Physical and Surface Properties of Insect Apolipophorin III*

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Apolipophorin III (apoLp-III) from Manduca sexta has a molecular weight of 18,100. Based on its hydrodynamic properties (sedimentation and diffusion coefficients, frictional ratio, intrinsic viscosity) and its behavior during gel permeation chromatography, we concluded that apoLp-III is a prolact ellipsoid with an axial ratio of about 3. The circular dichroic spectrum of apoLp-III suggests that the protein contains approximately 50% α-helix. At the air-water interface, apoLp-III forms a monolayer which is gaseous at surface pressures ≤1 dyne/cm. The isotherm of this phase yields an excluded molecular area of 3800 Å²/molecule (23 Å²/amine acid). At a surface pressure of 22.1 dyne/cm, the monolayer undergoes a phase transition reminiscent of a first-order phase transition of pure lipids. The monolayer can be compressed in this surface pressure range to an area per molecule of 480 Å² (2.9 Å²/amine acid). Since a globular protein of molecular weight 18,100 could occupy an area of only about 2000 Å² when bound to a surface, it is suggested that in the expanded state, apoLp-III must unfold on the surface, whereas in the compressed state, the molecule is oriented with its minor axis parallel to the water surface. ApoLp-III binds with high affinity (Kd = 1.9 × 10⁻¹⁰ M) to both phosphatidylcholine- and diacylglycerol-coated polystyrene beads. All of these results are consistent with the proposal that apoLp-III plays a key role in increasing the capacity of the insect lipoprotein, lipophorin, to transport diacylglycerol by stabilizing the increment of lipid-water interface that results from diacylglycerol uptake.

Lipids are transported in insect hemolymph by an abundant lipoprotein, lipophorin (Gilbert and Chino, 1974; Chino et al., 1981). In the adult tobacco hornworm, Manduca sexta, lipophorin is a high density lipoprotein (density = 1.076 g/ml) which contains 48% protein and 52% lipid, mainly phospholipid and diacylglycerol (Ryan et al., 1986). The protein moiety consists of three apolipoproteins: apolipophorin I (M, ≈ 250,000), apolipophorin II (M, ≈ 78,000), and apoLp-III (M, ≈ 17,000) (Pattnaik, et al., 1979; Shapiro et al., 1984; Kawooya et al., 1984).

Whereas apolipoporphins I and II are integral constituents of lipophorin (Ryan et al., 1986), apoLp-III appears mostly free in the hemolymph, with only a small fraction associated with lipophorin (Kawooya et al., 1984). During flight, diacylglycerols are mobilized from the fat body, a process stimulated by a peptide hormone, adipokinetic hormone, secreted from the corpus cardiacum (Beenakkers et al., 1978; Shapiro and Law, 1983). In flying animals and in animals treated with adipokinetic hormone, the lipid content of lipophorin increases to about 60% and the density of the lipoprotein decreases to 1.030 g/ml, which lies within the low density lipoprotein range. Therefore, this lipid-enriched form is termed LDLp (Shapiro and Law, 1983; Ryan et al., 1986). During the conversion of adult high density lipophorin to LDLp, there is considerable increase in the apoLp-III content of the lipoprotein, presumably resulting from the adsorption of free apoLp-III from the hemolymph (Wheeler and Goldworthy, 1983a, 1983b; Shapiro and Law, 1983). Thus, it appears that the binding of apoLp-III during the conversion of high density lipophorin to LDLp is linked to diacylglycerol uptake. We felt that apoLp-III would play a key role in increasing the capacity of lipophorin to carry diacylglycerol by stabilizing the increment of lipid-water interface that results from diacylglycerol uptake and that a determination of the molecular properties of apoLp-III would be essential in understanding this important physiological role of apoLp-III.

In this paper, we present the results of studies on the physical and surface properties of apoLp-III, which are consistent with this proposed role.

