Exchangeable apolipoproteins of insects share a common structural motif

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Abstract Elucidation of the secondary structure of the exchangeable apolipoproteins has been hindered by the difficulty in producing crystals suitable for X-ray spectrographic analyses. Consequently, in order to analyze potential structure-function relationships in the family of insect exchangeable apolipoproteins, apolipophorins-III (apoLps-III), two apoLps-III cDNA clones, one from the palo verde beetle (*Derobrachus geminatus*) and one from the house cricket (*Acheta domesticus*), have been isolated and sequenced. Multiple sequence alignments of the deduced protein sequences with two previously reported apolipophorins-III from *Manduca sexta* and *Locusta migratoria* reveal low sequence identity, suggesting that these proteins are very old and are highly divergent. Computer-assisted predictions of protein structure and subsequent analyses, using the known secondary structure of *Locusta migratoria* apolipophorin-III as a control, indicate that these insect proteins are composed of five amphipathic helices with characteristics similar to those of the helical domains of the mammalian exchangeable apolipoproteins. Thus, although insect and vertebrate exchangeable apolipoproteins share a common function in assisting lipid transport, precise amino acid identity is less important than the common structural feature of multiple amphipathic helices. Moreover, because these proteins occur widely among insect species, even in those where flight is limited or absent, we hypothesize that apolipophorin-III has a more generalized function in lipid metabolism than had been previously proposed.—**Smith, A. F., L. M. Owen, L. M. Strobel, H. Chen, M. R. Kanost, E. Hanneman, and M. A. Wells.** Exchangeable apolipoproteins of insects share a common structural motif. *J. Lipid Res.* 1994. **35:** 1976–1984.

Supplementary key words lipophorin • amphipathic helices • secondary structure • lipid transport

Apolipophorin-III (apoLp-III), an analog of the vertebrate exchangeable apolipoproteins, is a low molecular weight (17–20 kDa), insect hemolymph protein (1). The current view of the physiological role of apoLp-III is assistance in the translocation of lipid stores required to fuel prolonged flight (1). Lipophorin, the major insect hemolymph lipoprotein, acquires diacylglycerol from fat body stores of triacylglycerol. With increasing diacylglycerol content, lipid patches appear on the surface of the lipophorin particle, decreasing its solubility in the aqueous hemolymph. Free apoLps-III attach to these nascent hydrophobic surfaces via the nonpolar faces of the amphipathic helices, thereby restoring the solubility of the lipid-loaded lipophorin.

Despite the diversity of arthropod species, only two apoLps-III, one from the tobacco hornworm (*Manduca sexta*) and one from the migratory locust (*Locusta migratoria*), have been characterized to any great extent. These two proteins have been intriguing, because although they are functionally equivalent (2), they bear little amino acid sequence similarity (< 10%). *L. migratoria* apoLp-III is the only full-length, exchangeable apolipoprotein to have been characterized by X-ray crystallography (3). The protein is composed of five amphipathic α-helices, connected by short loops, and arranged as simple up-and-down helical bundles. Most hydrophobic residues are oriented facing toward the interior of the protein and charged residues face outward to the hemolymph. Although apoLps-III of the distantly related locust and tobacco hornworm exhibit little conservation in amino acid sequence, the pattern of distribution of hydrophobic and hydrophilic residues appears similar (4).

In order to analyze potential structure-function relationships in this family of proteins, it is necessary to examine apoLps-III from other insect species. To this end, we have cloned and sequenced apoLp-III cDNA from the flightless house cricket *Acheta domesticus* (Orthoptera: Gryllidae), a species relatively closely related to *L. migratoria*

Abbreviations: apoLp-I, apolipophorin-I; apoLp-II, apolipophorin-II; apoLp-III, apolipophorin-III; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; apoA-IV, apolipoprotein A-IV; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoE, apolipoprotein E; $<\mu_H>$, hydrophobic moment.

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(Orthoptera: Acrididae), and from the nonfeeding adult palo verde beetle *Derobrachus geminatus* (Coleoptera: Cerambycidae), a representative from a major group of insects distantly related to both the Orthoptera and Lepidoptera (*M. sexta*).

MATERIALS AND METHODS

Insects and diet

Crickets were purchased from a commercial vendor (Flucker's Cricket Farm, Baton Rouge, LA) and were provided with water, Purina dog chow, and potato slices. Palo verde beetles (*D. geminatus*) were field collected in the Tucson area of Arizona. Adult males and females of both species were used in the purification of their respective apoLp-III proteins.

