



Isolation and Characterization of Apolipoprotein-III from the Giant Water Bug (*Lethocerus medius*)

MICHAEL R. KANOST,*† KIMBERLY A. SPARKS,* MICHAEL A. WELLS*‡

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Upon injection of synthetic adipokinetic hormone, lipophorin from *Lethocerus medius* decreased in density and became associated with apolipoprotein-III (apoLp-III). ApoLp-III isolated from hemolymph of *Lethocerus medius* had a $M_r = 19,000$ and an amino acid composition high in methionine, in comparison with other apoLp-IIIs. Its circular dichroism spectrum was consistent with a protein with secondary structure of predominantly α -helix. NH₂-terminal sequence alignment with apoLp-III sequences from other species showed a conservation of the hydrophobic or hydrophilic properties of residues at each position rather than of specific amino acids. ApoLp-III from *Lethocerus medius* has the potential to form amphipathic α -helices, similar to those found in the three-dimensional structure of *Locusta migratoria* apoLp-III. A portion of the apoLp-III molecules that are not associated with lipophorin contained the blue chromophore, biliverdin.

Lipophorin Apolipoprotein-III Apolipoprotein Biliverdin Giant water bug *Lethocerus medius*

INTRODUCTION

In insect hemolymph, lipids are transferred between tissues while bound to the lipoprotein lipophorin (for reviews see Ryan, 1990; Van der Horst, 1990; Soulages and Wells, 1994). Lipophorin particles are composed of two apoproteins, apolipoprotein-I (apoLp-I, $M_r \approx 250,000$) and apolipoprotein-II (apoLp-II, $M_r \approx 80,000$) along with phospholipids, diacylglycerols, and small amounts of hydrocarbons and sterols. A third apoprotein, apolipoprotein-III (apoLp-III, $M_r \approx 18,000$), present in some insect species, exists either free in hemolymph or associated with the lipophorin particle. The peptide adipokinetic hormone (AKH) can cause release of diacylglycerol from the fat body into the hemolymph, where it associates with lipophorin. ApoLp-III then binds to the lipophorin particle, increasing its lipid-carrying capacity by covering hydrophobic surfaces resulting from diacylglycerol uptake (Kawooya *et al.*, 1986; Wells *et al.*, 1987).

ApoLp-III has been best studied in a lepidopteran, *Manduca sexta*, and an orthopteran, *Locusta migratoria*, from which the primary structures have been deduced from cDNA sequences (Cole *et al.*, 1987; Kanost *et al.*,

1988). The degree of similarity of these two sequences is low, with only 29% identical residues. The three-dimensional structure of *Locusta migratoria* apoLp-III has been determined by X-ray crystallography and found to be composed of five amphipathic α -helices in a cylindrical arrangement, with the hydrophobic residues pointing toward the interior of the protein and the hydrophilic residues directed outward (Breiter *et al.*, 1991). This structure is consistent with a model in which the protein binds to lipid surfaces through a change in conformation in which the cylinder opens to bring the hydrophobic side chains of the helices into contact with the surface, leaving the hydrophilic side chains in contact with water.

As one approach to understanding the relationship between apoLp-III structure and function, we have begun to characterize apoLp-III from a variety of insect species (Wells *et al.*, 1985; Strobel *et al.*, 1990; Smith *et al.*, 1995), with a goal of identifying common structural features among apoLp-III proteins. Through examining these natural experiments (proteins that have diverged during evolution to have quite different amino acid sequences, yet retain the same function) it may be possible to gain some understanding of the structural constraints on apoLp-III molecules.

In this study we have isolated and characterized apoLp-III from a hemipteran, *Lethocerus medius*. This insect, a giant water bug, was shown to form low density lipophorin (LDLp) with associated apoLp-III in response

*Department of Biochemistry and Center for Insect Science, University of Arizona, Tucson, AZ 85721, U.S.A.

†Present address: Department of Biochemistry, Kansas State University, Manhattan, KS 66506, U.S.A.

‡Author for correspondence.

to injection of AKH. A portion of the apoLp-III in the hemolymph had the unusual characteristic of carrying the blue chromophore, biliverdin.

