Insect Apolipophorin III

PURIFICATION AND PROPERTIES*

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The hemolymph of adult Manduca sexta (tobacco hornworm) contains a 17,000-dalton protein that can associate reversibly with the insect lipoprotein lipophorin. The protein is abundant in the hemolymph of the adult, but is found in larval hemolymph in only small amounts, and does not associate with larval lipophorin. On the basis of its association with adult lipophorin, we have designated the protein apolipophorin III. Apolipophorin III was dissociated from adult lipophorin by guanidinium chloride treatment and isolated by gel permeation and ion exchange chromatography. The unassociated apolipophorin III was also purified from lipophorin-free hemolymph by gel permeation, ion exchange, and lectin chromatography. Both preparations have identical isoelectric points and amino acid composition as well as the following properties. Apolipophorin III is a non-glycosylated polypeptide lacking cysteine and tryptophan. The 17,000-dalton polypeptide dimerizes in solution to a protein of Mr = 34,000.

Purification—ApoLp-III was purified both from intact adult lipophorin and from the subnatant obtained following KBr density gradient separation of lipophorin from other hemolymph proteins (Shapiro et al., 1984). We prepared adult lipophorin also by the low ionic strength method of Chino and Kitazawa (1981), and found that the proportion of apoLp-III in this material was similar to that in lipophorin prepared in concentrated salt solutions, demonstrating that the salt does not disrupt the binding of apoLp-III. Indeed, apoLp-III cannot be dissociated from lipophorin in 3 M urea. It was found that 6 M guanidinium chloride was necessary for dissociation. Separation of apoLp-III from intact adult lipophorin was achieved by gel permeation chromatography in 6 M guanidinium chloride. The elution profile of the three

1 The abbreviations used are: apoLp-III, apolipophorin III; apoLp-I, apolipophorin I; apoLp-II, apolipophorin II; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

2 Portions of this paper (including "Experimental Procedures" and Figs. 4-6) 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, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-3383, cite the authors, and include a check or money order for $3.60 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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C. The contaminating proteins at this stage were shown by reaction with the periodate-Schiff reagent to be glycoproteins, and they could be removed by adsorbing them on a column of concanavalin A-Sepharose. Homogeneous preparations of apoLp-III were obtained from male hemolymph by using these procedures. However, in the female hemolymph an additional protein, microtubillogenin (M, = 31,000), was observed. The female-specific protein could be separated from apoLp-III by an additional chromatographic step on hydroxylapatite (Kawooya and Law, 1983). The products of the various purification steps were subjected to SDS-polyacrylamide gel electrophoresis, and the results are shown in Fig. 3. The two preparations had identical mobilities on native or SDS-polyacrylamide gel electrophoresis. Each showed a characteristic reddish color when stained with Coomassie Blue.

We observed that dialysis of apoLp-III solutions against distilled water resulted in loss of the protein through binding to the dialysis membrane. This could be circumvented by dialysis against dilute acetic acid.

**Molecular Weight**—Gel permeation chromatography of apoLp-III on a calibrated Sephadex G-75 column gave an estimated M, = 34,000, while chromatography on a TSK 125 high performance liquid chromatography column gave an estimate of M, = 31,000. However, SDS-polyacrylamide gel electrophoresis, with or without 2-mercaptoethanol, yielded an estimated M, = 17,000. Calculation of a minimum molecular weight, based upon the amino acid composition (see Table I), gave a value of 17,000.

**Carbohydrate**—Bands of apoLp-III on acrylamide gels did not react with the periodate-Schiff reagent or fluorescein isothiocyanate-conjugated concanavalin A, and the protein...
and polypeptides in the lower density lipophorin particles is determined by rocket immunoelectrophoresis. From the results, the molar ratio of apoproteins was calculated to be 2 apoLp-III per lipophorin particle. The number of apoLp-III polypeptides in the lower density lipophorin particles is obviously much higher as judged by Coomassie Blue staining of gels, but it was not possible to make an estimate with the same techniques because the absorbance due to apoLp-III now becomes significant.

