Structural Studies on Lipophorin, an Insect Lipoprotein*

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An insect high density lipoprotein, lipophorin, can be rapidly isolated from larval Manduca sexta (tobacco hornworm) hemolymph by single vertical spin density gradient ultracentrifugation. The two apolipoproteins (Mₚ = 245,000 and 78,000; designated apoLp-I and apoLp-II, respectively) were readily dissociated and separated in 6 m guanidine HCl by gel permeation chromatography. ApoLp-I and apoLp-II showed no immunological cross-reactivity on electrophoretic blots of sodium dodecyl sulfate-polyacrylamide gels. ApoLp-I and apoLp-II from lipophorin of adult M. sexta behaved identically to their larval counterparts. Amino acid compositions of larval apoLp-I and apoLp-II were similar except with respect to tryptophan and cysteine; apoLp-I contained 32 residues/mol of tryptophan (1.5 mol %) and 22 residues/mol (1.1 mol %) of cysteine; apoLp-II contained 2 residues/mol of tryptophan (0.2 mol %) and 14 residues/mol of cysteine (2.1 mol %). In double immunodiffusion tests, antisera against apoLp-I or whole lipophorin strongly precipitated lipophorin, while antisera against apoLp-II caused only minor precipitation. This indicates relatively greater exposure of apoLp-I to the aqueous environment.

Unlike mammalian blood, insect hemolymph contains a single major class of low- to high-density lipoproteins, called lipophorins because of their role in lipid transport (Chino et al., 1981a). Lipophorins can reversibly load lipid, increasing in size and decreasing in density from the high to the low lipid density lipoprotein class upon association with a small soluble protein (Mwangi and Goldsworthy, 1977, 1981; van der Horst et al., 1981; Wheeler and Goldsworthy, 1985a, 1985b; Shapiro and Law, 1983). Lipophorin appears to be the major carrier of hydrophobic natural products (Gilbert and Chino, 1974; Katase and Chino, 1982) and xenobiotics (Winter et al., 1975; Skalsky and Guthrie, 1975; Fell et al., 1976) in insect hemolymph. Though present in all life stages, lipophorin usually has been isolated from hemolymph of pupal and adult insects (Chapman, 1980). Recently, it was isolated and characterized from the hemolymph of larval Manduca sexta (Pattnaik et al., 1979).

Compared to mammalian lipoproteins, lipophorins are unusual in both lipid and apoprotein content. Lipids, which constitute 39 to 48% of lipoprotein mass, consist of 33 to 56% diacylglycerol, 19 to 36% phospholipid, and 5 to 6% unesterified cholesterol (Chapman, 1980). Furthermore, lipophorins contain two relatively large apoproteins (Mₚ = 250,000 and ~80,000). In contrast, mammalian lipoproteins contain little diacylglycerol and a much higher proportion of nonpolar lipid (primarily triacylglycerol and cholesterol esters). Most mammalian lipoproteins also contain numerous small (Mₚ < 40,000) apoproteins, with the exception of the low density lipoproteins. These compositions suggest a difference in structural organization between mammalian and insect lipoproteins.

Little is known about the structure of native lipophorin. Improved methods of lipophorin and apoprotein isolation from larval M. sexta reported here permit structural studies using immunological probes. We also report complete amino acid compositions of the two apoproteins, since cysteine and tryptophan contents were not previously reported (Pattnaik et al., 1979). The marked similarity in amino acid content of the apoproteins indicated possible homology, and a recent report on locust lipophorin (Gellissen and Wyatt, 1981) suggested that a large apoprotein, not consistently present in lipophorin preparations, represented an aggregate of small apoproteins. We have, therefore, compared the apoproteins of M. sexta lipophorin, which we designate apoLp-11₂ (Mₚ = 245,000) and apoLp-II (Mₚ = 78,000), and demonstrate unequivocally that they are not homologous.

EXPERIMENTAL PROCEDURES

RESULTS

Isolation of Lipophorin—Flotation in a KBr gradient using a single vertical spin at 50,000 rpm yielded a highly purified preparation of lipophorin, as judged by SDS-polyacrylamide slab gel electrophoresis (Fig. 4). Fig. 2 illustrates the efficiency of separation of lipophorin from the major hemolymph protein manducin (Kramer et al., 1980), whose two subunits (Mₚ = 78,000 and 27,000) appear in fractions 14-20. A small amount of manducin was carried over into lipophorin fractions when tubes were fractionated from the bottom (Fig. 2). However, for routine preparations, the pure lipophorin was withdrawn.

