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Computational Analysis of Apolipophorin-III in *Hyphantria cunea*

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Recently a cDNA clone of apoLp-III from *Hyphantria cunea* was isolated and subjected to computational analysis to compare with other available sequences. Multiple sequence alignments were carried out using the amino acid sequences of apoLp-III from six insects. It was found that the *H. cunea* apoLp-III has relatively high sequence identities to *Spodoptera litura* (69.5%), *Manduca sexta* (66.8%), *Galleria mellonella* (65.1%), *Bombyx mori* N4 (54.3%) but less identity to *Locusta migratoria* (18.3%). The amino acid composition was compared with other insects using EXPASY tools; it shows that alanine (Ala), glutamine (Gln), leucine (Leu) and lysine (Lys) are the major amino acid components of apoLp-III in *H. cunea* as well as other lepidopterans. Homology modeling performed using PSI-BLAST (PDB template *M. sexta*) reveals that the apoLp-III molecules consist of five, long amphipathic alpha helical bundles with short loops connecting the helices and shows homology with other insects. Phylogenetic analysis shows that the orthopteran apoLp-III represented by locust was most distantly related to the lepidopteran insects.

Key words: ApoLp-III, cDNA clone, Amphipathic α-helix, *Hyphantria cunea*

Introduction

In insects, apoLp-III serves as an excellent model system for exploring the molecular basis of the interactions of water-soluble exchangeable apolipoproteins with lipid surfaces. During flight, the major resting insect lipoprotein, high-density lipophorin (HDLp), takes up diacylglycerol, which is mobilized from the fat body in response to a peptide adipokinetic hormone (AKH), resulting in the transformation of this lipophorin into a low-density lipophorin (LDLp). Concomitant with diacylglycerol loading of the HDLp, which contains a single molecule of apolipophorin-I (apoLp-I, ~200 KDa) and apolipophorin-II, (apoLp-II, ~80 KDa), several molecules of low-molecular apolipoprotein, apolipophorin-III (apoLp-III, 18-20 KDa) become associated with the particles (Wells *et al.*, 1987; Ryan *et al.*, 1990 and Van der Horst *et al.*, 2002). In muscles used for flight, diacylglycerol carried by LDLp is hydrolyzed by a lipoprotein lipase (Van der Horst *et al.*, 2002) to produce fatty acid that is taken up into the muscle cell. The HDLp and the apoLp-III however are recycled (Van der Horst *et al.*, 1988, 2001) without internalization of the LDLp. ApoLp-III is an exchangeable insect apolipoprotein consisting of five amphipathic α-helices. This protein is able to open reversibly on association with hydrophobic surfaces and plays a role both in lipid transport and the induction of immune responses. The relationship between reduced lipid association and reduced immune stimulating activity support the hypothesis that apoLp-III induced immune activation is triggered by the conformation changes of the protein (Niere *et al.*, 2001). Therefore, the remarkable property of apoLp-III to associate with HDLp has aroused great interest as a result of its physical properties and biological functions.

Biology is the most useful and contribution to science and has the ability to recognize and match DNA and protein structures as well as sequences based on sequence data alone (Henikoff, 1996; Brenner *et al.*, 1997). The data are stored in an increasingly large variety of rapidly growing databases and have to be organized, displayed, analyzed, understood and exploited (Fredj, 2000). There are numerous computational techniques that can be used

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to identify the protein functions of newly sequenced genes, which stand between sequence alignments on the one hand, and the complete three-dimensional structure determination on the other hand (Wiesner *et al.,* 2000). Visualization and computer animation are useful for demonstrating the complex process in two or three dimensions, design, and analysis of molecular structures. For instance, it is now possible to predict structure with very high reliability (< 95%), where secondary structure prediction methods still only achieve up to 75% accuracy (Rost and Sander 1993; Mathew and Jonathan, 2004). We have recently cloned and sequenced apoLp-III from fall webworm *Hyphantria cunea* [molecular weight 20 KDa and 187 amino acids (561bp)]. This protein plays a key role in the regulation of lipid metabolism (Wells *et al.*, 1987), insect development and immune activation (Wiesner *et al.*, 1997; Niere *et al.*, 1999). Therefore, a number of studies concerning their biosynthesis, structure, functions, regulation and evolution have been conducted in recent years (Blaber *et al.*, 1993; Kim *et al.*, 1998, 2001, 2004; Soulages *et al.*, 1998; Narayanaswami and Ryan, 2000; Estela *et al.*, 2001; Halwani *et al.*, 2001; Kiss *et al.*, 2003). Hence the present study focuses on the computational analysis of apolipophorin-III in *Hyphantria cunea* and discusses the predicted structure and evolutionary aspect.

