1.42	0.68	8.26	0.92
apoLp-III·DMPC complexes	0.87	0.94	0.13	0.10	0.76	0.71

[a] The solution of KI contained 1 mM sodium thiosulfate to prevent the formation of free iodine. [b] Stern–Volmer quenching constant. [c] f_a: accessibility of quencher derived from modifier Stern–Volmer plots (Lehrer, 1971).

apoLp-III there was a loss of α-helical structure as detected by CD or fluorescence changes. apoLp-III displayed a guanidine hydrochloride denaturation midpoint of 0.60 M (Figure 4). On the assumption that denaturation of apoLp-III is a two-state process representing a reversible equilibrium between native and denatured states, the free energy of unfolding was determined from the guanidine hydrochloride experiments according to the method of Pace (1986). Table 3 reveals that lipid-free apoLp-III has $\Delta G_D^{H_2O}$ = 5.37 kcal/mol. Also shown in Table 3 are values obtained for

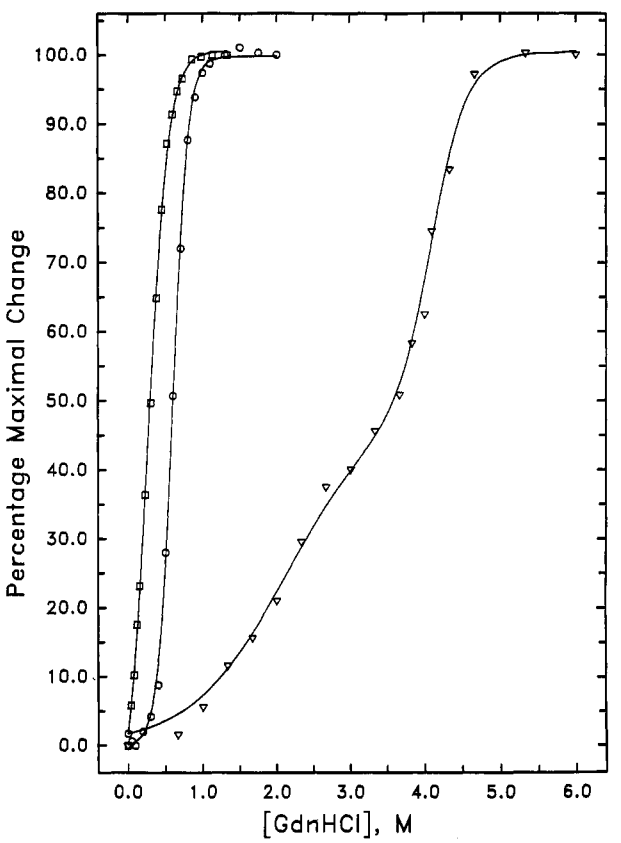

FIGURE 4: Denaturation of apoLp-III samples with guanidine hydrochloride: native lipid-free apoLp-III (○); deglycosylated apoLp-III (□) is apoLp-III·DMPC complexes (▽). Samples were incubated overnight with different concentrations of guanidine hydrochloride and the extent of denaturation was monitored by CD or fluorescence. The data for apoLp-III·DMPC complexes were fit to two sigmoidal curves to generate the line shown.

Table 3: Thermodynamic Parameters for the Reversible Denaturation of apoLp-III

		$[GdnHCl]_{1/2}$[a] (M)	$\Delta G_D^{H_2O}$ [b] (kcal/mol)	m[c] (kcal mol^{-1} M^{-1})
apoLp-III		0.60	5.37	3.46
deglycosylated apoLp-III		0.27	1.17	4.33
apoLp-III·DMPC	transition 1	2.13		
	transition 2	4.09		

[a] $[GdnHCl]_{1/2}$ is the transition midpoint, the molar concentration of guanidine hydrochloride required to give a 50% decrease in ellipticity at 221 nm. [b] $\Delta G_D^{H_2O}$ is the free energy of unfolding in the absence of guanidine hydrochloride, determined according to Pace (1986). [c] m is a measure of the solvent-accessible area of the denaturated state minus the solvent-accessible area of the native state (Shortle, 1989).

deglycosylated apoLp-III, which yielded a lower guanidine hydrochloride denaturation midpoint and a substantially lower $\Delta G_D^{H_2O}$. On the other hand, complexation with DMPC caused a significant resistance to guanidine hydrochloride induced denaturation of apoLp-III and resulted in an apparent bimodal denaturation curve with transition midpoints centered at 2.1 and 4.1 M guanidine hydrochloride, respectively (Figure 4).