Purification and characterization of apoLp-III

Palo verde beetle hemolymph was obtained by the flushing-out method (5) with a buffer (110 mM KCl, 15 mM $MgCl_2$, 4 mM $CaCl_2$, 4 mM NaCl, 5 mM KH_2PO_4, 10 mM EDTA, 10 mM glutathione, and 1 mM diisopropylfluorophosphate). Hemocytes were removed by low-speed centrifugation and lipophorin was isolated by KBr gradient ultracentrifugation (50,000 *g;* 16 h) with a Ti-60 fixed-angle rotor (6). The pure lipophorin was dialyzed against 50 mM ammonium bicarbonate, heated in a boiling water bath for 5 min, and subjected to low-speed centrifugation (5,000 *g,* 10 min) to remove precipitated proteins and associated lipids. In this procedure, little, if any, lipid remains associated with apoLp-III, in contrast to the behavior of most of the vertebrate apolipoproteins, and does not interfere with subsequent amino acid analyses or the generation of antibodies. The supernatant was lyophilized and the purity of the remaining apolipoprotein, apoLp-III, was confirmed by 3–15% gradient SDS-PAGE (7). Amino acid analyses and amino-terminal sequencing were conducted as previously described (8). Palo verde beetle apoLp-III was tested for glycosylation by two methods. Lipophorin was separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with fluorescein isothiocyanate-conjugated concanavalin A (9); purified apoLp-III was also analyzed by phenol–sulfuric acid colorimetry (10). Cricket apoLp-III was purified and characterized as previously described (8).

RNA isolation, cDNA library construction, and screening

Total RNA was prepared from the fat body of a single male *D. geminatus* and from pooled fat bodies of *A. domesticus* by a urea–SDS/phenol procedure (11). Polyadenylated mRNA was isolated from total RNA with oligo(dT)-cellulose spin columns (Invitrogen; San Diego, CA).

Five μg of fat body polyadenylated mRNA from the two species was used to prepare cDNA and two directional libraries from a commercial kit (ZAP-cDNA Synthesis Kit, Stratagene; La Jolla, CA). Antisera to the cricket and palo verde beetle apoLps-III were produced in New Zealand White rabbits by intramuscular injections of the protein-adjuvant mixtures (Ribi ImmunoChem Research; Hamilton, MT). The cDNA libraries were screened with rabbit antisera using goat anti-rabbit IgG and an alkaline phosphatase color development system (Bio-Rad; Hercules, CA). Positive plaques were purified and the cDNA was subcloned into pBluescript SK plasmid by an in vivo excision protocol (Stratagene; La Jolla, CA). Sequencing was performed on both strands by the dideoxy chain termination method (12).

Sequence alignments and computer analyses

Protein sequences were aligned using a progressive sequence alignment algorithm (13). Significance of the similarity between sequences was evaluated by the RDF2 program (14). Computer analyses of deduced protein sequences were made with the University of Wisconsin GCG computer package (15), the PHD program (16, 17), and the WHEEL, CONSENSUS, and COMBO programs (18, 19). The latter programs were used with the kind permission of Dr. Jere P. Segrest of the Atherosclerosis Research Unit at the Department of Medicine, University of Alabama Medical Center. The sequences discussed in this paper are available from the GenBank™/EMBL Data Bank with accession numbers L25277, L25278, M17286, and J03888 for the apoLps-III of *D. geminatus, A. domesticus, M. sexta,* and *L. migratoria,* respectively (20).

RESULTS AND DISCUSSION

Insect lipophorin, in addition to lipids, is composed of two apolipoproteins, apoLp-I and apoLp-II. In many species, a third apolipoprotein, apoLp-III, is also associated with lipophorin, as well as being found free in the hemolymph. This low molecular weight protein has been isolated from a number of insect species representing five orders and 12 families (21). Identification has been made by either its characteristic association with lipophorin in the low density fractions after KBr ultracentrifugation or by its induced-association with lipophorin through the action of administered adipokinetic hormone, the lipid-mobilizing hormone of insects. A typical SDS-PAGE analysis, demonstrating that the low-density hemolymph fractions from *D. geminatus* contain three proteins of similar molecular weight to the other insect apolipoproteins, is illustrated in **Fig. 1.**

Of the 70,000 *A. domesticus* and 16,000 *D. geminatus* recombinants immunologically screened, 16 and 7 positive plaques, respectively, were purified and the N-