Larval hemolymph contained 0.46 mg/ml of apoLp-III, none of which was associated with the larval lipophorin.

**Isoelectric Point**—Both preparations of apoLp-III have identical isoelectric points of 6.1.

**Amino Acid Composition**—The amino acid composition of apoLp-III isolated either from lipophorin or lipophorin-free hemolymph is shown in Table I. The values are in good agreement, suggesting that the preparations are identical. The lack of tryptophan is notable; this accounts for the low absorbance of apoLp-III at 280 nm (0.04 absorbance units/mg of protein). The ratio of the absorbance at 230 to 280 nm is approximately 30.

**DISCUSSION**

Mammalian blood contains a variety of lipoproteins that serve different lipid transport functions and perform these functions in different ways. Chylomicrons deliver dietary fat to adipose tissue without being taken up by cells, while low density lipoprotein delivers cholesterol esters and other potential membrane components to cells through an endocytotic uptake mechanism involving specific receptors. In general, insects have a single lipoprotein species, which may serve several different functions and may even change its size, density, and apoprotein complement as it alters its function (Chino et al., 1981a; Wheeler and Goldsworthy, 1983a, 1983b; Shapiro and Law, 1983). This lipoprotein has been given the name lipophorin in recognition of its function as a lipid carrier. The hemolymph of the *M. sexta* larva contains a lipophorin with about 60% protein, 40% lipid, and a density of 1.13 g/ml, which is typical of high density lipoproteins (Pattnaik et al., 1979; Shapiro et al., 1984). The particle contains two glycosylated apoproteins. ApoLp-I, a large (245,000 daltons) polypeptide, has some properties that suggest similarity to human apolipoprotein B, although no definitive evidence for a relationship is available. The other apoprotein apoLp-II, is of moderate molecular weight (Mr = 78,000) and its properties have not been correlated with those of mammalian apolipoproteins (Shapiro et al., 1984). We do not know the functions of larval lipophorin, but we believe that it may be primarily responsible for the transport of digested fats from the mid-gut to storage depots in the fat body.

After metamorphosis, the hemolymph of the adult moth contains chiefly a lipophorin of density 1.11 g/ml with a smaller amount of a second form of lower density, 1.06 g/ml. Each of these contain not only apoLp-I and -II, but in addition they are associated with variable amounts of a third apoprotein, apoLp-III. The lower density form can be increased markedly by injecting the animal with a synthetic decapetide, the adipokinetic hormone of the migratory locust, *Locusta migratoria* (Shapiro and Law, 1983). The decrease in density to 1.06 g/ml is accompanied by association of increased amounts of lipid and apoLp-III with the low density lipophorin. This is quite analogous to events in locust hemolymph when adipokinetic hormone is released from the corpus cardiacum into the hemolymph to prepare the animal for flight (Mwangi and Goldsworthy, 1977, 1978; Van Der Horst et al., 1981; Wheeler and Goldsworthy, 1982, 1983a, 1983b).

ApoLp-III can be isolated either from lipophorin or from crude hemolymph. The two preparations are identical in chromatographic and electrophoretic behavior as well as in amino acid composition and isoelectric point. They are immunologically cross-reactive, but there is no immunological cross-reactivity between apoLp-I, apoLp-II, and apoLp-III. Therefore, apoLp-III is not a proteolytic degradation product of apoLp-I or apoLp-II. It should also be noted that apoLp-III, unlike apoLp-I and -II, is not glycosylated. The fact that

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**Table I**

*Table I*

Amino acid analyses of apoLp-III residues per Mr = 17,000

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Substant-derived protein</th>
<th>Lipophorin-derived protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Serine</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Alanine</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Threonine</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Arginine</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Lysine</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Methionine</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Proline</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Valine</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Hipoxaline</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Histidine</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cystine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>167</td>
<td>165</td>
</tr>
</tbody>
</table>

