

## Role of Glycosylation in the Lipid-Binding Activity of the Exchangeable Apolipoprotein, Apolipoprotein-III

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**Non-glycosylated recombinant *Locusta migratoria* apolipoprotein-III, apoLp-III, was expressed in *E. coli* and its physical-chemical properties were compared to those of the glycosylated native apoLp-III. Fluorescence quantum yield and acrylamide quenching studies indicated a slightly higher accessibility of the Trp residues in the recombinant apoLp-III. Far-UV CD spectroscopy indicated that the recombinant apoLp-III has a lower  $\alpha$ -helical content than the glycosylated apoLp-III. Both proteins spontaneously formed discoidal recombinant lipoprotein particles when incubated with dimyristoylphosphatidylcholine (DMPC). Interaction with lipid promotes an increase in  $\alpha$ -helical content. CD and fluorescence studies indicate that both proteins adopt the same conformation in the lipid-bound state. However, the kinetics of association of the recombinant protein with DMPC is 5-fold faster than that of the native protein. The results suggest that glycosylation inhibits the lipid binding activity by preventing the exposure of hydrophobic domains and/or decreasing the conformational flexibility of the protein.** © 1998 Academic Press

Apolipoprotein-III (apoLp-III) is an exchangeable apolipoprotein found in the hemolymph of many insect species (1-2). *Locusta migratoria* apoLp-III (17kD) is the only full-length, exchangeable apolipoprotein whose structure has been determined by x-ray crystallography (3). Its structure is described as a bundle of five amphipathic  $\alpha$ -helices. *Locusta migratoria* apoLp-III has two glycosylated Asn residues, Asn 16 and Asn 83. The structure of the N-linked oligosaccharides has been recently reported (4), but the biological role of the glycosylation has not been elucidated. ApoLp-III shares a large number of physical-chemical properties with the exchangeable apolipoproteins of humans and other vertebrates. They are rich in  $\alpha$ -helical structure in the

lipid-bound and water-soluble states (5-7) and, according to the hydrophobic moment and hydrophobicity of the  $\alpha$ -helices, they have been classified as typical exchangeable-apolipoproteins (8). They interact with the lipoprotein lipid-surface stabilizing the lipoprotein particle and can be interchanged with the human counterparts in binding to human or insect lipoproteins (9).

The study of the structure-function relationship of *Locusta migratoria* apoLp-III is of interest for several reasons: **1)** apoLp-III plays an essential role in the transport of large amounts of diacylglycerol, DG, which is the major source of energy for flight in many insects (10); **2)** The knowledge of the crystal structure of apoLp-III offers the possibility of relating the structure of an apolipoprotein to its function; **3)** The similarity between the properties of the insect and vertebrate exchangeable apolipoproteins (8) suggests that elucidation of the mechanism of interaction of apoLp-III with lipids would be directly applicable to human apolipoproteins.

The understanding of the relationship between the physical-chemical properties of apolipoproteins and their function is a major goal that has not been achieved yet.

Given the relevance of *Locusta migratoria* apoLp-III as an exchangeable-apolipoprotein model, the characterization of the bacteria-expressed non-glycosylated recombinant apoLp-III and the comparison of its properties with those of the glycosylated native protein are important for future site-directed mutagenesis studies. The observations made in this study showed significant structural and functional differences between the glycosylated and the non-glycosylated proteins.

### METHODS

*Expression and purification of Locusta migratoria ApoLp-III.* *Locusta migratoria* apoLp-III cDNA was first cloned and sequenced by Kanost et al. (11) in this lab. In order to express *Locusta migratoria* apoLp-III using the pET-32a expression system Novagen (Madison, WI), the original full length cDNA was modified by PCR to introduce

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Nco I and BamH I sites, using as template the original cDNA (p-Bluescript).

The PCR product was cut with Nco I and BamH I and the digested product ligated into Nco I/Bam HI predigested pET-32a plasmid, using T4 DNA ligase. Novablue competent cells were transformed with the ligation reaction according to the manufacturer's instructions. The plasmid DNA of the transformed bacteria was screened for the correct sized insert and sequenced from the S-tag and T7-terminator sites with S-tag and T7-terminator primers. AD494 expression host cells were transformed with the pET-32a-apoIII plasmid.