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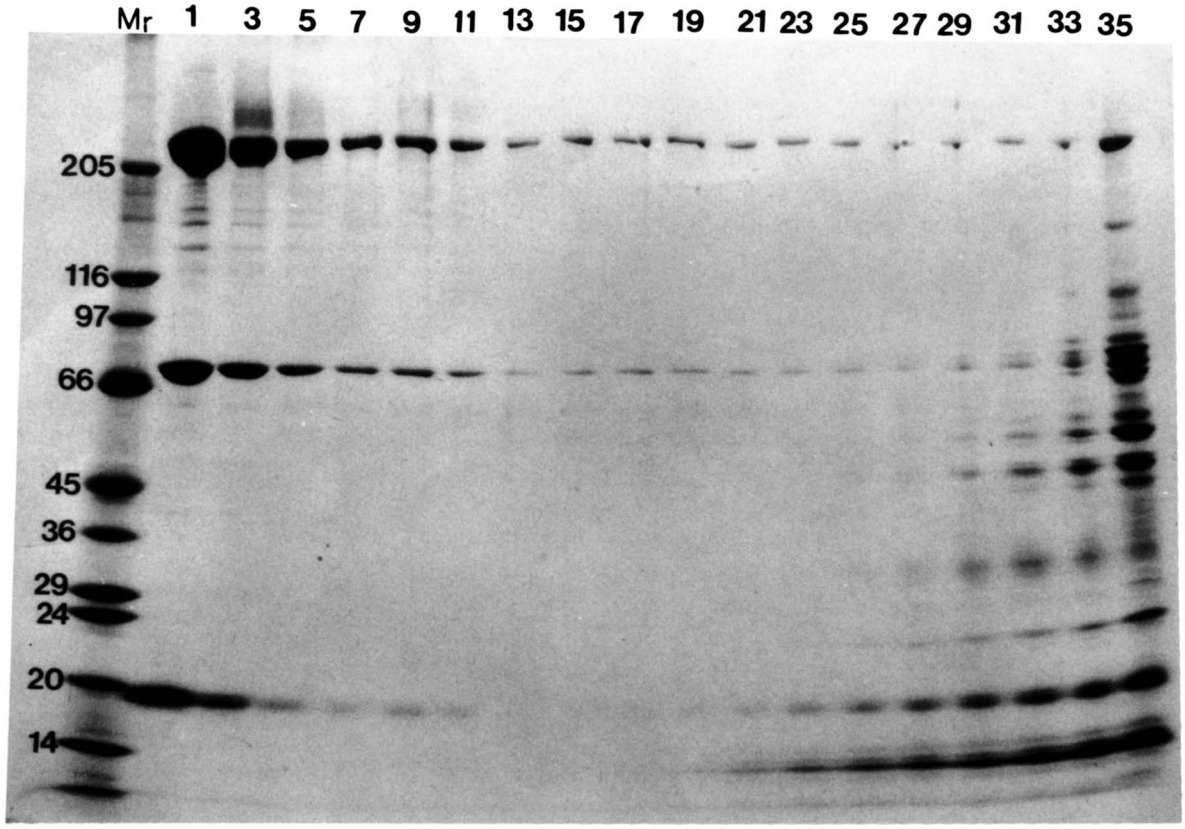

Fig. 1. SDS-PAGE analysis (3–15% acrylamide) of KBr gradient ultracentrifugation fractions of *D. geminatus* hemolymph. Lipophorin is found in the low density fractions (1–11) and is composed of three apolipoproteins: apoLp-I (220 kDa), apoLp-II (75 kDa), and apoLp-III (18 kDa). Lane M_r = molecular weight standards (kDa).

terminal coding regions were sequenced to determine potential full-length clones. One cDNA clone from each species was completely sequenced and confirmation of the identity of each was made by comparison with the amino-terminal protein sequences. The molecular weights, amino acid compositions, and predicted isoelectric points were determined from the deduced amino acid sequences (**Table 1**) and compared with those values from the *M. sexta* and *L. migratoria* apoLp-III proteins (4, 22). All four proteins are similar in length and molecular weight (161–166 AA, 17.2–18.0 kDa). The high isoelectric point of *D. geminatus* apoLp-III is probably derivative of the high lysine and arginine content. Based on the cDNA sequences, the apoLp-III mRNAs encode putative signal peptides of 18–23 amino acids.

TABLE 1. Characteristics of the four insect apoLp-III proteins

ApoLp-III	Deduced Molecular Weight (Da)	No. Amino Acids	pI[b]	Glycosylation[c]
A. domesticus	17,247	161	4.8	No
D. geminatus	17,964	165	8.1	Yes
M. sexta[a]	18,364	166	5.6	No
L. migratoria	17,310	162	5.1	Yes

[a] cDNA sequence from ref. 22.
[b] Predicted pI values based on the amino acid sequences.
[c] Glycosylation of *D. geminatus* apoLp-III determined by colorimetry (10). Other glycosylation assays from previously published references (4, 8, 24).

While using site-directed mutagenesis to produce mutant *L. migratoria* apoLp-III in a related project, several disparities in the cDNA sequence were detected. Re-sequencing the original cDNA clone confirmed several errors in the original reported sequence (4). The corrected version shows 7 nucleotide changes resulting in 13 alterations in the deduced amino acid sequence, including an extension in the total length by one residue. These minor modifications result in an improvement of the fit between the amino acid sequence and the X-ray structural data (H. Holden, personal communication). These corrections have been added as an update to the GenBank™/EMBL file (J03888).

The amino-terminal amino acid is the acidic residue, aspartic acid, in three of the species (*A. domesticus, M. sexta, L. migratoria*), whereas in the beetle *D. geminatus,* the initial residue is the basic amino acid, lysine. Aspartic acid has also been described as the initial residue in the five other insect apoLps-III that have been subjected to

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amino-terminal protein sequencing (21). The significance of a charged residue in the initial position, other than exposure to an aqueous environment, is not known at present.