1 The abbreviations used are: apoLp-I, apolipophorin I; apoLp-II, apolipophorin II; apoLp-III, apolipophorin III; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.

2 We have used Roman numerals to designate these insect apolipoproteins to avoid premature comparison to the lettered mammalian apolipoproteins.

3 Portions of this paper (including "Experimental Procedures," Figs. 1-3, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-369, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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through the side of the tube with a hypodermic syringe (see Fig. 4, lanes 1 and 6). Recentrifugation of isolated lipophorin and comparison with a density gradient reference tube yielded a buoyant density of 1.13 g/ml (Fig. 1). Gel permeation HPLC demonstrated a single major peak, with a small amount (<10%) appearing at the void volume as aggregated material.

**SDS-Polyacrylamide Gel Electrophoresis: Molecular Weight Determination and Carbohydrate Content**—Earlier molecular weight determinations on SDS-polyacrylamide gels were made by comparison to cross-linked standards (Pattnaik et al., 1979). Our more recent experience with these standards has been less satisfactory than with non-cross-linked standards. We, therefore, slightly revised our molecular weight estimates, using non-cross-linked standards (Bio-Rad high molecular weight standards, plus ferritin (220,000) and thyroglobulin (330,000), not shown) on 3–8% SDS-polyacrylamide gradient slab gels (Fig. 4). Molecular weights of apoLp-I and apoLp-II were calculated as 245,000 ± 7,000 and 78,000 ± 5,000 (M, ± S.D., n = 6).

Scanning densitometry of stained apoproteins yielded an approximate apoLp-I:apoLp-II ratio of 3:1 by weight, corresponding to the 1:1 molar ratio previously observed (Pattnaik et al., 1979). The stoichiometric ratio was further confirmed by separation of the two apoproteins by preparative SDS-gel electrophoresis. The fractions containing the two apoproteins were identified by analytical SDS-slab gel electrophoresis and the amounts of protein present in pooled fractions of each apoprotein were determined quantitatively by the Folin-phenol method of Peterson (1983), using SDS solutions of the pure apoproteins as standards. In two runs, apoLp-I/apoLp-II molar ratios of 1:13 and 0.87 were determined.

After separation on slab gels, both apoLp-I and apoLp-II stained with periodate-Schiff reagent, and both bound fluorescein-labeled concanavalin A.

**Amino Acid Composition and UV Absorbance**—Apoproteins were analyzed for content of amino acids, including tryptophan and cysteine (Table II). Spectrophotometric determination of tryptophan yielded values of 29 and 2 residues/mol of protein in apoLp-I and apoLp-II, respectively, compared to 32 and 2 residues/mol when determined by mild hydrolysis and chromatographic analysis. Specific absorbances for apoLp-I and apoLp-II at 280 nm in 6 M guanidine HCl were 0.716 mg⁻¹ ml⁻¹ and 0.300 mg⁻¹ ml⁻¹, respectively. A visible wavelength scan of native lipophorin in PBS revealed a maximum absorbance at 455 nm (due to the presence of carotenoids) and profile identical to that observed in Locusta migratoria lipophorin (Feled and Tietz, 1975).

**Immunological Comparison of Apoproteins**—Immunological reaction of nitrocellulose-bound proteins was used to determine whether the apoproteins have common structural features. Lipophorin was subjected to electrophoresis on SDS-polyacrylamide slab gels, transferred electrophoretically to nitrocellulose, and reacted with antisera specific to whole lipophorin or to either apoprotein. IgG-bound proteins were located by reacting blotted proteins with Staphylococcus aureus protein A and located by autoradiography. Clearly, the anti-lipophorin serum reacted with both apoLp-I and apoLp-II (Fig. 4). However, anti-apoLp-I did not bind to apoLp-II, and anti-apoLp-II did not bind to apoLp-I. ApoLp-I and apoLp-II were the only proteins in fifth instar larval hemolymph that reacted with anti-lipophorin.