IPTG induced cells were pelleted, resuspended in buffer and lysed with lysozyme and deoxycholate. After removing the DNA with DNase I, the lysate was centrifuged for 1 hour at 50,000 rpm. The supernatant contained 100% of the target protein. The supernatant was loaded into a nickel affinity column and eluted with increasing concentrations of imidazole. The fusion protein with an apparent mass of 33 kDa elutes as a single band in the 60mM imidazole fraction. Recombinant Locust apoLp-III was cleaved from the rest of the fusion protein with enterokinase (Novagen, Madison, WI) according to the manufacturer's instructions. ApoLp-III does not have any cleavage sites for enterokinase. After the cleavage reaction, the solution was dialyzed and loaded onto an activated Nickel affinity column and the proteins separated with a discontinuous concentration gradient of imidazole in 20mM Tris.Cl buffer pH 7.9.

Native ApoLp-III was purified from the low-density lipophorin as previously described (12). *Locusta migratoria* low-density lipophorin was a generous gift from Dr. Dick van der Horst.

**Circular dichroism.** CD spectra were acquired in an 62A DS AVIV CD instrument (Lakewood, NJ) equipped with a Peltier temperature controller. The CD spectra were measured every 0.25 nm with 1 s averaging time per point and a 1nm band-pass. CD spectra were analyzed for secondary structure using the Contin program with the original Provencher and Glockner (13) structure-data set. Protein was determined spectrophotometrically (14).

**Fluorescence spectroscopy.** Fluorescence spectra were collected at 24 °C. Total emission intensity was obtained by integration of the spectra between 305nm and 400nm. In acrylamide quenching experiments, excitation was performed at 295 nm. The total intensity was corrected for the inner filter effect due to acrylamide using an extinction coefficient of  $0.25 \text{ M}^{-1} \text{ cm}^{-1}$ . The data were then corrected for the blank and the dilution. Aliquots of a 7M stock solution of acrylamide were added to 2.5 ml of the protein solution in 20mM Naphosphate buffer pH 7.4. Effective Stern-Volmer quenching constants were obtained by regression analysis of the data using the approximation proposed by Lehrer (15):  $F_0/\Delta F_0 = 1/f_1 + 1/(f_1 * K_s * v * Q)$

**Lipid-protein interaction.** The kinetics of the apoLp-III-DMPC association was followed by the decrease in absorbance at 325 nm that accompanies the transformation of the multilamellar liposomes into discoidal lipoprotein particles as described by Pownall et al., (16). The kinetics was performed at 23.9 °C in a Hewlett Packard diode array spectrophotometer equipped with a temperature-controlled cell holder. The reaction was performed at a 17:1 lipid to protein molar ratio by adding 50  $\mu\text{l}$  of a liposome suspension (2mg/ $\mu\text{l}$ ) to 1950 $\mu\text{l}$  of the apoLp-III solution.