MATERIALS AND METHODS

Insects and hemolymph collection

Adult *Lethocerus medius* were collected from ponds in southern Arizona and generously provided by Dr Robert Smith (Department of Entomology, University of Arizona, AZ). The animals were anesthetized on ice, and hemolymph was collected by the "flushing out" method (Chino *et al.*, 1987).

Isolation and characterization of lipophorins

Insects were injected with 100 pmol of synthetic cicada (*Cacama valvata*) AKH (sequence = pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-Gly-Asn-NH₂, provided by Jan Veenstra, Department of Entomology, University of Arizona, AZ) in 10 μ l water or with water alone as a control. Hemolymph was collected 90 min after injection, and lipophorin was isolated by density gradient centrifugation and analyzed as described by Prasad *et al.* (1986). Lipophorin was located in the gradient by measuring the absorbance at 450 nm, which detects carotenoids associated with lipophorin.

Isolation of apoLp-III

ApoLp-III was isolated by three different methods described below.

Method 1. After density gradient centrifugation of hemolymph, the fractions containing high density proteins were pooled and heated at 100°C for 10 min. Precipitated protein was removed by centrifugation (30 min, 10,000g, 25°C). The supernatant was desalted by gel filtration (PD-10 column, Pharmacia), and apoLp-III was purified by reversed phase HPLC (Cole *et al.*, 1987).

Method 2. Hemolymph proteins were fractionated by gel filtration on a column (1.5 \times 180 cm) of Sephacryl

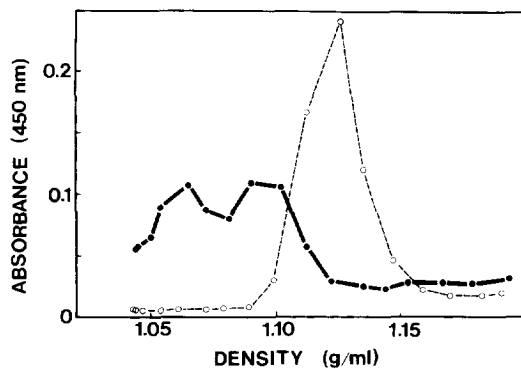


FIGURE 1. Adipokinetic response in *Lethocerus medius*. Hemolymph proteins collected from insects 90 min after injection of AKH (●) or water (○) were separated by density gradient centrifugation. After fractionating the gradient, lipophorin was detected by absorbance at 450 nm.

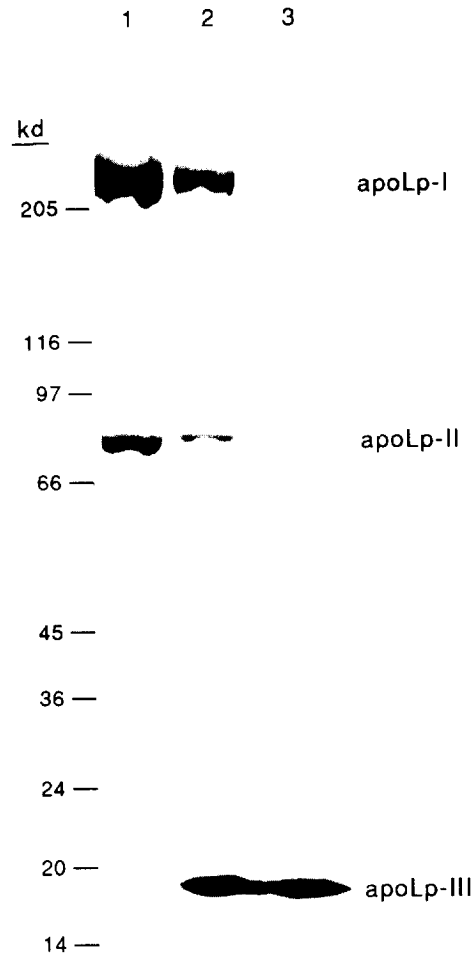


FIGURE 2. SDS-PAGE analysis of the apoproteins of *Lethocerus medius* lipophorins. Lane 1, HDLp isolated from control insects; lane 2, LDLp from insects 90 min after injection of AKH; lane 3, apoLp-III isolated by method 1.