As reported previously, *L. migratoria* apoLp-III is a glycoprotein (23), whereas *M. sexta* apoLp-III is neither glycosylated (24), nor does it contain any N-linked glycosylation sites (22). The deduced protein sequences of *A. domesticus* and *D. geminatus* each have a potential glycosylation site (Asn-X-Thr; **Fig. 2**) near the amino-terminus at a location approximate to that observed in the glycosylated apoLps-III of *L. migratoria* and other members of the short-horned grasshopper family Acrididae (4, 25). Although *A. domesticus* is closely related to the grasshoppers, its apoLp-III is not a glycoprotein (8). However, apoLp-III of the phylogenetically distant *D. geminatus* is glycosylated. The protein contains 10% carbohydrate by mass, as determined by colorimetry. Moreover, as has been reported for the *L. migratoria* protein (26), *D. geminatus* apoLp-III does not bind to concanavalin A. The biological significance of glycosylation of this protein is not known. What is known is that glycosylation of apoLp-III does not appear to play a role in its physiological function. The non-glycosylated apoLp-III of *M. sexta* can associate with locust lipophorin and accept diacylglycerol from the fat body in an in vitro system (2).

L. migratoria apoLp-III is the only full-length, exchangeable apolipoprotein to have been structurally characterized by X-ray diffraction analysis (3). The molecule contains five long amphipathic helices with the hydrophobic residues oriented inward away from the aqueous environment of the hemolymph. The model for the association of apoLp-III with lipophorin, as presently proposed (3), consists of the protein binding to a nascent lipid patch on the surface of a lipophorin particle via the two leucines that project into the hemolymph between helices 1 and 2 and helices 3 and 4 (Fig. 2; positions 32 and 93). Subsequently the molecule undergoes a conformational change: it opens and spreads upon the lipophorin surface, such that the nonpolar residues are in contact with the hydrophobic patch of the lipophorin particle and the polar residues face the hemolymph. Analysis of the four sequences following alignment by the progressive algorithm (13) reveals a conservation of leucine residues at or near positions 32 and 93 among the four apoLp-III proteins. Moreover, a review of amino-terminal protein sequences determined by Edman degradation from five other insect apoLps-III (21) illustrates the same pattern: leucine at or near position 32 is conserved.

A number of computer algorithms exist to deduce the secondary structure of proteins, although interpretation is seldom unequivocal (27). However, because the structure of *L. migratoria* apoLp-III is known, the amino acid sequence of this protein could serve as a control in estimating the locations of the amphipathic helices in the other

Fig. 2. Comparative alignments and predicted secondary structures of the four apoLp-III proteins. The amino acid sequences were aligned by the progressive method of Feng and Doolittle (13). Sequences and GenBank™/EMBL Data Bank assession numbers are *A. domesticus* (Adapo3; L25278), *D. geminatus* (Dgapo3; L25277), *M. sexta* (Msapo3; M17286), and *L. migratoria* (Lmapo3; J03888). The leucines that are putatively involved in the initial contact of the lipophorin surface are indicated by an * above the residue. The glycosylation sites in *L. migratoria* and *D. geminatus* apoLps-III are each marked with an arrow (↓); the corresponding, but unglycosylated, site of *A. domesticus* apoLp-III is marked with (♦). The solid lines under the *L. migratoria* sequence designate the actual locations of the five amphipathic helices of this protein as previously determined by X-ray crystallography (3). Dashed lines under the other three sequences denote estimated locations of the five amphipathic α-helical domains. Conserved residues that are probably involved in breaks and/or turns between the helices are designated with a (■) above the residue.

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three apolipoproteins. Using several programs available in the GCG Sequence Analysis Software Package (e.g., PepPlot, PlotStructure, Moment) as well as the profile neural network prediction program (16, 17), the four proteins were examined for potential turns and amphipathic helices. The programs generally produced the same predictions: the proteins consist largely of α-helices with helical contents ranging from 54.3 to 84.5%, depending on the program being used and the sequence being analyzed.

Comparison of the three aligned sequences with *L. migratoria* apoLp-III revealed a relatively conserved distribution of three to four prolines and a glycine (Fig. 2), residues often associated with breaks in α-helices or turns (28), suggesting that these proteins may also be composed of five amphipathic helices. This hypothesis is consistent with the empirical evidence that free exchangeable apolipoproteins (i.e., human apoA-IV, apoA-I, apoA-II, apoE; *M. sexta* apoLp-III) have a minimum structural requirement of four amphipathic helical segments for the initial phases of cholesterol translocation to the liver (29). Using the locations of these residues as a guide and the results of the structural predictions, we conservatively estimated the locations of the five amphipathic helical domains of the three proteins for further refinement.

A set of computer programs has been developed to identify and analyze the amphipathic helical domains of the mammalian exchangeable apolipoproteins (19). Because the structure of *L. migratoria* apoLp-III had been determined by X-ray crystallography (3), it was used by the programs' developers to test the accuracy of secondary structure prediction (18). Although not perfect, the amphipathic helices were located with 90% accuracy; had the corrected *L. migratoria* protein sequence been available, the precision would likely have been higher. We used these programs to analyze the insect apoLps-III. Helical wheel diagrams (30) were constructed for each of the predicted regions, as well as for segments with lengths different and/or shifted from the initial predictions using the WHEEL program. The refined, deduced helical regions are depicted in Fig. 2, the locations of which are consistent with the *L. migratoria* apoLp-III structure, the previously noted structural predictions, and the conserved residues of the aligned sequences.