Lipophorin was isolated from adult *M. sexta* hemolymph and analyzed in the same manner, using antisera against larval lipophorin and apoproteins (Fig. 5). ApoLp-I and apoLp-II were not detected by any of the antisera used.
Insect Lipophorin

**Amino acid composition of lipophorin apoproteins**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>ApoLp-I</th>
<th>ApoLp-II</th>
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<tr>
<td></td>
<td>Residues/mol</td>
<td>Mol %</td>
</tr>
<tr>
<td>Aspartate</td>
<td>232</td>
<td>11.2</td>
</tr>
<tr>
<td>Threonine</td>
<td>93</td>
<td>4.5</td>
</tr>
<tr>
<td>Serine</td>
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<td>Glutamate</td>
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<td>Proline</td>
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<tr>
<td>Glycine</td>
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</tr>
<tr>
<td>Valine</td>
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</tr>
<tr>
<td>Methionine</td>
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</tr>
<tr>
<td>Isoleucine</td>
<td>102</td>
<td>4.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>170</td>
<td>8.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>71</td>
<td>3.4</td>
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<td>Phenylalanine</td>
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<tr>
<td>Histidine</td>
<td>68</td>
<td>3.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>185</td>
<td>8.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>92</td>
<td>4.4</td>
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</tbody>
</table>

* Calculated on the basis of Ala = 153 for apoLp-I and Ala = 56 for apoLp-II. Three replicates were run; a representative analysis is shown.

Insect lipophorin is thought to be a lipid shuttle, reutilized many times without new synthesis or degradation (Chino and Kitazawa, 1981). In the adult locust, the lipophorin particle can absorb additional diacylglycerol from the fat body under the influence of the decapeptide adipokinetic hormone (Chino and Goldsworthy, 1981). Lipophorin thereupon increases in size and decreases in density to the low density lipoprotein class. These changes are reversed as diacylglycerol is deposited for use as fuel in the flight muscles. Thus, unlike mammalian lipoproteins, which may change in density but not fluctuate, insect lipophorin fluctuates between lipid density classes in the course of its transport function. Diacylglycerol transport is apparently facilitated by association with a small protein, designated C protein (Mwangi and Goldsworthy, 1981; van der Horst et al., 1981; Wheeler and Goldsworthy 1983a, 1983b), though the protein and nature of its association with lipophorin have not been characterized. While not present in larvae, a similar protein has recently been isolated from adult M. sexta. We propose to call this small apoprotein apoLp-III, in accordance with our proposed terminology and to avoid premature comparison with mammalian apolipoproteins C.

In order to understand how insect lipophorin functions during lipid transport, we need to know more about the structure of the particle and of its apoprotein components. We have developed a gentle and rapid density gradient procedure for isolating the larval lipophorin from the hemolymph of M. sexta and an efficient gel permeation chromatography procedure for separating the apoproteins. The larval lipophorin is homogeneous with respect to molecular weight and density, while lipophorin from adult M. sexta is polydisperse and less dense than the larval form and thus resembles the diacylglycerol-loaded lipophorin of the adult locust (Shapiro and Law, 1983).

We have substantially improved separation of the apoproteins by gel permeation chromatography through use of guanidine hydrochloride as a chaotrope rather than SDS, which was used previously (Pattnaik et al., 1979). We performed complete amino acid analysis on the isolated apoproteins and found that the cysteine and tryptophan contents, not previously determined, were of particular interest. ApoLp-I contained 29-32 residues/mol (1.5 mol %) of tryptophan, compared to only 2 residues/mol (0.2 mol %) for apoLp-II. This observation accounts for the low absorbance of apoLp-II at 280 nm seen during chromatography in 6 M guanidine hydrochloride (Fig. 3). Cysteine content of the apoproteins also differed; apoLp-I contained 22 residues/mol (1.1 mol %), while apoLp-II contained 14 residues/mol (2.1 mol %). Assuming that most cysteine residues are involved in disulfide linkages, the higher percentage of cysteine in apoLp-II may contribute to a more compact structure.

Some studies on the lipophorin of Locusta migratoria have demonstrated a single apoprotein that is comparable in molecular weight to apoLp-II of Manduca lipophorin (Gellisse and Emmerich, 1990; Gellisse and Wyatt, 1981), while others have found both apoproteins corresponding to those of M. sexta (Chino et al., 1981b; Chino and Kitazawa, 1981). Chino and Kitazawa (1981) found a definite difference in the apoproteins of locust lipophorin; apoLp-I stained for carbohydrate in SDS-polyacrylamide gels, while apoLp-II did not. We have isolated two distinct apoproteins from Manduca lipophorin, both of which are stained by the periodate-Schiff reagent and both of which bind fluorescein labeled concanavalin A. Harnish and Law, 1982). We also showed that two apoproteins found in adult lipophorin are immunologically identical to those in larval lipophorin (Fig. 5).