MATERIALS AND METHODS

ApoLp-III was isolated from the hemolymph of adult male or female M. sexta as previously described (Kawooya et al., 1984; Wells et al., 1985). Purity of the protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Radioactive sn-1,2-diacylglycerol was prepared by mixing 0.2 μCi of 1-palmitoyl-2-[1-¹⁴C]oleoyl-sn-glycero-3-phosphocholine (Amersham Corp.) with 30 μmol of egg yolk phosphatidylcholine (Avanti Polar Lipids, Inc, Birmingham, AL) and converting the phospholipid to diacylglycerol with phospholipase C (Boehringer Mannheim). The diacylglycerol was purified as described by Crawford and Wells (1979). Unlabeled sn-1,2-dipalmitoylglycerol was obtained from NuCheck Prep, Inc. (Ely, MN). Polystyrene beads of 5.7-μm diameter were obtained from Dow Diagnostics, Indianapolis, IN. All other chemicals were of the highest purity available.

Analytical Ultracentrifugation—A Beckman Model E analytical ultracentrifuge with electronic speed control and a photoelectric scanner was used for all studies. In all cases, the protein was dissolved in 5 mM sodium phosphate buffer, pH 7.0, containing 0.1 M KCl. The measurements were carried out at 20.0 °C. The sedimentation and diffusion coefficients of apoLp-III were measured in the concentration range of 2.5–10 mg/ml. Sedimentation velocity experiments were carried out at 60,000 rpm using a double sector cell, and the diffusion measurements were performed at 6,000 rpm using a synthetic boundary cell. The data were analyzed and extrapolated to standard con-
ditions according to Svedberg and Petersen (1940). Sedimentation equilibrium was carried out at 22,000 rpm using protein concentrations of 0.175 and 2.32 mg/ml.

Viscosity—Viscosity was measured in 5 mM sodium phosphate buffer, pH 7.0, containing 0.1 M KCl at 20.0 ± 0.2 °C with a protein concentration range from 1 to 20 mg/ml. A Cannon-Ubbelohde semimicro dilution viscometer (Cannon Instrument Co., State College, PA) was used with a Mechrolab 5901A Auto-Viscometer to determine efflux times. The intrinsic viscosity, [η], and the Sihma factor, ω, were calculated in the usual manner (Van Holde, 1971).

Circular Dichroism—The circular dichroic spectrum of apoLp-III was measured under N2 between 190 and 240 nm, using a Cary 60 spectropolarimeter fitted with electronics from Aviv Associates Inc. (Lakewood, NJ) and a 0.05-mm quartz cell at 25 °C. The protein concentration was 0.075 mg/ml in 1 mM Tris-HCl buffer, pH 7.4. The data were analyzed according to Chang et al. (1978), using a mean residue weight of 100.

Monolayer Studies—Monolayers of apoLp-III were formed at the interface between air and a 10 mM potassium phosphate buffer, pH 6.80, containing 0.2 mM KCl. Thirty-eight μg of apoLp-III in 60 μl of buffer was spread onto 600 cm2 of the buffer using a partially immersed, acid-washed microscope coverslip inclined at 30° from the horizontal and applying the protein solution at least 30 cm from the buffer surface. The monolayer was compressed at rates not exceeding 40 mg/cm2/s. The concentration range from 0.05-5.0 mg/ml with no indication of self-association. The values for the Sihma factor, ω, which corresponds to the intrinsic viscosity when concentration is expressed as volume fraction (Mehl et al., 1940), the frictional coefficient (f/f0), and the Scheraga and Mandelkern (1953) β value correspond closely to the theoretical values for a nonhydrated prolate ellipsoid with an axial ratio of 5 (Schachman, 1959). Assuming a hydration level of 0.3 g of water/g of protein, which is commonly used (Richards, 1977), the axial ratio would be 3.3. This would correspond to a minor axis of 12.5 Å and a major axis of 40 Å (Van Holde, 1971).