S-200 HR (Pharmacia) equilibrated with 0.1 M ammonium bicarbonate. The column was eluted with the same buffer at 0.3 ml/min, and fractions of 4 ml were collected. Fractions containing proteins of approx. 19,000 Da, as determined by SDS-PAGE, were pooled, concentrated (SpeedVac, Savant) and desalted using a

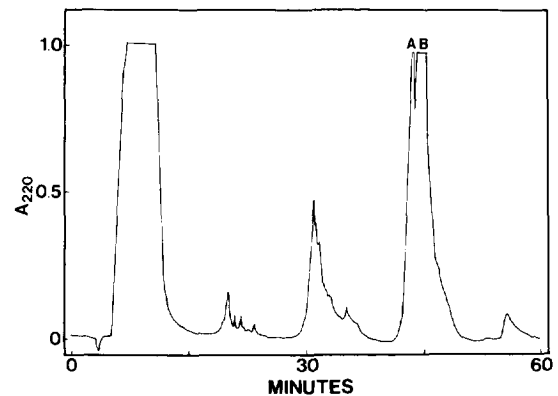


FIGURE 3. Isolation of apoLp-III by reversed phase HPLC. Heat stable proteins from hemolymph of one insect (approx. 0.5 ml) were applied to a C8 column (Vydac, 10 \times 250 mm) and eluted with an acetonitrile gradient as described by Cole *et al.* (1987).

TABLE 1. Amino acid composition of *Lethocerus medius* apoLp-III compared with apoLp-III from other insect species

	<i>Lethocerus</i>	<i>Thasus</i> *	<i>Locusta</i> †	<i>Acheta</i> ‡	<i>Manduca</i> §	<i>Derobrachus</i> ‡
Asx	22	24	18	14	19	23
Glx	28	30	34	35	34	33
Ser	4	10	11	8	12	7
Gly	9	9	4	3	5	5
His	7	4	7	4	4	2
Arg	2	4	3	6	2	1
Thr	8	7	11	11	7	10
Ala	14	17	29	33	24	18
Pro	4	4	7	3	3	3
Tyr	2	2	0	0	1	0
Val	14	15	9	9	9	17
Met	11	2	0	0	2	0
Ile	4	9	5	3	2	7
Leu	10	16	15	11	11	17
Phe	3	9	2	6	8	1
Lys	18	29	7	4	24	20
Cys	0	0	0	0	0	0
Trp	—	0	2	—	0	0

Data are in residues per molecule.

*Wells *et al.* (1985).

†Kanost *et al.* (1988).

‡Smith *et al.* (1995).

§Cole *et al.* (1987).

PD-10 column. ApoLp-III was then isolated by HPLC as in method 1.

Method 3. LDLp, isolated by density gradient centrifugation, from animals injected with AKH as described above, was heated at 100°C for 10 min. The precipitated apoLp-I and apoLp-II were removed by centrifugation. The supernatant was desalted (PD-10 column) and apoLp-III was isolated by HPLC as in method 1.

Characterization of apoLp-III

The molecular weight of apoLp-III was determined by SDS-PAGE (Laemmli, 1970) carried out in a 5–15% acrylamide gradient gel stained with Coomassie Blue. For amino acid analysis, protein samples were hydrolyzed in 6 N HCl at 100°C *in vacuo* for 24 h and analyzed on a Beckman 7300 amino acid analyzer. NH₂-terminal amino acid sequence was determined by automated Edman degradation using an Applied Biosystems 47A protein sequencer with online phenylthiohydantoin amino acid

analyzer 120A. Carbohydrate was assayed by the phenol-sulfuric acid method (Ashwell, 1966). The circular dichroism (CD) spectrum of apoLp-III was measured with an AVIV spectropolarimeter model 60 DS at 28°C from 250 to 190 nm in a quartz cuvette with a 1 mm pathlength. The protein concentration was 50 µg/ml in water.

Characterization of the chromophore

The blue chromophore from apoLp-III (purified by method 1) was extracted with 1-butanol. The solvent was evaporated under a stream of nitrogen, and the chromophore was dissolved in methanol. The absorbance spectrum from 800 to 250 nm was measured with a Beckman DU-40 spectrophotometer. For comparison, absorbance spectra were measured for biliverdin dihydrochloride (Sigma) dissolved in methanol and from apoLp-III purified by methods 1 and 3 and dissolved in water.