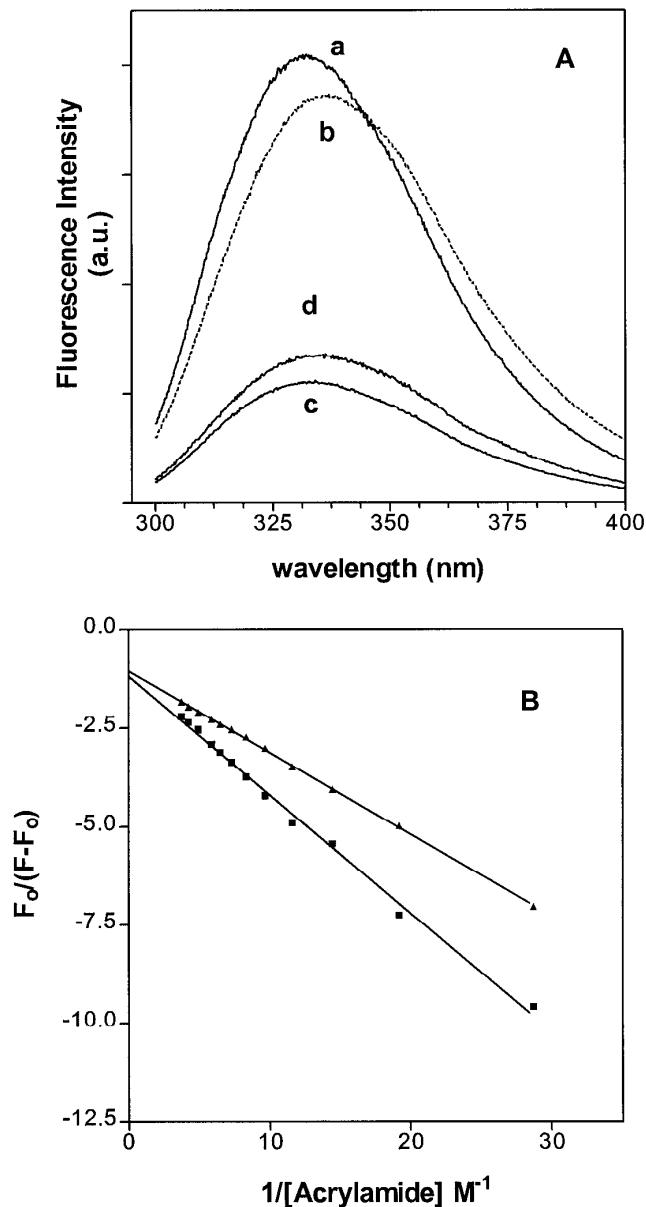
## RESULTS AND DISCUSSION

**ApoLp-III purification.** The free apoLp-III was separated from the enterokinase cleavage products of the recombinant thioredoxin/apoLp-III by Ni-affinity chromatography. ApoLp-III elutes as a sharp single-band in the fraction containing 15 mM imidazole. The amino acid composition of this fraction matched the compo-

sition of the insect apolipoprotein. Two isoforms of apoLp-III have been found in the hemolymph *Locusta migratoria*. One of them contains two additional amino acids, Arg-Pro, added at the N-terminal (17). We expressed the shorter apoLp-III form (166 aa residues) which is the most abundant form in adult insects and probably represents the mature form of apoLp-III (17). Edman degradation analysis gave the sequence of the first six amino acids of apoLp-III (DAAGHV..) confirming the specificity of the enterokinase-cleavage. The insect-apoLp-III was also sequenced and rendered only one sequence, which matched that of the expressed protein. Electrospray ionization mass spectrometry of the purified apoLp-III revealed a molecular mass of 17,331 Da, which corresponds very well with the molecular mass that is deduced from the amino acid sequence of *Locusta migratoria* apoLp-III, 17,333 Da.

**Spectroscopic characterization.** Because *Locusta migratoria* apoLp-III has two Trp residues, two Phe and no Tyr residues, the environment of these two Trp residues defines the absorption and emission characteristics of the protein in the near UV range. According to the crystal structure of the native apoLp-III (3), the indole groups of the two Trp residues are oriented toward the protein core. Figure 1A shows the fluorescence spectra of recombinant and native proteins. The wavelengths of maximum fluorescence of both proteins, the insect ( $\lambda = 333 \text{ nm}$ ) and the recombinant (336 nm), are consistent with the location of the indole groups observed in the crystal structure and indicate that, in solution and in both proteins, the Trp residues reside in a medium of relatively low polarity (18). However, the quantum yield of the insect protein is 30% higher than that of the recombinant apoLp-III (Figure 1A and Table 1). The accessibility of Trp residues to acrylamide was studied by fluorescence quenching experiments (Figure 1B). At high concentrations of acrylamide, the y-intercept approaches unity suggesting that both Trp residues are accessible to the quencher. At low concentration of acrylamide, Effective Stern-Volmer quenching constants of  $4.83 \text{ M}^{-1}$  and  $3.33 \text{ M}^{-1}$  and accessibility factors of 0.95 and 0.85 were obtained for the recombinant and the native protein, respectively. This result, as well as the small differences in position of the maxima and quantum yields, indicate that the Trp residues of the recombinant protein are more exposed to solvent than those of the native protein.