1. Duplicate samples hydrolyzed for 24 h in 3 N mercaptoethanesulfonic acid in vacuo, 110 °C.
2. Duplicate samples were performic acid-oxidized, then hydrolyzed for 24 h in 6 N HCl, in vacuo, 110 °C.
apoLp-III from the hemolymph associates rapidly (≤ 1 h) with lipophorin on treatment with adipokinetic hormone (Shapiro and Law, 1983) further indicates that the two polypeptides, that associated with lipophorin and that free in the hemolymph, are identical and distinct from apoLp-I and -II.

The total amount of apoLp-III in adult hemolymph is 17 ± 5 mg/ml. About half of this is free and half associated with lipophorin, especially with the lowest density (1.06 g/ml) form. The higher density form has two apoLp-III peptides/particle, or an apoprotein ratio of 2 apoLp-III per apoLp-I and apoLp-II.

The monomeric apoLp-III has a molecular weight of about 17,000 as shown by SDS-polyacrylamide gels and by calculation of a minimum molecular weight based upon the single tyrosine residue. Under native conditions, the protein can interact of a hydrophobic binding site on apoLp-III. The 17,000 dalton form dimers, as indicated by gel permeation chromatography. The higher density form has two apoLp-III peptide spots, that associated with lipophorin and that free in the hemolymph, are identical and distinct from apoLp-I and -II.

The tendency to dimerize may result from intermolecular interaction of a minimum molecular weight based upon the single tyrosine residue. Under native conditions, the protein can associate with the lipid loaded form of lipophorin or as a recognition signal at muscle cells to identify it as a loaded carrier, or both. Although lipophorin is considered to act as a reutilizable shuttle for lipid transport (Chino et al., 1981a), there is no evidence as to whether it enters insect cells in the course of its function (other than the oocyte, which it certainly does enter (Telfer and Kulakosky, 1984)). Further investigation into the function of apoLp-III may shed light on these questions.

Acknowledgments—We thank Dale Dalenberg for performing immunodiffusion on apoLp-III preparations, Mary Gonzalez for animal rearing, and Dr. Michael A. Wells for much helpful advice and discussion.

REFERENCES


Insect Apolipoporin III

Supplementary Notes

Insect Apolipoporin III: Purification and Properties

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EXPERIMENTAL PROCEDURES

Animals. Apis M. larvae were reared in the apiary kindly supplied by D. J. P. BERTHOUX and Dr. J. B. BUCKNER, U. S. Department of Agriculture, Fairview, Mo. The nectar was supplied according to TRAMFORD et al. (1970).

Collection of insect saliva. Approximately 1,000 1-day-old worker honeybees were used in each hemolymph preparation. Hemolymph procedures were performed as described previously by Chino et al. (1981a) and Law and LAW (1981). The hemolymph was centrifuged at 12,000 g for 5 min to remove hemocytes.

Purification of the Protein

The protein was purified from adult hemolymph as described by Shapiro et al. (1981), employing ultracentrifugation in a 12.1 density gradient. The 12.1-SLipophorin was isolated from the top, one third of the way from the top, by means of a hypodermic syringe. Subunits from the 12.1 density gradient ultracentrifugation were collected from the bottom of the centrifuge tube by means of a hypodermic syringe. It could be identified by its blue color (Fig. 3).

a) From the Substrate:

The column was packed to a total weight of 2.0 ml by ultracentrifugation through a 0.5 M NaCl ammonium sulfate solution. The sample was diluted to a total weight of 2.0 ml with 0.5 M NaCl ammonium sulfate and the pH adjusted to 7.0. The proteins were then diluted to a final volume of 2.0 ml with 0.5 M NaCl ammonium sulfate and the pH 7.0. The proteins were then diluted to a final volume of 2.0 ml with 0.5 M NaCl ammonium sulfate and the pH 7.0.

b) From the Substrate:

The column was packed to a total weight of 2.0 ml by ultracentrifugation through a 0.5 M NaCl ammonium sulfate solution. The sample was diluted to a final volume of 2.0 ml with 0.5 M NaCl ammonium sulfate and the pH adjusted to 7.0.