Effect of Buffer pH on the Stability of apoLp-III. Locust apoLp-III contains 34 charged amino acid residues (17 positive and 17 negative) and, conceivably, its structure may be stabilized by ion pairing between oppositely charged side chains (Marqusee & Baldwin, 1987). The stability of lipid-free apoLp-III as a function of solvent pH was investigated by monitoring the ellipticity at 221 nm (Figure 5) at various pH intervals. The data indicate that, even at pH values below 2.0 and above 11.5, apoLp-III retains considerable negative

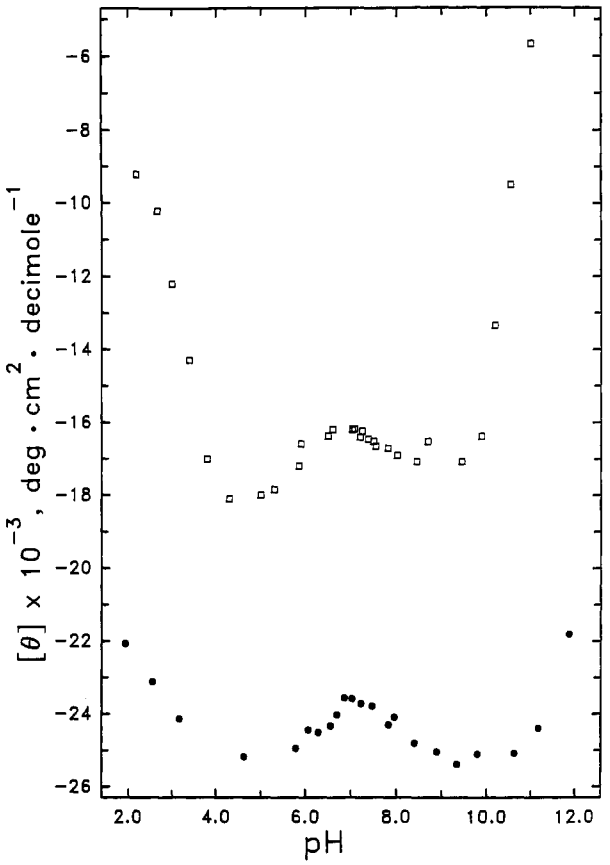

FIGURE 5: Effect of pH on the ellipticity of *L. migratoria* and *M. sexta* apoLp-III's at 221 nm. A sample of native, lipid-free *L. migratoria* apoLp-III (●) was adjusted with respect to pH over the range 1.5–11.7, and CD scans were obtained at various intervals. The data for *M. sexta* apoLp-III (□) are from Ryan et al. (1993) and are presented for comparison.

ellipticity, indicating maintenance of structure. At pH values below 4.0, protonation of the side-chain carboxyl moiety of the 13 glutamate and 4 aspartate residues present in apoLp-III will occur. Likewise, above pH 10 the 7 lysine residues in apoLp-III will be deprotonated. Comparison of the data for *L. migratoria* apoLp-III with that for *Manduca sexta* apoLp-III reveals that, at all pH values, *L. migratoria* apoLp-III displays much greater negative ellipticity (221 nm) values and is considerably more resistant to pH-induced denaturation. As has been observed previously with other apolipoproteins, including *M. sexta* apoLp-III (Weinberg & Spector, 1985; Ryan et al., 1993; Kiss et al., 1993), *L. migratoria* apoLp-III displays a slight decrease in negative ellipticity at 221 nm between pH 6.0 and 7.0.