RESULTS AND DISCUSSION

AKH-induced formation of LDLp

Hemolymph from untreated adult *Lethocerus medius* contained lipophorin with an average density of 1.125 g/ml (Fig. 1), which contained apoLp-I and apoLp-II but not apoLp-III (Fig. 2). After injection of AKH, the density of lipophorin decreased, and two peaks of lipophorin with densities of 1.063 and 1.095 g/ml were detected after density gradient centrifugation (Fig. 1). The LDLps contained apoLp-III in addition to apoLp-I and apoLp-II (Fig. 2).

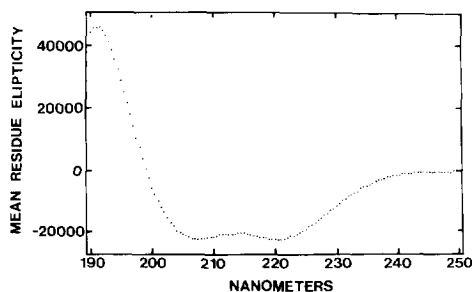


FIGURE 4. Circular dichroism spectrum of apoLp-III. The measurements were performed at 28°C using 50 µg/ml protein in water. Data shown are the average of five spectra.

Isolation and characterization of apoLp-III

Like apoLp-III from *Locusta migratoria* and *Acheta domestica* (Kanost *et al.*, 1988; Strobel *et al.*, 1990), apoLp-III from *Lethocerus medius* was very stable to heat, making possible an efficient separation from most other hemolymph proteins by heat-induced precipitation. Reversed phase HPLC of the heat stable hemolymph proteins resulted in two peaks with retention times similar to those observed for apoLp-III from other species (Fig. 3) (Strobel *et al.*, 1990). Peak A contained a protein of $M_r = 15,000$, which was not apoLp-III. Peak B contained apoLp-III, which had a $M_r = 19,000$ (Fig. 2). ApoLp-III isolated by three methods (see Materials and Methods) had the same apparent molecular weight, retention times on HPLC, and amino acid compositions. ApoLp-III isolated by methods 1 and 2 had a blue color, which will be discussed below.

The amino acid composition of *Lethocerus medius* apoLp-III (Table 1) is similar to that from another hemipteran, *Thasus acutangulus*, but is lower in lysine. The *Lethocerus medius* apoLp-III also has a much higher methionine content than any other previously studied apoLp-III. No carbohydrate could be detected in *Lethocerus medius* apoLp-III. The CD spectrum (Fig. 4) indicates an α -helix content of 55% with no β structure, consistent with the crystal structure of *Locusta migratoria* apoLp-III (Breiter *et al.*, 1991) and with CD measurements of *M. sexta* apoLp-III (Kawooya *et al.*, 1986).

The NH₂-terminal amino acid sequence of *Lethocerus medius* apoLp-III is shown in Fig. 5. An alignment

of this sequence with the same region of apoLp-III from *A. domestica* (Orthoptera), *Derobrachus geminatus* (Coleoptera), *M. sexta* (Lepidoptera), and *Locusta migratoria* (Orthoptera) demonstrates that the percentage identity with these sequences is low, but that the positions of hydrophobic and hydrophilic residues are conserved. Analysis of the regions of *Lethocerus medius* apoLp-III that align with the first two α -helices of *Locusta migratoria* apoLp-III indicate that *Lethocerus medius* apoLp-III has the potential to form similar amphipathic helices (Fig. 6). These observations are consistent with our hypothesis that insect apoLps-III have in common a structural motif of amphipathic α -helices in which the distribution of polar and nonpolar residues is conserved (Smith *et al.*, 1995). Residue 31 (Leu) in *Lethocerus medius* apoLp-III aligns with Leu 30 in *Locusta migratoria* apoLp-III, and with Leu or Val in the other apoLp-III sequences. The hydrophobic side chain of this Leu residue is not buried between the helices in *Locusta migratoria* apoLp-III (Breiter *et al.*, 1991). It has been proposed that this residue may be important in the binding of apoLp-III to diacylglycerol in lipophorin (Breiter *et al.*, 1991; Soulages and Wells, 1994).