We propose that the apoproteins serve two general roles, maintenance of particulate structure and facilitation of lipid transfer. In mammalian systems, lipoprotein structure is maintained by a combination of hydrophilic surface interactions
FIG. 5. Cross-reactivity of larval and adult apoproteins to antisera against larval apoproteins and lipophorin. Lipophorin (lanes 1, 4, and 7) or hemolymph (lanes 2, 5, and 8) from larvae or lipophorin from adults (lanes 3, 6, and 9) was applied to a 5% SDS-polyacrylamide gel, electrophoresed, transferred electrophoretically to nitrocellulose, and reacted with anti-lipophorin (lanes 1–3), anti-apoLp-I (lanes 4–6), or anti-apoLp-II (lanes 7–9) as above.

FIG. 6. Double immunodiffusion of lipophorin and hemolymph against antisera to apoproteins or whole lipophorin. 10 μl of antiserum against (A) whole lipophorin (5-fold dilution), (B) apoLp-I (2-fold dilution), or (C) apoLp-II (undiluted) was placed into center wells. Peripheral wells in A and B contained 10 μl of lipophorin (10 μg, well 1; 2 μg, well 2; 1 μg, well 3), whole hemolymph (2-fold dilution, well 4; 5-fold dilution, well 5; 10-fold dilution, well 6). Peripheral wells in C contained 10 μl of: apoLp-II in PBS (>10 μg, well 1; >5 μg, well 2), whole hemolymph (2-fold dilution, well 3), or lipophorin (20 μg, well 4; 10 μg, well 5; 5 μg, well 6). Samples were allowed to diffuse for 3 days at room temperature and 3 days at 4 °C. Plates were dried and stained as described under “Experimental Procedures.”

The former are contributed by polar apoprotein residues and lipid groups, the latter by apolar lipids. The small proportion of apolar hydrocarbons, triacylglycerols, and sterol esters in lipophorin may demand a greater role for apoproteins in formation of an apolar core. This concept is supported by our experiments on relative accessibility of the two apoproteins to proteases, immunoglobulins, and radiolabeling reagents.
An earlier report found apoLp-I to be much more susceptible to trypsin digestion than apoLp-II (Pattnaik et al., 1979) and radiiodination (Mundall et al., 1980). Here we have demonstrated that apoLp-I in the intact particle is more susceptible than apoLp-II to reaction with antibodies. Low accessibility of apoLp-II to bulky probes suggests that this polypeptide is sheltered from the aqueous environment and may lie partly within the particle, perhaps constituting a part of the core.

Though lipophorins readily exchange lipid (diacylglycerol and cholesterol) with tissue both in vitro (Chino and Gilbert, 1965; 1971) and in vivo (van der Horst et al., 1981), no exchange mechanism has yet been proposed (Chino and Kitazawa, 1981). However, the apoproteins, especially apoLp-I, may participate in exchange by reversible association with plasma membranes. In mammalian systems, polar lipids exchange from lipoproteins more readily than the apolar triacylglycerols and cholesterol esters found in the lipoprotein core (Morton and Zilversmit, 1982). Several soluble plasma proteins that facilitate triacylglycerol and cholesteryl ester exchange have been isolated (Rajaram et al., 1980; Zilversmit et al., 1975; Pattnaik et al., 1978; Ihm et al., 1980, 1982; Chajek and Fielding, 1978). Since insect lipophorins contain a larger proportion of polar lipid than mammalian lipoproteins, a larger proportion of lipid should be freely exchangeable. This does not exclude the presence of hemolymph proteins that facilitate lipid transfer, especially in life stages requiring rapid turnover of polar lipid. In adult M. sexta a new apoprotein, apoLp-III, is found in the hemolymph. ApoLp-III, which can reversibly associate with lipophorin, may serve a lipid transfer function during the adult stage, a period of great demand for lipid, both as flight fuel in many insects (Bailey, 1975) and as a constituent of egg yolk. Furthermore, apoLp-III might serve as a recognition signal to promote interaction between lipophorin and muscle cell membranes, or it might serve to activate lipolytic enzymes at the muscle to aid in assimilation of transported diacylglycerol, much as apolipoprotein C-II in human very low density lipoprotein serves to activate lipoprotein lipase in peripheral tissue (Smith et al., 1978).