Materials and Methods

Sequence searching

When a new sequence of a protein or a gene is determined, one immediate task is to search the databases to find whether the sequence is known and whether any related sequences exist from which structural and functional conclusions can be drawn. Using the WWW servers at EBI or NCBI nucleotide sequence (http://www.ncbi.nlm.nih.gov), data are collected in one of the three internationally collaborating databases: EMBL, Genbank (Benson *et al.*, 1998) or DDBJ. The following amino acid sequence access numbers AAQ24031 (*Hyphantria cunea*), AAC6337 (*Spodoptera litura*), AAA29301 (*Manduca sexta*), CAA07363 (*Galleria mellonella*), AAB58735 (*Bombyx mori* N4) and AAA29282 (*Locusta migratoria*) are used for the computational analysis of apoLp-III and compared with those of other insects.

Sequence similarity search

Sequence similarity searches use alignments to determine a "match" by BLAST (PSI-BLAST) or position specific scoring matrix, (PSSM) is constructed (automatically) from a multiple alignment of the highest scoring hits in an initial BLAST search (http://www.ncbi.nlm.nigh.gov/blast/). Calculating position-specific scores for each position in the alignment generates the PSSM. Highly conserved positions receive high scores and weakly conserved positions receive scores near zero.

Multiple sequence alignment

Clustal W is a general purpose multiple sequence alignment program (http://www.ebi.ac.uk/clustalw) for DNA or proteins (Thompson *et al.*, 1997). Sequences can be aligned across their entire length (global alignment) or only in certain regions (local alignment). This is true for pairwise and multiple alignments. Global alignments need to use gaps (representing insertions/deletions).

Physicochemical property

Freely available ProtParam tools are used to analyze physical and chemical parameters for a given protein stored in Swiss Port or TrEMBL (Appel *et al.*, 1994). Similarly hydropathy Kyte-Doolittle scale values and a power spectrum formula can easily analyze the properties of amphipathic area of the protein sequence by using Prot scale service (http:au.expasy.org/tools/protscale.html) on EXPASY tools (Kyte and Doolittle, 1996).

Homology modeling

Homology modeling was analyzed using Geno3D (http://geno3D-pbil.ibcp.fr & http://us.expasy.org/tools/#tertiary), an automatic server for protein molecular modeling. Starting with a query protein sequence, the server performs the homology modeling in the following steps : i) identify homologous proteins with known 3D structures using PSI-BLAST, ii) provide the user with all potential templates through a very convenient user interface for target selection (PDB template of 69% identity of known *Manduca sexta* structures), iii) perform the alignment of both query and subject sequences. Extract geometrical restraints (dihedral angles and distances) for corresponding atoms between the query and the template. iv) perform the 3D construction of the protein using a distance geometry approach and finally send the results by e-mail to the user.

Phylogenetic tree analysis

Several programs exist for making phylogenetic trees that display the relationship between sequences to calculate all possible tree topologies to find the one that fits best with the sequence data. We used Clustal X (Thompson *et al.*, 1997) to generate alignments of the sequences. Nucleotide /protein sequences were subsequently aligned manually in order to increase alignment. A bootstrapped, unrooted neighbor-joining tree was generated (pairwise) using the

same program. After getting the root distance format using the Clustal W, the code was submitted into the phylodraw software version 8.2 in njplot.

Results and Discussion

Sequence similarity and comparisons

Multiple sequence alignments were carried out with amino acid sequences of apoLp-III from 6 insects. They were found to have extensive homology with the exception of *L. migratoria*. The result of the amino acid alignment is illustrated in Fig. 1. It revealed two relatively conserved proline and glycine residues, which are involved in breaks or turns between alpha helices (Blaber *et al.*, 1993). The conserved pattern of leucine, glycine and proline in *H. cunea* apoLp-III is most similar to that of *Spodoptera litura*. According to the analysis of *S. litura*