The amphipathic helices of the vertebrate exchangeable apolipoproteins have been designated as class A and, in general, share a number of physical-chemical properties that distinguish them from the helical domains of other proteins (31). Because the insect apoLps-III are also exchangeable apolipoproteins, the properties of their amphipathic helices were determined in order to make comparisons with their vertebrate analogs, using the programs COMBO and CONSENSUS (19). After orienting the hydrophobic face of each helical wheel in the same direction, the CONSENSUS and COMBO programs superimpose and average a set of wheels, and then produce a number of physical parameters in addition to the consensus sequence information. The results of the analyses of the insect apoLps-III are presented in **Table 2** and **Fig. 3.** The predicted amphipathic helices of the apoLps-III share several features with the class A amphipathic helical domains of the mammalian exchangeable apolipoproteins, corroborating the earlier observations of *L. migratoria* apoLp-III (18). Both insect and class A amphipathic helical domains exhibit a high mean hydrophobic moment per residue and a high mean hydrophobicity per residue of the nonpolar face. The characteristic feature of Lys/Arg ratios greater than 1 is present in three of the four insect proteins. However, the amphipathic helices of the insect apoLps-III do differ from the class A amphipathic helical domains with regard to the distribution of charged amino acid residues. The clustering of negative residues at the center of the polar face, although well pronounced in the amphipathic helical domains of *L. migratoria* apoLp-III, is less so in the other three apoLps-III; the localization of positive residues at the polar-nonpolar interface is also less pronounced in the insect apoLps-III. Moreover, only the *M. sexta* apoLp-III helices exhibit a charge density that is generally ascribed to the class A amphipathic helices. However, individual amphipathic helices, both within and among the vertebrate exchangeable apolipoproteins, show considerable variation in these physical properties, prompting the division of class A amphipathic helical do-

TABLE 2. Properties of predicted amphipathic helical domains[a]

ApoLp-III	Mean $<\mu_H>$ per AA[b,c]	Hydrophobicity per AA of Nonpolar Face[c]	No. of + AA per 11 AA	No. of − AA per 11 AA	Lys/Arg Ratio
A. domesticus	0.35	0.75	0.9	1.2	0.4
D. geminatus	0.42	0.78	1.1	1.1	11.0
M. sexta	0.47	0.70	1.7	1.8	9.5
L. migratoria	0.40	0.76	0.7	1.3	7.0

[a] Calculations are based on the helical domains depicted in Fig. 4, using the CONSENSUS program (19) as described in the text.
[b] $<\mu_H>$ = hydrophobic moment.
[c] Calculated from a normalized GES hydrophobicity scale (31, 37).

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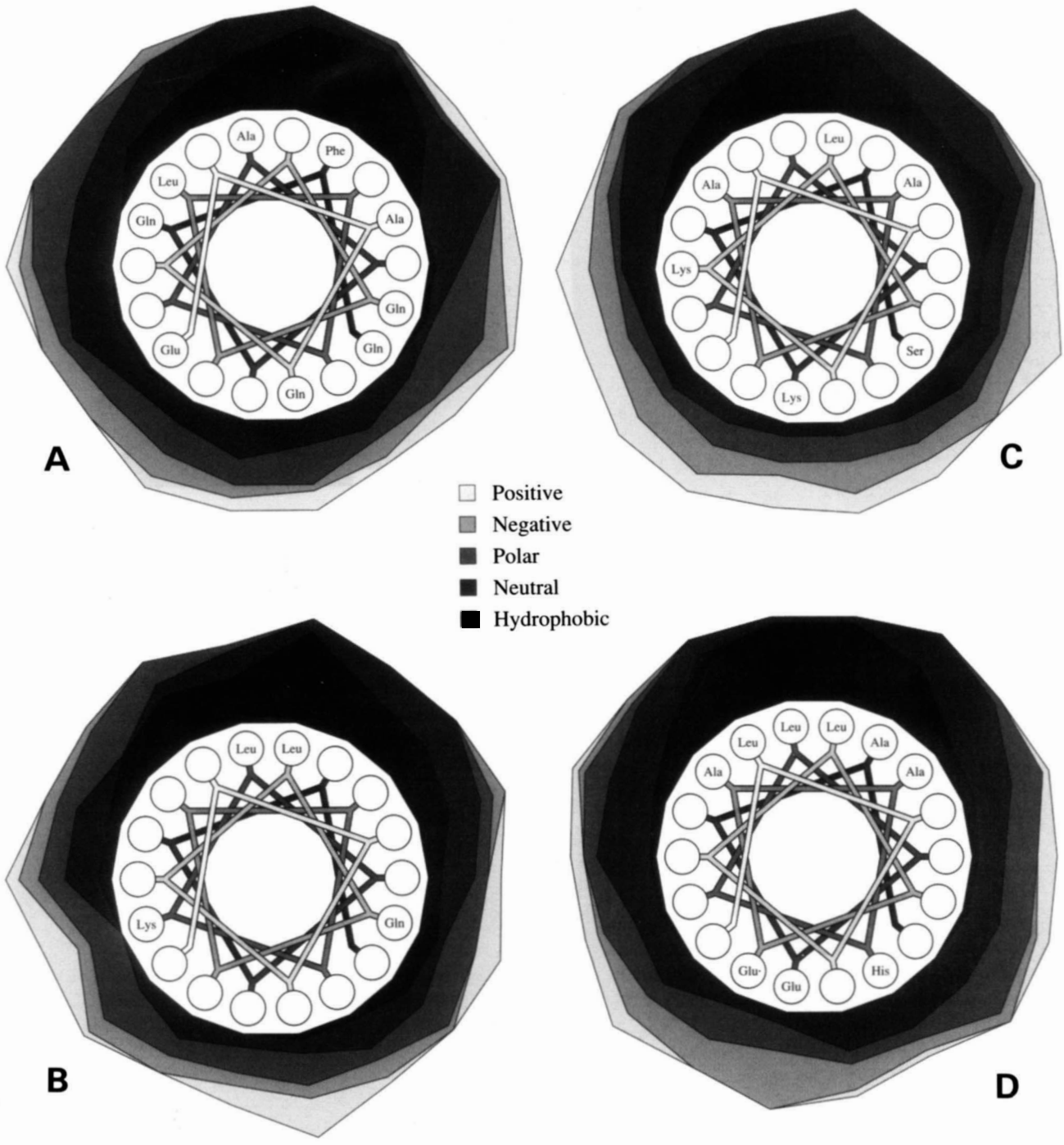