| **Table 1** |
| Physical properties of apolipopophorin III |
| s20,w | 1.95 ± 0.08 S |
| D20,w | 9.7 ± 1.3 × 10^-7 cm²/s |
| v | 0.728 ml/g |
| Mw | 17,400 ± 300 |
| η | 18,100 ± 250 |
| [η] | 4.25 ± 0.07 ml/g |
| f/f0 | 1.27 |
| β | 5.98 ± 0.09 |
| Ψ | 2.22 × 10^-3 |

*Calculated from the amino acid composition (Kawooya et al., 1984).
* Determined from sedimentation equilibrium data.
* Calculated from viscosity and diffusion coefficients.
* Calculated from [η] = ω (Mehl et al., 1940).
* β = (N/16,000) (f/f0) ω^1/3 (Scheraga and Mandelkern, 1953).

**RESULTS**

**Physical Properties of Apolp-III**—Table 1 presents values for the molecular weight and hydrodynamic properties of apoLp-III. The sedimentation equilibrium data show a constant molecular weight of 17,400 over the concentration range of 0.05-5.0 mg/ml with no indication of self-association. The values for the Sihma factor, ω, which corresponds to the intrinsic viscosity when concentration is expressed as volume fraction (Mehl et al., 1940), the frictional coefficient (f/f0), and the Scheraga and Mandelkern (1953) β value correspond closely to the theoretical values for a nonhydrated prolate ellipsoid with an axial ratio of 5 (Schachman, 1959). Assuming a hydration level of 0.3 g of water/g of protein, which is commonly used (Richards, 1977), the axial ratio would be 3.3. This would correspond to a minor axis of 12.5 Å and a major axis of 40 Å (Van Holde, 1971).

**Fig. 1.** Circular dichroic spectrum of apolp-III. The measurements were performed at 25 °C using 0.075 mg/ml protein solution in 1 mM Tris-HCl, pH 7.4.
Circular Dichroism of ApoLp-III—The circular dichroism spectrum of apoLp-III is shown in Fig. 1. The best estimate of the secondary structure after 5 iterations was 50% α-helix, 20% β-structure, and 30% random coil.

Gel Filtration—The gel permeation behavior of apoLp-III was anomalous in that the elution time was markedly dependent on flow rate, whereas those of the globular calibration proteins were not. The flow rate dependence of the apparent molecular weight is consistent with the pattern observed for asymmetric proteins (Meredith and Nathans, 1982). At infinity slow flow rate, the molecular weight of apoLp-III was approximately 6000. This corresponds to the molecular weight of a globular protein with a radius equal to that of the minor axis of the asymmetric protein (Meredith and Nathans, 1982). For apoLp-III, this gives a value of 12.0 Å, which corresponds well to the value calculated for the minor axis from hydrodynamic measurements (12.5 Å).

Behavior of ApoLp-III at the Air-Water Interface—ApoLp-III readily forms an insoluble monolayer at the air-water interface. The monolayer is stable during repeated compressions and expansions and is stable for at least 30 min. Below surface pressures of 1 dyne/cm, the surface isotherm is consistent with that of a gaseous phase (Fig. 2), i.e. it obeys the equation (Adamson, 1976): \[ \pi = nRT/(A - nA_e) \]
where \( \pi \) is surface pressure, \( A \) is area of the monolayer, \( A_e \) is the molar exclusion area, \( n \) is number of moles of protein in the monolayer, \( R \) is gas constant, and \( T \) is absolute temperature. Nonlinear least squares fitting of the data to this equation yields a molecular weight of 18,000 and a limiting molecular area of 3795 Å², which corresponds to a mean residue area of 23 Å²/amino acid.