Characterization of the chromophore

ApoLp-III isolated by method 1 (boiling, HPLC) had a distinct blue color, but apoLp-III isolated from LDLp (method 3) was colorless. To test whether the coloration of apoLp-III might be an artifact of heat treatment, we substituted gel filtration, a more gentle method, as a first step in the purification. On a column of Sephacryl S-200

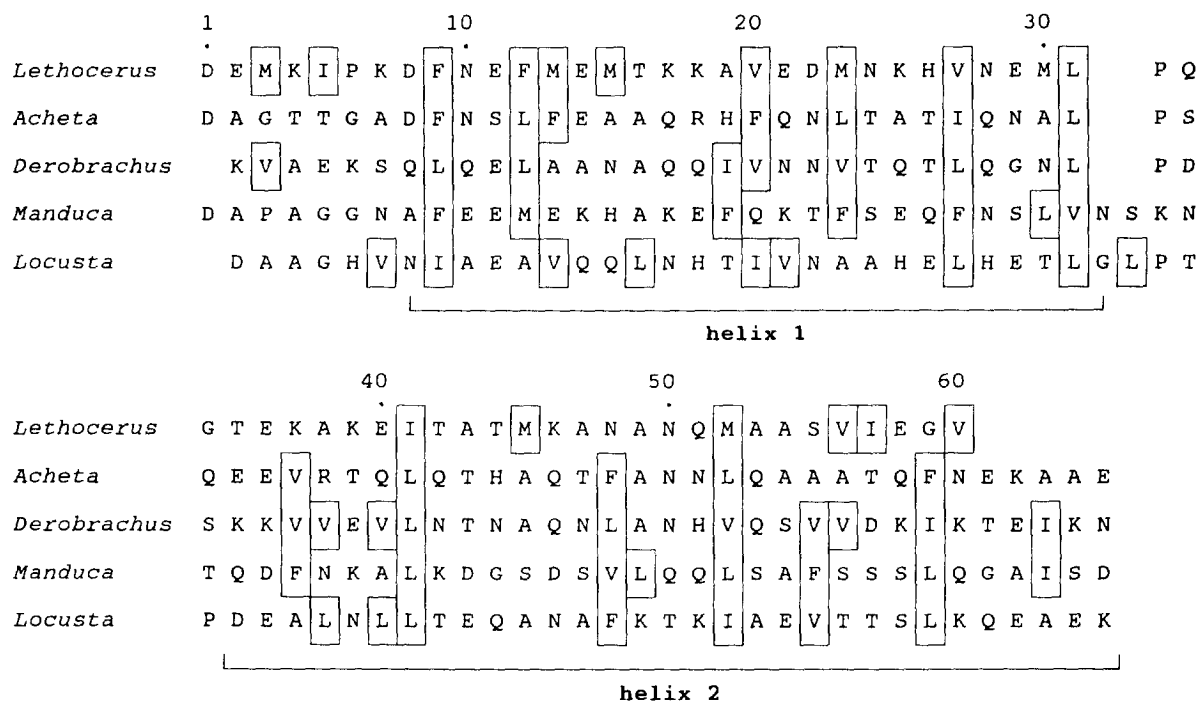


FIGURE 5. NH₂-terminal sequence of *Lethocerus medius* apoLp-III. Residues 1–60 were determined using protein purified by method 1. The sequence of residues 1–10 was also determined from protein isolated from LDLp (method 3) with identical results. The sequence was aligned with the NH₂-terminal sequences of four other apoLp-III proteins as described previously (Smith *et al.*, 1995). The regions that form α -helices 1 and 2 in *Locusta migratoria* apoLp-III are indicated below the alignment. Hydrophobic amino acids (Val, Ile, Leu, Phe and Met) are boxed to show that the positions of these residues are conserved.