Shapiro et al. (1981a) employed ultracentrifugation in a 12.1 density gradient. The 12.1-SLipophorin was isolated from the top, one third of the way from the top, by means of a hypodermic syringe. Subunits from the 12.1 density gradient ultracentrifugation were collected from the bottom of the centrifuge tube by means of a hypodermic syringe. It could be identified by its blue color (Fig. 3).

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Molecular Weight Estimation. The molecular weight of apo-III was estimated by gel permeation chromatography at 4°C using a Sephadex G-75 column calibrated with protein standards, and by high performance liquid chromatography on a Bio-Rad TSK 250 column (0.15 M Hepes, 0.002 M NaCl, 0.50% methanol, pH 7.0) and a Hitachi L-8500 spectrophotometer equipped with a D-241 detector. The molecular weight estimated from its electrophoretic mobility was relative to that of Bio-Rad protein standards on SDS-PAGE.

Analytical Gel Electrophoresis. Two-dimensional electrophoresis was carried out as described by [17]. Gel electrophoresis was performed in 8% polyacrylamide gels at pH 8.6 (pH 4.5) at 4°C. Densitometric scans were performed on a Bio-Rad laser densitometer.

Isolation and Puriﬁcation. Apo-III (0.5 ml) from either hippo-III or from hippo-III free hamster was dissolved in 0.5 ml 0.1 M Hepes, pH 7.0, and applied to a column of concanavalin A-Sepharose and eluted with buffer containing 2 M methyl-α-D-mannoside. Fractions were collected and assayed for absorbance at 280 nm. SDS-PAGE of selected fractions from the concanavalin A-Sepharose column shown at the top.

Fig. 4. Gel permeation chromatography. Top: Chromatography of subnatant from KBr density gradient centrifugation on Sephadex G-75 (see text). Closed circles, absorbance at 230 nm; open circles, absorbance at 280 nm. Bottom: SDS-PAGE of selected fractions from the gel permeation chromatography shown at the top.

Fig. 5. Ion exchange chromatography. Top: Pooled fractions from the ion exchange column (Fig. 4) were applied to a column of SP-Sephadex C-25 and eluted with buffer (see text). At the arrow, buffer containing 0.5 M sodium chloride was added (see text). Closed circles, absorbance at 230 nm; open circles, absorbance at 280 nm. Bottom: SDS-PAGE of selected fractions from the ion exchange chromatography shown at the top.

Protein Blotting. Various proteins were extracted from various organs and subjected to SDS-PAGE and transferred to a nitrocellulose membrane [18]. The membranes were incubated with specific polyclonal antibodies followed by [125]I-labeled protein A. Cross-reactivity was detected by autoradiography.

Rocket Immunoelectrophoresis. A rocket immunoelectrophoresis was performed according to Vennes [19] using a barbital-sodium citrate buffer (0.035 M barbital, 0.15 M sodium citrate, pH 8.6) at 6°C and 2.0% agarose with rabbit anti-apo-III antisera (v/v). Immune complexes were visualized by Coomassie blue staining.

Fig. 6. Lectin affinity chromatography. Top: Pooled fractions from the ion exchange column (Fig. 5) were applied to a column of concanavalin A-Sepharose and eluted with buffer (see text). At the arrow, buffer containing 2 M methyl-α-D-mannoside was added (see text). Closed circles, absorbance at 230 nm; open circles, absorbance at 280 nm. Bottom: SDS-PAGE of selected fractions from the concanavalin A-Sepharose column shown at the top.