DISCUSSION

An important feature of apoLp-III is its dual existence as a water-soluble globular protein and a lipid surface binding apolipoprotein (Van der Horst, 1990). This unique property suggests that apoLp-III may possess some novel structural characteristics that permit alternate functional interactions. The determination of the three-dimensional structure of *L. migratoria* apoLp-III, in the lipid-free state, revealed an elongated globular structure that is organized into a helix bundle, wherein the hydrophobic faces of its amphipathic α-helices orient toward the center of the bundle (Breiter et al., 1991). It has been postulated that interaction with a lipid surface would require a major conformational change in the protein, resulting in the exposure of its hydrophobic interior, which would interact directly with the surface of lipoproteins. From inspection of the three-dimensional structure, and on the basis of monolayer balance studies conducted at the air–water interface (Kawooya et al., 1986), it has been postulated that apoLp-III opens about hinge regions located between helices 2 and 3 and between 4 and 5. Recently, however, Demel et al. (1992) have reported additional monolayer balance studies that are not consistent with this model. On the other hand, Wientzek et al. (1994) have provided spectroscopic and structural evidence in support of a major conformational change. In addition, key questions exist with regard to the factor(s) that mediates the initial interaction of apoLp-III with the surfaces of lipoproteins. It is known that apoLp-III binding to lipophorin particles is directly correlated with an increase in particle diacylglycerol content (Wells et al., 1987). It has been postulated that solvent-accessible hydrophobic amino acids located in the loop region between helices 1 and 2 could be responsible for initiating interactions with lipid surfaces, followed by unfolding of the protein about the hinges (Breiter et al., 1991). Recent studies of the interactions of apoLp-III with a variety of phospholipids, however, have led to an alternate hypothesis in which positively charged amino acid side chains on apoLp-III interact with the negatively charged phosphate moiety of lipophorin phospholipids (Zhang et al., 1993). Unfolding of the molecule on the lipoprotein surface follows as new lipid surface is created, leading to a stable binding interaction with surface-associated diacylglycerol (Wang et al., 1992). Since it is known that apoLp-III association with lipophorin is a reversible process, it is reasonable to consider that lipolysis of diacylglycerol and removal of the product fatty acids from the particle may induce refolding of the helix bundle structure as hydrophobic interactions between diacylglycerol and apoLp-III are lost.

From the above discussion, it is clear that a balance must exist with respect to the relative stability of the lipid-free and lipid-bound states of apoLp-III. We have investigated the conformational stability of *L. migratoria* apoLp-III in the lipid-free helix bundle structure versus that when complexed to phospholipid. These results can be compared to the corresponding properties of the homologous protein from *M. sexta* in the lipid-free (Ryan et al., 1993) and lipid-bound states (Wientzek et al., 1994). Compared to *M. sexta* apoLp-III, *L. migratoria* apoLp-III appears to be more stable in the lipid-free state. This conclusion is based on the observed $\Delta G_D^{H_2O}$, of 5.37 versus 1.29 kcal/mol for the *M. sexta* protein. Likewise, the midpoint of guanidine hydrochloride induced denaturation was 0.60 M for *L. migratoria* apoLp-III versus 0.35 M for *M. sexta* apoLp-III. The biochemical basis for this difference is not known, but it could conceivably be related to differences in amino acid sequence or the oligosaccharide moiety of *L. migratoria* apoLp-III, which is lacking in the *M. sexta* protein (Kawooya et al., 1984). Indeed, enzymatic deglycosylation of *L. migratoria* apoLp-III resulted in a decrease in $\Delta G_D^{H_2O}$ to 1.17 kcal/mol. Far-UV CD spectra of apoLp-III revealed a significant loss of helical structure upon deglycosylation, suggesting that the protein may adapt to this perturbation by altering its overall helix folding motif. Consistent with this observation is the fact that deglycosylated apoLp-III was unstable [see Demel et al. (1992)] as a function of time following deglycosylation and was essentially denatured following storage in buffer for 5 days at 4 °C. These results suggest that the oligosaccharide moieties of apoLp-III, which are located on Asn_{16} and Asn_{83} (Kanost et al., 1988), play a key role in the maintenance of apoLp-III stability in the lipid-free state. Interestingly, the structures of these oligosaccharide

moieties have recently been determined and were shown to contain fucose, mannose, and *N*-acetylglucosamine, as well as carbohydrate-linked (2-aminoethyl)phosphonate (Hård et al., 1993). In view of the fact that previous hypotheses about the role of *L. migratoria* apoLp-III oligosaccharide moiety involvement in receptor interaction or lipase activation have not been verified experimentally, the present data indicate that the function of this unusual carbohydrate moiety may be in the stabilization of the protein in its lipid-free conformation. Interestingly, Demel et al. (1992) reported that the CD spectrum of lipid-associated apoLp-III is unchanged upon deglycosylation of the protein, suggesting that removal of the carbohydrate moiety of *L. migratoria* apoLp-III does not preclude interaction with lipid.