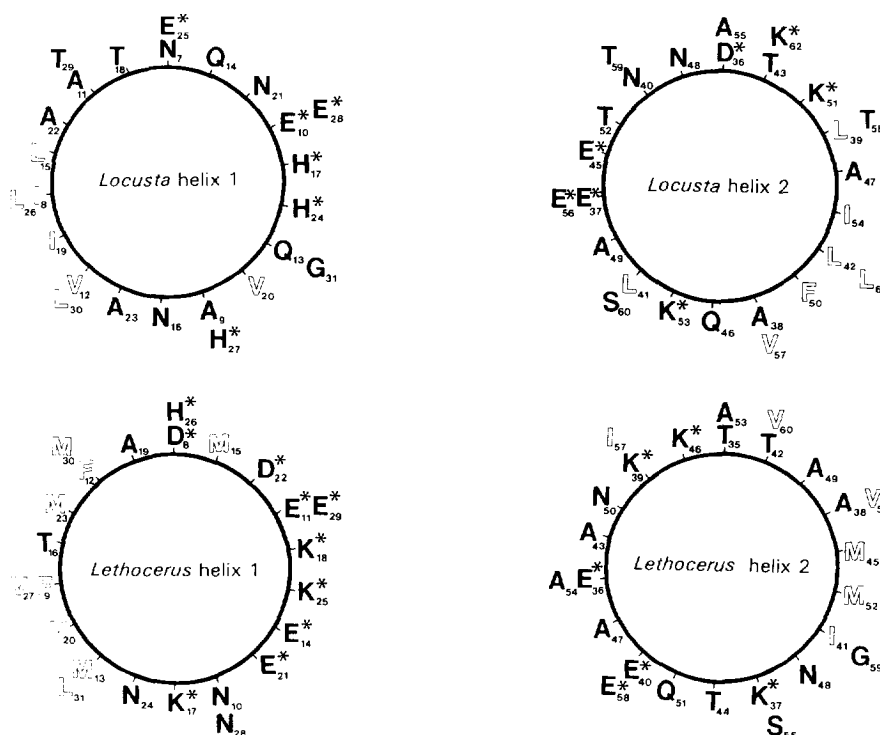


FIGURE 6. Potential for formation of amphipathic helices by *Lethocerus medius* apoLp-III. Helical wheel diagrams were constructed for *Lethocerus medius* apoLp-III sequences corresponding to the first two α -helices of *Locusta migratoria* apoLp-III (Fig. 5). These are compared with similar diagrams for the *Locusta migratoria* regions known to form amphipathic helices (Breiter *et al.*, 1991). Similar patterns of localization of hydrophobic residues (Val, Ile, Leu, Phe and Met, shown in outline characters) and charged residues (identified with asterisks) are present in the *Lethocerus medius* and *Locusta migratoria* sequences. The NH₂-terminal sequence for *Lethocerus medius* apoLp-III was determined up to residue 60, which may not include all of helix 2.

HR, two peaks of apoLp-III were observed: a peak containing colorless apoLp-III eluted slightly ahead of a peak containing apoLp-III and blue color (data not shown). When these proteins were further purified by reversed phase HPLC they had the same retention time, molecular weight (SDS-PAGE) and amino acid composition. The blue color remained associated with the apoLp-III protein from the second gel filtration

peak. Thus, association of the blue chromophore with apoLp-III was not a result of heat treatment.

The chromophore could not be removed from apoLp-III by dialysis or by treatment with EDTA. It could be extracted with 1-butanol and was also soluble in methanol. The absorbance spectrum of the isolated chromophore was very similar to that of biliverdin (Fig. 7), a chromophore present in several other insect hemolymph proteins (Kanost *et al.*, 1990). Thus, it appears that the chromophore associated with *Lethocerus medius* apoLp-III is a biliverdin, although the isomer is not known.

These data suggest that biliverdin is bound to a portion of the apoLp-III molecules in hemolymph and that these molecules do not participate in the formation of LDLp. The difference between blue and colorless apoLp-III in mobility on gel filtration chromatography suggests that binding biliverdin may alter the conformation of the apoLp-III molecule.

Biliverdin has been observed to associate with several different classes of insect hemolymph proteins (Kanost *et al.*, 1990) but not previously with apoLp-III. Haunerland *et al.* (1992) found that in hemolymph from another hemipteran, *Podisus maculiventris*, biliverdin is associated with lipophorin. In this case, biliverdin was bound to the lipophorin particle, which included apoLp-I, -II, and -III. This differs from *Lethocerus medius*, in which biliverdin was associated only with apoLp-III. It appears that in these two hemipteran

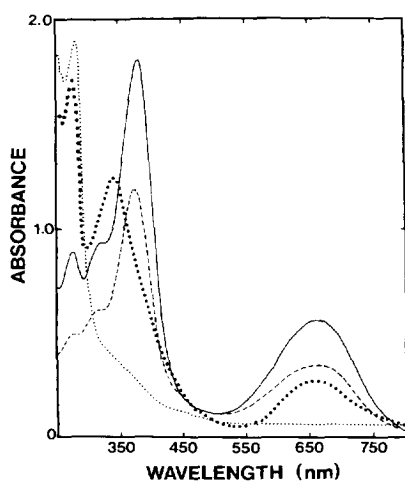


FIGURE 7. Absorption spectra of apoLp-III and the extracted chromophore. ••••• apoLp-III (Method 1); apoLp-III from LDLp (method 3); — extracted chromophore in methanol; - - - biliverdin standard in methanol.

species, lipophorin or apoLp-III has been adapted for transport of this hydrophobic molecule, although the biological function of biliverdin in these species remains unknown.