Fig. 3. CONSENSUS analyses of the amphipathic helical domains of the insect apoLps-III. After orienting the nonpolar faces of the five helical wheels to the top of the page, a composite figure is generated for each apoLp-III. The locations of the amphipathic helical domains are indicated in Fig. 2. Amino acids have been grouped into five physical-chemical classes as denoted by the key. A three-letter amino acid code appears for the residue that occurs most often (minimum = 3) at a given position. A. *A. domesticus* apoLp-III; B. *D. geminatus* apoLp-III; C. *M. sexta* apoLp-III; D. *L. migratoria* apoLp-III.

mains into several subclasses (18). The insect apoLps-III appear to be similar in this regard, with a given protein being composed of different subclasses of class A amphipathic helices. Unfortunately, because the volume of vertebrate exchangeable apolipoprotein sequence information, in terms of both the lengths and number of helices, far exceeds that of the insect apoLps-III, a comparable analysis of potential apoLp-III amphipathic helical subclasses is not feasible.

Based on the information from these analyses, the ob-

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TABLE 3. Pairwise % amino acid identities of the aligned apoLp-III protein sequences

	D. geminatus	*M. sexta*	*L. migratoria*
A. domesticus	25.0	18.6	17.8
D. geminatus		16.6	16.9
M. sexta			9.9

Multiple sequence alignments were performed with a progressive algorithm (13).

served conservation of structurally and functionally important residues, and the experimental evidence that *M. sexta* apoLp-III is functionally equivalent to *L. migratoria* apoLp-III (2), we propose that the insect apoLps-III share the common structural motif of five amphipathic helices with properties similar to the class A amphipathic helical domains of the vertebrate exchangeable apolipoproteins. In addition, precise amino acid identity appears less important than the distribution of polar and nonpolar residues.

Although the percent identity of these apoLp-III sequences is low (**Table 3**), statistical analysis of their similarity indicates a high probability that they share a common origin (**Table 4**). The similarity of the sequences from *A. domesticus, D. geminatus,* and *M. sexta* is very significant, with alignment scores greater than 8 standard deviations higher than the mean score obtained with randomized sequences of the same amino acid compositions. The *L. migratoria* sequence is most significantly similar to that of *A. domesticus,* its closest phylogenetic relative in this comparison. The significance of the similarity with *D. geminatus* and *M. sexta* is lower, with alignment scores around 3 standard deviations above the mean for randomized sequences, which is at the borderline of marginal significance (32). Proteins that give significant similarity scores due to similar secondary structures or hydropathic profiles, instead of evolutionary relatedness, can be detected with a more stringent evaluation. Sequences are shuffled in small blocks of residues, a process that alters the order of the sequence, but not local amino acid composition. Pairs of proteins that have a common evolutionary ancestor should have similar z values when both uniform and local shuffles are performed; proteins that are similar only by chance, due to analogous secondary structure, often have much lower z values when evaluated with local shuffling (14). We tested the apoLp-III sequences with the RDF2 program, shuffling one sequence in blocks of ten residues. The results of the comparisons with local shuffling were very similar to those obtained with uniform shuffling, providing further evidence that these four apoLp-III proteins are evolutionarily related.