Complexation of apoLp-III with DMPC results in the formation of relatively uniform disklike structures that have an average molecular mass of 588 500 Da. This suggests that discrete particles have multiple copies of apoLp-III. This was confirmed by chemical cross-linking studies, which provided evidence of five apoLp-III molecules per disk. When considered in light of the DMPC:apoLp-III molar ratio of 122:1, the data are similar to the complexes formed upon complexation of *M. sexta* apoLp-III with lipid (Wientzek et al., 1994). Furthermore, calculations based on the dimensions of apoLp-III (Breiter et al., 1991) are consistent with the concept that the protein adopts an open, extended conformation in which the helices orient in a perpendicular manner with respect to the phospholipid acyl chains around the perimeter of a disk-shaped DMPC bilayer. In comparing the guanidine hydrochloride denaturation midpoints between apoLp-III in the lipid-free and -bound states, it is apparent that complexation with phospholipid confers considerable stability to this protein. Furthermore, lipid-associated *L. migratoria* apoLp-III displays a two-stage denaturation curve that may suggest the existence of a relatively stable denaturation intermediate. Similar results have been observed for *M. sexta* apoLp-III (Wientzek et al., 1994) and human apoA-I (Reijngoud & Phillips, 1982). The CD spectrum of apoLp-III in the lipid-free state indicated the presence of considerable α-helix (78%), consistent with structural data obtained by X-ray crystallography. Complexation of apoLp-III with DMPC did not induce major changes in the CD spectrum or the estimated α-helix content. The spectrum obtained with apoLp-III·DMPC complexes revealed values of negative ellipticity that were considerably greater than those reported by Demel et al. (1992) for apoLp-III bound to lipid monolayers and deposited on quartz disks.

The CD aromatic spectrum of apoLp-III in the native lipid-free state gives rise to a set of well-distinguished maxima that correspond to the two tryptophan and two phenylalanine residues present in this protein. It is noteworthy that this spectrum is less complicated than that observed for human apoA-I (Lux et al., 1972). The spectra of *L. migratoria* apoLp-III show distinct extrema that are similar to those observed for tryptophan model compounds, while the weaker peaks at lower wavelengths are attributable to phenylalanine. The lack of tyrosine or cysteine in this protein is likely responsible for the unusually well-defined spectra obtained (Strickland, 1974). Deglycosylation of apoLp-III induced a decrease in the overall intensity of the extrema present, but did not alter the sign of these peaks. By contrast, upon complexation with DMPC, the spectrum became entirely negative and extrema were shifted to longer wavelengths, consistent with a reciprocal repositioning of helical segments. Similar observations have been made with apoA-I, and it has been proposed that such spectral changes indicate that the tryptophan and phenylalanine residues reside in a new average conformational environment. From the crystal structure, it is known that both phenylalanine and tryptophan residues in native apoLp-III are oriented with their side chains directed toward the center of the helix bundle. The observed complete reversal of sign for extrema assigned to these phenylalanine and tryptophan residues, together with their locations within the helix bundle structure, is consistent with the concept that the helix bundle opens to expose its hydrophobic interior, thereby permitting interaction of these residues with the lipid surface.

The tryptophan fluorescence emission maximum of apoLp-III decreased as a function of deglycosylation as well as complexation with DMPC. In the latter case, the observed shift from 347 to 321 nm indicates that these residues now reside in a more hydrophobic environment. Examination of the crystal structure of apoLp-III reveals that Trp_{113} is located in an α-helical segment, with its side chain oriented toward the center of the five-helix bundle. By contrast, Trp_{127} is found in the loop region between helices 4 and 5. The observed large blue shift in the tryptophan fluorescence emission maximum suggests that these residues may be in direct contact with the lipid surface. This would be difficult to accomplish, however, if the helix bundle structure was maintained in the lipid-associated state. Conversely, if the bundle opened so as to expose these residues, it is conceivable that they could contact the lipid surface directly. In the case of deglycosylation the observed blue shift was less dramatic (332 nm). These data suggest a reorganization of apoLp-III structure such that, on average, the two tryptophan residues are more shielded from the aqueous environment. This alteration in structural organization upon deglycosylation was detectable by CD, which indicated a loss of α-helix and an increase in β-sheet, β-turn, and random structure. Importantly, however, this conformation is considerably less stable than that of native apoLp-III, and thus, the data support the concept that deglycosylation induces a conformational change to a less stable structure.