Further compression of the apoLp-III monolayer initially leads to the expected increase in surface pressure, but when the surface pressure reaches 22.1 dyne/cm, the monolayer undergoes a phase transition (Fig. 3). This behavior is unusual for protein monolayers which usually collapse near this pressure (Shen and Scanu, 1980). The apoLp-III monolayer, however, can be compressed until the limiting area is reduced to 2.9 Å²/amino acid, which corresponds to a molecular area of 480 Å². This limiting area is determined by the geometrical constraints of the film balance and therefore represents an upper limit for the molecular area of the protein in the compressed state. After expansion of the highly compressed monolayer, recompression yields a force-area curve identical to the first compression showing that no molecules of protein were lost from the surface in the highly compressed state and that the transition is reversible. We infer that the highly compressed monolayer has a high viscosity since during expansion there is a large hysteresis, which takes about 10 min to relax.

Binding of ApoLp-III to Lipid-coated Beads—The adsorption isotherm for the binding of apoLp-III to phospholipid and diacylglycerol-coated polystyrene beads is shown in Fig. 4. There was no significant difference between the two sets of data. The binding constant \( K_b = 1.9 \pm 0.2 \times 10^{-7} \) M and maximum binding \( (3.8 \pm 0.3 \times 10^{-12} \) mol of apoLp-III/cm² of surface) were determined by nonlinear least squares fitting of the data. A theoretical curve based on these parameters is shown in Fig. 4. The area occupied by apoLp-III on the surface of the lipid-coated beads corresponds to 26.2 ± 2.0 Å²/amino acid residue or a molecular area of 4300 ± 300 Å².

Cross-linking Experiments—Under conditions which lead to extensive cross-linking of the female specific protein from M. sexta, no evidence could be found for cross-linking of apoLp-III. This is in marked contrast to human apolipoprotein...
tein A-I, which does undergo cross-linking under these conditions (Swaney and O'Brien, 1978).

**DISCUSSION**

The above results show that apoLp-III is a highly α-helical protein with a shape approximating that of a prolate ellipsoid. The protein has a high affinity for amphiphilic surfaces and when adsorbed to the air-water interface can exist in two strikingly different conformations. The surface conformations which occur at the lipid-coated bead-water interface and at the air-water interface, at surface pressures below 22 dynes/cm, are both characterized by a molecular exclusion area of about 4000 Å², and we conclude that this state probably represents the state of the protein when adsorbed to the fully loaded lipoprotein particle.

However, regardless of its exact shape, a globular protein with \( M_p = 18,100 \) could occupy an area of only about 2,000 Å² when bound to a surface. Thus, it is apparent that apoLp-III cannot maintain its solution structure when adsorbed to an interface, but must unfold onto the surface in some manner that permits the molecule to occupy the observed area. Since both the monolayer and lipid-coated bead data were calculated on a per molecule basis, dimer formation cannot be invoked to explain this discrepancy. Furthermore, there are no data to support dimerization of apoLp-III in solution. An earlier report (Kawooya et al., 1984) that apoLp-III behaved as a dimer on gel permeation chromatography can be explained by the unexpected asymmetry of the protein.

Of all the physical properties of apoLp-III, perhaps the most remarkable is its ability to remain at the air-water interface at surface pressures beyond those which force other amphiphilic proteins into the aqueous phase. The limiting area at these high pressures approaches a value consistent with the minor axis of the molecule oriented parallel to the interface. One possible structural feature which would account for this strong binding to the interface would be the presence of amphiphilic \( \beta \)-strand. Such structures have been shown to possess very high surface affinities (Osterman et al., 1984). The presence of this amphiphilic \( \beta \)-strand could represent the site for initial adsorption of the protein at the interface. However, the exact nature of the two surface conformations must await further experimental data. All of the results reported in this paper are consistent with the suggestion that the role of apoLp-III in the conversion of adult high density lipophorin to LDLp is to bind to the newly created interface, resulting from diacylglycerol uptake, and to thus stabilize LDLp. Whether apoLp-III has any role to play in delivery of the diacylglycerol to the flight muscle, where its fatty acids are used as an energy source (Beenakkers et al., 1981), must await further experimentation.

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