REFERENCES

- Ashwell G. (1966) New colorimetric methods of sugar analysis. *Meth. Enzym.* **8**, 85–95.
- Breiter D. R., Kanost M. R., Benning M. M., Law J. H., Wells M. A., Rayment I. and Holden H. M. (1991) Molecular structure of an apolipoprotein determined at 2.5 Å resolution. *Biochemistry* **30**, 603–608.
- Chino H., Hirayama Y., Kiyomoto Y., Downer R. and Takahashi K. (1987) Spontaneous aggregation of locust lipophorin during hemolymph collection. *Insect Biochem.* **17**, 89–97.
- Cole K. E., Fernando-Warnakulasuriya G. J. P., Boguski M. S., Freeman M., Gordon J. I., Clark W. A., Law J. H. and Wells M. A. (1987) Primary structure and comparative sequence analysis of an insect apolipoprotein. Apolipophorin-III from *Manduca sexta*. *J. Biol. Chem.* **262**, 11794–11800.
- Haunerland N. H., Ortego F., Strausfeld C. M., and Bowers W. S. (1992) Blue lipophorin from *Podisus maculiventris*. *Archs Insect Biochem. Physiol.* **20**, 49–59.
- Kanost M. R., Boguski M. S., Freeman M., Gordon J. I., Wyatt G. R. and Wells M. A. (1988) Primary structure of apolipophorin-III from the migratory locust, *Locusta migratoria*. Potential amphipathic structures and molecular evolution of an insect apolipoprotein. *J. Biol. Chem.* **263**, 10568–10573.
- Kanost M. R., Kawooya J. K., Law J. H., Ryan R. O., Van Heusden M. C. and Ziegler R. (1990) Insect haemolymph proteins. *Adv. Insect Physiol.* **22**, 141–147.
- Kawooya J. K., Meredith S. C., Wells M. A., Kezdy F. J. and Law J. H. (1986) Physical and surface properties of insect apolipophorin-III. *J. Biol. Chem.* **261**, 13588–13591.
- Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **226**, 680–685.
- Prasad S. V., Ryan R. O., Law J. H. and Wells M. A. (1986) Changes in lipoprotein composition during larval-pupal metamorphosis of an insect, *Manduca sexta*. *J. Biol. Chem.* **261**, 558–562.
- Ryan R. O. (1990) Dynamics of insect lipophorin metabolism. *J. Lipid Res.* **31**, 1725–1739.
- Smith A. F., Owen L. M., Strobel L. M., Chen H., Kanost M. R., Hanneman E. and Wells M. A. (1995) Insect apolipophorins-III share a common function, but minimal amino acid sequence identity. Are amphipathic α -helices the common structural motif? *J. Lipid Res.* Submitted.
- Soulaiges J. L. and Wells M. A. (1994) Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Adv. Protein Chem.* **45**, 371–415.
- Strobel L. M., Kanost M. R., Ziegler R. and Wells M. A. (1990) Adipokinetic hormone causes formation of a low density lipophorin in the house cricket, *Acheta domestica*. *Insect Biochem.* **20**, 859–863.
- Van der Horst D. J. (1990) Lipid transport function of lipoproteins in flying insects. *Biochim. Biophys. Acta* **1047**, 195–211.
- Wells M. A., Ryan R. O., Kawooya J. K. and Law J. H. (1987) The role of apolipophorin-III in *in vivo* lipoprotein interconversions in adult *Manduca sexta*. *J. Biol. Chem.* **262**, 4172–4176.
- Wells M. A., Ryan R. O., Prasad S. V. and Law J. H. (1985) A novel procedure for the purification of apolipophorin III. *Insect Biochem.* **15**, 565–571.

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