The fluorescence properties of apoLp-III were investigated further by quenching studies. Deglycosylation of apoLp-III resulted in an increase in quencher accessibility for all quenchers studied. The largest increase in K_{sv} observed upon deglycosylation was with acrylamide, which increased from 1.72 to 8.26 M^{-1}. Thus, although deglycosylation induced a blue shift in the tryptophan fluorescence emission maximum, these residues became more accessible to all three quenchers investigated. An interesting feature of the quenching results obtained with deglycosylated apoLp-III pertains to CsCl. The K_{sv} values for this quencher were very low (0.04 M^{-1}) in the native protein, yet increased dramatically upon deglycosylation. Since the corresponding K_{sv} and f_a values for either KI or acrylamide indicated greater accessibility, it was possible that access of the positively charged quencher might be restricted by ionic repulsion from positively charged amino acid side chains in the vicinity of either or both tryptophan residues in the folded structure. The hypothesis that histidine residues may be playing a role in such a charge repulsion of the quencher was evaluated by determining the effect of pH changes on the K_{sv} value for CsCl. At pH values that would be expected to deprotonate apoLp-III histidine residues, no effect on tryptophan fluorescence quenching by CsCl was observed. Thus, at present, the structural reason for the low accessibility of CsCl to the tryptophan residues in native apoLp-III remains unclear. However, the dramatic increase in quencher accessibility to these tryptophan residues provides

further support for the concept that deglycosylation induces a change in the structure of the protein.

In quenching studies employing apoLp-III complexed with DMPC, comparison with lipid-free apoLp-III revealed a decrease in K_{sv} for KI and acrylamide, while that for CsCl displayed a small increase. In contrast to the K_{sv} results obtained with KI, f_a increased from 0.60 to 0.94 upon complexation with DMPC. At present we are unable to explain these apparently conflicting data. The K_{sv} data for KI and acrylamide, however, are consistent with the concept that tryptophan residues may interact with the lipid surfce of the disk particles and thereby reduce the accessibility of these quenching agents. In the case of CsCl, although the K_{sv} value increased slightly from 0.04 to 0.13 M^{-1}, the f_a value decreased to 0.10. The lower value of f_a suggests that accessibility to this quencher is decreased upon interaction of apoLp-III with DMPC.

In *M. sexta* apoLp-III, numerous oppositely charged amino acid side chains are arranged in an $i + 4$ sequence configuration. On the basis of changes in apoLp-III α-helix content as a function of solvent pH, it has been suggested that some of these pairs may form salt bridges in the native protein (Ryan et al., 1993). *L. migratoria* apoLp-III, however, possesses far fewer oppositely charged amino acid residues in such a configuration (5 versus 22 in the *M. sexta* protein), suggesting that other factors may contribute to the overall stabilization of this protein in the lipid-free state. Consistent with this conclusion is the observation that *L. migratoria* apoLp-III is relatively resistant to pH-induced denaturation.

In summary, the present data reveal important differences in structural stability and physical properties between *L. migratoria* and *M. sexta* apoLp-III's that exist in spite of the functional similarities between these two proteins (Van der Horst et al., 1988). Through further characterization of these differences, it may be possible to define the structural features of these proteins that are required for their dual existence as water-soluble hemolymph proteins and lipid surface binding apolipoproteins. Furthermore, given the similarity in molecular architecture between apoLp-III and human apolipoprotein E (Wilson et al., 1991), it is likely that basic information obtained will be relevant to apolipoprotein structure and function in higher organisms as well.

ACKNOWLEDGMENT

We thank Leslie D. Hicks for performing the analytical ultracentrifugation experiments and Roger Bradley for electron microscopy and photography.

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