The CD spectra of both proteins showed the minima at 208 nm and 222 nm commonly observed for predominantly  $\alpha$ -helical peptides (Figure 2). The analysis of the CD spectrum indicated that the recombinant apolipoprotein has a lower  $\alpha$ -helical content (49%) than the native protein (59%). For both proteins the deduced  $\alpha$ -helical content was lower than that observed in the crystal structure (72%). Storage of the protein solutions



**FIG. 1.** (A) Effect of the lipid protein interaction on the fluorescence of *Locusta migratoria* apoLp-III. (a) Fluorescence spectrum of a 1.6  $\mu\text{M}$  solution of insect apoLp-III in buffer 10mM pH 7.8. (b) Recombinant wild type apoLp-III (2.1  $\mu\text{M}$ ) in 10mM Na-phosphate buffer pH 7.8. (c) Same as in "b" but containing 0.5% SDS. (d) Same as in "a" but containing 0.5% SDS. (B) Quenching of apoLp-III fluorescence by acrylamide. Data for insect ( $\blacksquare$ ) and recombinant ( $\blacktriangle$ ) apoLp-III were obtained in sodium phosphate buffer pH 7.8 at 25°C and fitted to the Lehrer equation.

at 4°C, in buffer at pH 7.8 containing 500mM NaCl, did not have a significant effect on the CD-spectrum for at least 3 months. A previous study on the spectroscopic properties of the native and enzymatically deglycosylated *L. migratoria* apoLp-III reported that the native apoLp-III displays a fluorescence maximum at 347 nm,

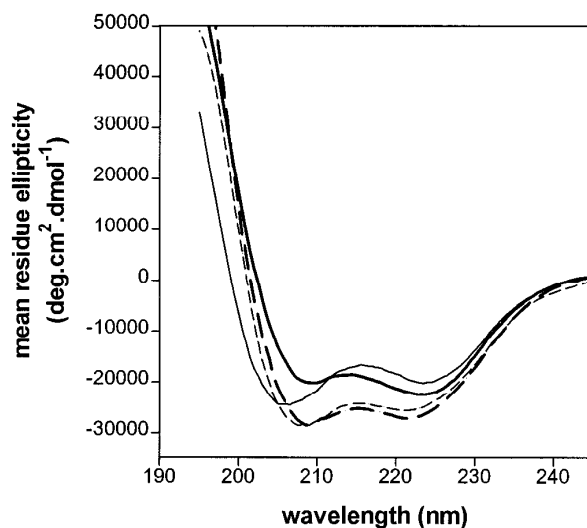
TABLE 1

Physical-Chemical Properties of Native and Recombinant ApoLp-III from *Locusta migratoria*

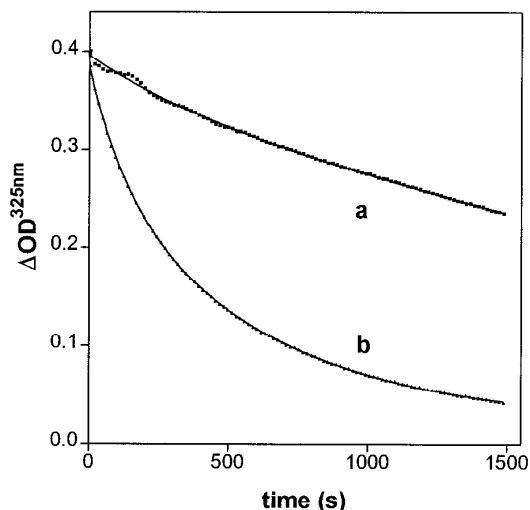
	Insect <sup>a</sup> apoLp-III	Wild type <sup>a</sup> apoLp-III
Fluorescence maxima ( $\lambda_{\text{max}}$ )		
In buffer	333 nm	336 nm
DMPC-apoLp	331 nm	332 nm
+0.5% SDS	334 nm	335 nm
Relative quantum yield		
In buffer	1	0.77
+DMPC-apoLp	0.33	0.35
+0.5% SDS	0.28	0.28
Effective Stern-Volmer K <sub>sv, eff</sub>		
	$3.32 \pm 0.07 \text{ M}^{-1}$	$4.83 \pm 0.03 \text{ M}^{-1}$
$f_i$ (fraction of accessible emitters)		
	$0.85 \pm 0.06$	$0.95 \pm 0.02$
Molecular Weight		
	17333	17331
% $\alpha$ -helix		
Buffer	59	49
DMPC-apoLp	73	71