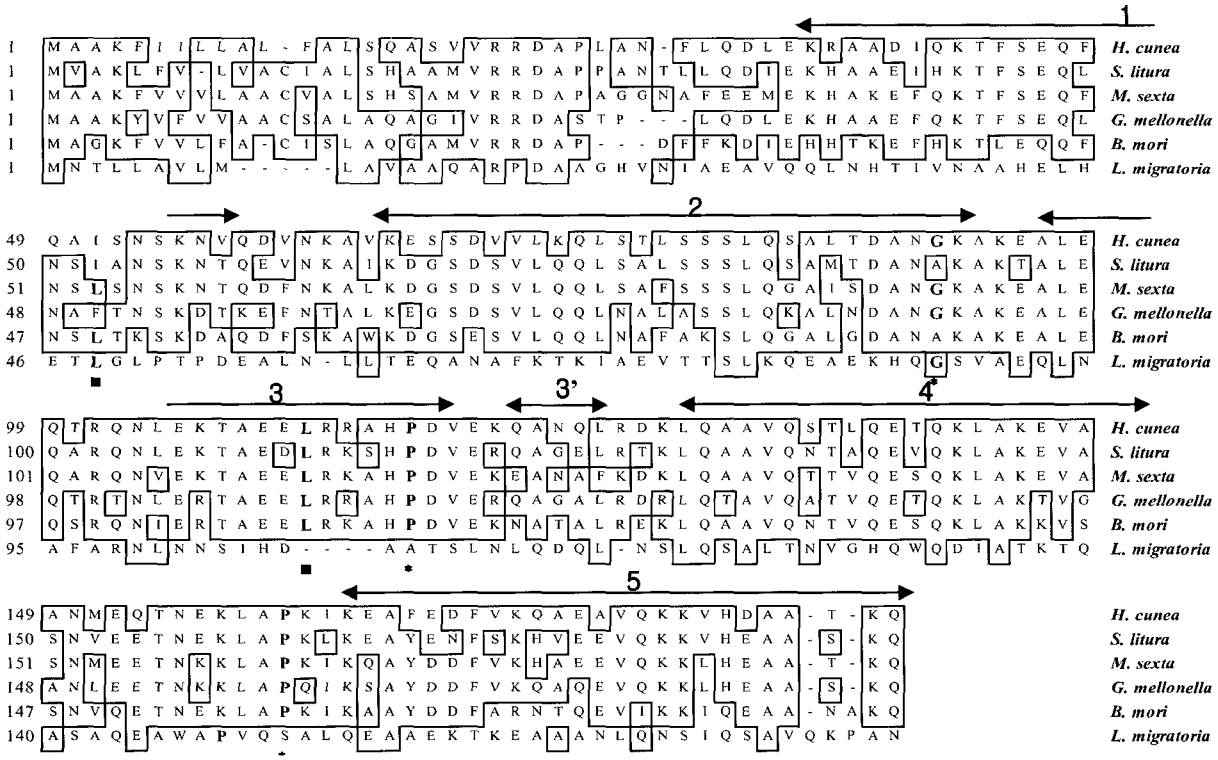

Fig. 1. Alignment of the amino acid sequences of insect apoLp-IIIs. Aligned amino acid sequences for apoLp-IIIs are from *Hyphantria cunea* (AAQ24031), *Spodoptera litura* (AAC63377), *Manduca sexta* (AAA29301), *Galleria mellonella* (CAA07363), *Bombyx mori* N4 (AAB02852) and *Locusta migratoria* (AAA29282). Five amphipathic α-helical domains are indicated by arrow line. The leucines that are putatively involved in the initial contact of the lipophorin surface are indicated by a solid box and bold face. Conserved residues that are probably involved in breaks and/or turns between the helices are designated with an asterisk below the residue.

Table 1. Sequence homology in the insect apoLp-III*

Percent identity							
1	2	3	4	5	6		
	69.5	66.8	65.1	54.3	18.3	1	*H. cunea*
		70.2	65.1	60.8	20.6	2	*S. litura*
			68.8	65.6	18.9	3	*M. sexta*
				60.8	17.8	4	*G. mellonella*
					18.3	5	*B. mori*
						6	*L. migratoria*

*Identities were determined by pairwise alignment using MEGALIGN.

apoLp-III, this protein is expected to have an α-helix content of 89.8% and have five amphipathic α-helices. Because of the high sequence identity, the overall *H. cunea* apoLp-III is almost the same as *S. litura* apoLp-III. To retrieve protein sequences similar to that apoLp-III, relevant databases were searched using BLAST & PSI-BLAST according to Altschul *et al.* (1990). It clearly indicates that the *H. cunea* apoLp-III showed a relatively high degree of sequence identity to those of other lepidopteran insects such as *S. litura* (69.5%), *M. sexta* (66.8%), *G. mellonella* (65.1%), and *B. mori* N4 (54.3%) (Table 1). A substantial difference was found when the orthopteran *L. migratoria* was compared to lepidopteran insects (17.8 – 20.6%).