The *L. migratoria* apoLp-III has diverged further from those of *M. sexta* and *D. geminatus* than has the apoLp-III of *A. domesticus,* even though the locust and cricket should be about the same evolutionary distance from the other two species (33). One possible biological explanation for this difference is that long distance migratory flight, up to 500 km in a single night, is an important component of the life history of locusts (34). Because a major role of apoLp-III is to assist in lipid transport during flight, there may be a difference in selection pressure for apoLp-III

TABLE 4. Statistical analyses of the similarity of apoLp-III amino acid sequences

	Uniform Shuffle[a]		
ApoLp-III	*D. geminatus*	*M. sexta*	*L. migratoria*
A. domesticus	13.85	8.40	5.59
D. geminatus		9.00	3.38
M. sexta			2.45
	Local Shuffle[a]		
A. domesticus	14.73	7.95	5.06
D. geminatus		8.25	3.32
M. sexta			2.82

Pairs of sequences were compared with the RDF2 program (14). In this analysis a score is determined for the alignment of two sequences. Then one of the sequences is randomized (shuffled), and a score is determined for the new alignment of one intact sequence with the other shuffled sequence. This process is repeated (in this case 200 shuffled comparisons for each pair of sequences, ktup ' 1) and the distribution of alignment scores obtained from the shuffled sequences is compared with the score for the alignment of the two intact sequences. From this distribution a z value is calculated: $\frac{\text{actual similarity score - mean of random scores.}}{\text{standard deviation of random scores}}$

The z value represents the number of standard deviations greater than the mean for random scores obtained by the score of the actual alignment. For uniform shuffles, the sequence was randomized over its full length. For localized shuffles, the sequence was randomized in blocks of ten amino acid residues to preserve local sequence composition.

[a] Z values.

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function in locusts compared with the other species.

It has been estimated that the ancestral apoLp-III arose over 480 million years ago (35), thus predating the emergence of insects and the development of flight. And yet, considerable attention has focused on the role of apoLp-III in assisting the delivery of diacylglycerol to fuel extended flight (1). We hypothesize that because apoLp-III occurs in a diversity of insect species, many of which are non- or poor flyers, that the function of this protein is simply to assist in the delivery of diacylglycerol to tissues for various physiological functions and that flight muscle in certain species is just one type of tissue requiring lipid. For example, we do know that although apoLp-III is found in the hemolymph of larval *M. sexta,* it is not bound to lipophorin. However, under conditions of physiological stress, such as starvation, apoLp-III will bind to lipophorin (K. Tsuchida, R. Ziegler, and M. Wells, unpublished observations), presumably to assist in the mobilization and transport of diacylglycerol to metabolically impoverished tissues. Moreover, adult *D. geminatus,* although capable fliers, do not engage in the prolonged flight requiring lipid mobilization as do adult *L. migratoria* and *M. sexta.* The beetle likely has a more fundamental requirement of apoLp-III: *D. geminatus* do not feed as adults, and presumably rely on fat body stores acquired during the 3 years of larval development for their energy needs. We speculate that the relatively short-lived adults are able to survive by mobilizing and transporting fat body reserves of lipid by using lipophorin and apoLp-III.

In the case of secondarily flightless insects, the function of apoLp-III may have been lost. ApoLp-III is present in the hemolymph of the flightless grasshopper *Barytettix psolus,* but it does not associate with lipophorin, likely through a failure of the fat body to respond to adipokinetic hormone (36). In the secondarily flightless cricket *A. domesticus* (8), the function of apoLp-III may have reverted to its original role of protection from starvation and perhaps in the mobilization of lipids required for oogenesis in females.

Future endeavors, including X-ray crystallographic characterizations and observations of the occurrence and distribution of apoLp-III from other species under varying physiological conditions, will increase our understanding of their function and structure.

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REFERENCES

1. Van der Horst, D. J. 1990. Lipid transport function of lipoproteins in flying insects. *Biochim. Biophys. Acta.* **1047:** 195–211.
2. Van der Horst, D. J., R. O. Ryan, M. C. Van Heusden, T. K. F. Schulz, J. M. Van Dorn, J. H. Law, and A. M. T. Beenakkers. 1988. An insect apolipoprotein hybrid helps define the role of apolipophorin III. *J. Biol. Chem.* **263:** 2027–2033.
3. Breiter, D. R., M. R. Kanost, M. M. Benning, G. Wesenberg, J. H. Law, M. A. Wells, I. Rayment, and H. M. Holden. 1991. Molecular structure of an apolipoprotein determined at 2.5-A resolution. *Biochemistry.* **30:** 603–608.
4. Kanost, M. R., M. S. Boguski, M. Freeman, J. I. Gordon, G. R. Wyatt, and M. A. Wells. 1988. Primary structure of apolipophorin-III from the migratory locust, *Locusta migratoria. J. Biol. Chem.* **26:** 10568–10573.
5. Chino, H., H. Katase, R. G. H. Downer, and K. Takahashi. 1981. Diacylglycerol-carrying lipoprotein of hemolymph of the American cockroach: purification, characterization, and function. *J. Lipid Res.* **22:** 7–15.
6. Shapiro, J. P., P. S. Keim, and J. H. Law. 1984. Structural studies on lipophorin: an insect lipoprotein. *J. Biol. Chem.* **259:** 3680–3685.
7. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **226:** 680–685.
8. Strobel, L. M., M. R. Kanost, R. Ziegler, and M. A. Wells. 1990. Adipokinetic hormone causes formation of a low density lipophorin in the house cricket, *Acheta domesticus. Insect Biochem.* **20:** 859–863.
9. Furlan, M., B. A. Perret, and E. A. Beck. 1979. Staining of glycoproteins in polyacrylamide and agarose gels with fluorescent lectins. *Anal. Biochem.* **96:** 208–214.
10. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28:** 350–356.
11. Savakis, C., M. Ashburner, and J. H. Willis. 1986. The expression of the gene encoding for alcohol dehydrogenase during development of *Drosophila melanogaster. Dev. Biol.* **114:** 194–207.
12. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
13. Feng, A-F., and R. F. Doolittle. 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *J. Mol. Evol.* **25:** 351–360.
14. Pearson, W. R., and D. J. Lipmann. 1988. Improved tools for biological sequence comparisons. *Proc. Natl. Acad. Sci. USA.* **85:** 2444–2448.
15. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12:** 387–395.
16. Rost, B., and C. Sander. 1993. Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232:** 584–599.
17. Rost, B., and C. Sander. 1992. Jury returns on structure prediction. *Nature.* **369:** 540.
18. Segrest, J. P., M. K. Jones, H. De Loof, G. G. Brouillette, Y. V. Venkatachalapathi, and G. M. Anantharamaiah. 1992. The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. *J. Lipid Res.* **33:** 141–166.