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REFERENCES

Insect Lipophorin

Supplement to: "Biolipophorin as an Insect Lipophorin" by Jeffrey F. Shaw, Hana E. Neld and John M. Law

EXPERIMENTAL PROCEDURES

Animals-Manduca sexta (tobacco hornworm) eggs were obtained from the U.S. Department of Agriculture, Fargo, North Dakota. Eggs were hatched and larvae reared as previously described (Traver et al., 1993).

Lipophorin Preparation-Lipophorin was isolated from whole larval hemolymph by single stage anion-exchange chromatography on a Beckman 605/10-anion-exchange column, using a linear gradient of sodium chloride as described by Traver et al. (1993). Fifth instar larvae were blotted dry and weighed. Hemolymph was removed by centrifugation for 10 min at 4000 x g (4°C), the supernatant collected and 15% PEG-8000 (1000 x g, 1 h) was added. The hemolymph mixture was placed into Beckman 39 ml (quicksafe) tubes (19.5 ml, 1 h), and overlaid with 15% PEG-8000 (density 1.070) to sink the 15% PEG-8000 (density 1.060). Tubes were then placed on an Optima XL 100 rotator (Beckman Instruments Inc., Fullerton, CA) for 2 h (r.p.m. 5400). Lipophorin-containing fractions were then fractionated by heparin-agarose chromatography as previously described (Naumov et al., 1971) in the presence of sodium chloride. Fractions containing lipophorin were pooled and concentrated by ultrafiltration.

Under these conditions the gradient ranged from 1.03 to 1.30 g/ml in density (Fig. 1). Lipophorin, which appeared as a bright yellow band in the center of the tube, separated from other hemolymph proteins by sodium chloride, as indicated by the bright blue color of the blue-stained column, and was recovered by the use of sodium chloride. Fractions containing lipophorin were pooled and concentrated by ultrafiltration.

Fig. 1. Density gradient ultracentrifugation of hemolymph proteins and isolated lipophorin. Hemolymph was subjected to ultracentrifugation at 35,000 rpm. Data are expressed as absorbance at 280 nm (tubes with lipophorin or lipophorin fragments) or refractive index (tubes with crude hemolymph) from 1 to 50% elution. ---, lipophorin absorbance; ---, total absorbance from crude hemolymph.

Preparative Gel Permeation Chromatography on a Bio-Gel P-300 Column. Each protein was separated by preparative gel permeation chromatography as described. Absorbance at 280 nm was monitored on a Gilford Molecular monitor and a linear strip chart recorder.

Lipophorin was isolated as an tandem antigen and catalyzed in the carbohydrate gel chromatography as described. Absorbance at 280 nm was monitored on a Gilford Molecular monitor and a linear strip chart recorder.

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Table 1. Recovery of lipophorin from gel permeation chromatography on a Bio-Gel P-300 column.

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<tr>
<th>Purification Step</th>
<th>PEG-8000 (ml)</th>
<th>Volume (ml)</th>
<th>App. Frac.</th>
<th>Total Frac.</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanidine HCl</td>
<td>Lipophorin</td>
<td>1.779</td>
<td>24.0</td>
<td>60.7</td>
<td>65.6</td>
</tr>
<tr>
<td></td>
<td>App. Frac. I</td>
<td>1.226</td>
<td>40.0</td>
<td>126.5</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td>App. Frac. II</td>
<td>0.171</td>
<td>10.0</td>
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Preparative gel permeation chromatography utilized the same anion-exchange system as in the preparative apparatus. Gel permeation chromatography of the gel of 0.8 ml of gel was eluted with different elution buffer. Gel permeation chromatography of the gel of 0.8 ml of gel was eluted with different elution buffer. Gel permeation chromatography of the gel of 0.8 ml of gel was eluted with different elution buffer. Gel permeation chromatography of the gel of 0.8 ml of gel was eluted with different elution buffer. Gel permeation chromatography of the gel of 0.8 ml of gel was eluted with different elution buffer.

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