<sup>a</sup> Insect and wild-type refer to the proteins isolated from the insect hemolymph (glycosylated) and the bacterial expression system, respectively.

which upon deglycosylation is blue shifted to 332 nm (7). The same report indicated that enzymatic deglycosylation resulted in a large increase in the exposure of the Trp residues, and a concomitant loss in secondary structure and stability upon storage. The discrepancies



**FIG. 2.** CD spectra of the native *Locusta migratoria* apoLp-III, thick traces, in solution (—) and in DMPC-apoLp complexes (---) and of the recombinant locust apoLp-III, thin traces, in solution (—) and in DMPC complexes (---). Spectra were acquired at 25°C in 10 mM Na-phosphate buffer pH 7.8.



**FIG. 3.** Kinetics of the association of native (curve a) and recombinant (curve b) locust apoLp-III with multilamellar DMPC liposomes. After 24 h of incubation, a complete clearance of turbidity is achieved with both proteins.

between the spectroscopic studies carried out with the enzymatically deglycosylated protein and the recombinant apoLp-III could be due to undetected proteolysis of the protein during enzymatic deglycosylation.

**Lipid-protein interaction.** The kinetics of the association of native and recombinant *Locusta migratoria* apoLp-III with DMPC, as followed by the clearance of liposomal turbidity, was studied at 23.9 °C. The interaction of DMPC with exchangeable apolipoproteins is characterized by a decrease in light scattering due to the disruption of the large liposomes and the formation of small discoidal apolipoprotein-DMPC particles. Both proteins interact with DMPC promoting the formation of discoidal apoLp-lipid complexes. Figure 3 shows the turbidity decay obtained during the first 30 min of the reaction. A much faster association of the recombinant protein with DMPC was observed. The clearance curves follow an exponential decay that upon fitting yields pseudo first-order rate constants of  $0.039 \pm 0.001 \text{ min}^{-1}$  and  $0.203 \pm 0.011 \text{ min}^{-1}$  for the native and recombinant apoLp-III, respectively. These results indicate that glycosylation has an important inhibitory effect on the kinetics of formation of discoidal lipoprotein particles. The interaction of the native and recombinant proteins with SDS micelles and DMPC results in a large decrease in the quantum yield of the Trp fluorescence (Figure 1B and Table 1). Interestingly, the differences in quantum yield observed in the absence of lipid are not present in the presence of SDS or in the DPMC-apoLp-III complexes (Table 1).

Both proteins undergo a significant increase in  $\alpha$ -helical content when bound to lipid in DMPC discoidal recombinant particles (Figure 2, Table 1). Mimicking

the fluorescence results, the increase in the  $\alpha$ -helical content of the recombinant protein (from 49% to 73%) was larger than that observed in the native protein (from 59 to 71%) and, both proteins appear to have the same  $\alpha$ -helical content in the lipid bound state. The large reduction in quantum yield and the fact that in the lipid bound state both proteins have the same quantum yield and  $\alpha$ -helical content suggests that similar conformational changes and lipid-bound conformations take place upon the interaction of the native and recombinant lipoprotein with lipid.

## CONCLUSIONS

The 5-fold increase in association rate that accompanies deglycosylation indicates that the apparently small differences in structural properties of the proteins in the lipid-free state have a large impact on the functional properties of the protein. The ability of an apolipoprotein to interact with lipid is dictated to a large extent by its conformational flexibility. The transient exposure of hydrophobic residues, or the ability of the protein to expose hydrophobic domains after an initial binding to the lipid surface (19), allows the interaction with the acyl-chains of the lipid matrix that leads to the insertion of the protein into the bilayer. The large impact of the glycosylation on the kinetics of binding to lipid suggests that the oligosaccharide moiety inhibits the lipid binding activity by preventing the exposure of hydrophobic domains and/or decreasing the conformational flexibility of the protein.

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