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19. Jones, M. K., G. M. Anantharamaiah, and J. P. Segrest. 1992. Computer programs to identify and classify amphipathic α helical domains. *J. Lipid Res.* **33:** 287–296.
20. Benson, D., D. J. Lipman, and J. Ostell. 1993. GenBank. *Nucleic Acids Res.* **21:** 2963–2965.
21. Soulages, J. L., and M. A. Wells. 1994. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Adv. Protein Chem.* **45:** 371–415.
22. Cole, K. D., G. J. P. Fernando-Warnakulasuriya, M. S. Boguski, M. Freeman, J. I. Gordon, W. A. Clark, J. H. Law, and M. A. Wells. 1987. Primary structure and comparative sequence analysis of an insect apolipoprotein. *J. Biol. Chem.* **262:** 11794–11800.
23. Van der Horst, D. J., J. M. Van Doorn, and A. M. T. Beenakkers. 1984. Hormone induced rearrangement of locust haemolymph lipoproteins: the involvement of glycoprotein C_2. *Insect Biochem.* **14:** 495–505.
24. Kawooya, J. K., P. S. Keim, R. O. Ryan, J. P. Shapiro, P. Samaraweera, and J. H. Law. 1984. Insect apolipophorin-III. Purification and properties. *J. Biol. Chem.* **259:** 10733–10737.
25. Ryan, R. O., R. Ziegler, D. J. Van der Horst, and J. H. Law. 1990. Characterization of apolipophorin III from *Barytettix psolus* and *Melanoplus differentialis*. *Insect Biochem.* **20:** 127–133.
26. Hard, K., J. M. Van Doorn, J. E. Thomas-Oates, J. P. Kamerling, and D. J. Van der Horst. 1993. Structure of the Asn-linked oligosaccharides of apolipophorin III from the insect *Locusta migratoria*. Carbohydrate-linked 2-aminoethylpyrophosphate as a constituent of a glycoprotein. *Biochemistry.* **32:** 766–775.
27. Nishikawa, K. 1983. Assessment of secondary-structure prediction of proteins comparison of computerized Chou-Fasman methods with others. *Biochim. Biophys. Acta.* **748:** 285–299.
28. Blaber, M., X-J. Zhang, and B. W. Matthews. 1993. Structural basis of amino acid α helix propensity. *Science.* **260:** 1637–1640.
29. Hara, H., H. Hara, A. Komaba, and S. Yokoyama. 1992. α-Helical requirements for free apolipoproteins to generate HDL and to induce cellular lipid efflux. *Lipids.* **27:** 302–304.
30. Schiffer, J., and A. B. Edmundson. 1967. Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. *Biophys. J.* **7:** 121–135.
31. Segrest, J. P., H. De Loof, J. G. Dohlman, C. G. Brouillette, and G. M. Anantharamaiah. 1990. Amphipathic helix motif: classes and properties. *Proteins.* **8:** 103–117.
32. Lipman, D. J., and W. R. Pearson. 1985. Rapid and sensitive protein similarity searches. *Science.* **227:** 1435–1441.
33. Kukalova-Peck, J. 1991. Fossil history and evolution of hexpod structures. *In* The Insects of Australia. CSIRO, editor. Melborne University Press, Melbourne, Australia. 141–179.
34. Farrow, R. A. 1990. Flight and migration in Acridids. *In* Biology of Grasshoppers. R. F. Chapman and A. Joern, editors. John Wiley and Sons, New York, NY. 227–314.
35. Cole, K. D., A. F. Smith, and M. A. Wells. 1990. The structure of the apolipophorin-III gene from *Manduca sexta*. *J. Biol. Chem.* **20:** 381–388.
36. Ziegler, R., R. O. Ryan, E. A. Arbas, and J. H. Law. 1988. Adipokinetic response of a flightless grasshopper (*Barytettix psolus*): functional components, defective response. *Arch. Insect Biochem. Physiol.* **9:** 255–268.
37. Engleman, D. M., T. A. Steitz, and A. Goldman. 1985. Identifying transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* **15:** 321–353.

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