Amino acid composition and physicochemical properties

From the deduced amino acid sequence of apoLp-III of *Hyphantria cunea,* the amino acid composition was determined and compared with apoLp-III from of others insects using ProtParam from EXPASY tools (Table 1). It revealed that *H. cunea* apoLp-III lacks cysteine (Cys), tryptophan (Trp) and tyrosine (Tyr), which make this protein show a high A230/A280 ratio (Kim *et al.*, 1998). Alanine (Ala), glutamine (Gln), leucine (Leu) and lysine (Lys) were the major amino acid components of this protein of *H. cunea* as well as other lepidopteran insects (Weise *et al.*, 1998; Yamauchi *et al.*, 2000), whereas in *L. migratoria* alanine (19%) and asparagine (8.9%) were high compared to lepidopteran insects (Table 2). Table 3 shows that the molecular weight of apoLp-III is about 20 kDa in lepidopteron insects, whereas it is 19 KDa in *L. migratoria.* Isoelectrofocusing points also show that they are neutral in *S. litura,* and *M. sexta,* whereas they are basic in *H. cunea, G. mellonella* and *B. mori,* but acidic in *L. migratoria.* In addition the total number of atoms is more or less similar (2888 – 2956) in lepidopteron insects, but shows a significant difference in *L. migratoria* due to a lower number of C, H and O atoms. The structure, functions and properties of biomolecules are often closely related, but it is usually difficult to determine the relationship from individual data. An important question, however, is whether hydrophobic interactions are responsible for the initiation of binding or whether ionic interactions localize apoLp-III at the particle surface, positioning the protein to "respond" to surface defects created by diacylglycerol portioning into the surface monolayer. Soulages and Wells (1994) have presented a "hydrophobic sensor" hypothesis to describe the initiation

Table 2. Comparison of amino acid composition of *Hyphantria cunea* apoLp-III with other insects

Amino acid	*H. cunea*	*S. litura*	*M. sexta*	*G. mellonella*	*B. mori* N4	*L. migratoria*
Ala	15.5	15.4	15.9	16.7	15.1	19.4
Arg	3.7	3.2	2.1	4.8	3.8	1.1
Asn	4.8	5.9	5.3	4.8	5.4	8.9
Asp	5.3	3.7	4.8	4.8	5.4	2.8
Cys	0.0	0.5	0.5	0.5	0.5	0.0
Gln	10.7	8.0	7.9	9.7	9.1	10.6
Glu	8.6	10.1	10.1	8.6	8.6	7.2
Gly	0.5	1.1	2.6	2.7	2.7	2.2
His	1.1	3.2	2.6	1.6	2.2	3.9
Ile	2.7	2.7	1.1	1.1	3.2	3.3
Leu	10.7	9.6	6.9	10.2	7.5	11.7
Lys	11.1	10.6	12.7	9.7	12.4	4.4
Met	1.1	1.6	2.1	0.5	1.1	1.1
Phe	3.7	1.6	4.8	3.2	4.8	1.1
Pro	1.6	2.1	1.6	1.6	1.6	2.8
Ser	7.0	8.0	7.4	5.4	5.9	5.6
Thr	4.8	4.8	3.7	7.0	4.3	7.2
Trp	0.0	0.0	0.0	0.0	0.5	1.1
Tyr	0.0	0.5	0.5	1.1	0.5	0.0
Val	7.0	7.4	7.4	5.9	5.4	5.6
Asx	0.0	0.0	0.0	0.0	0.0	0.0
Glx	0.0	0.0	0.0	0.0	0.0	0.0

Table 3. Comparison of physicochemical properties of *Hyphantria cunea* apoLp-III with other insects

Property	*H. cunea*	*S. litura*	*M. sexta*	*G. mellonella*	*B. mori* N4	*L. migratoria*
Molecular weight (kDa)	20745.5	20649.3	2079.3	20452.9	20711.4	19192.3
Theoretical pl	8.71	7.03	6.98	8.59	9.06	5.25
Negative charge	26	26	28	25	26	18
Positive charge	28	26	28	27	30	10
Carbon	903	888	902	883	907	828
Hydrogen	1498	1474	1458	1450	1468	1341
Nitrogen	262	264	260	264	266	245
Oxygen	291	292	293	289	286	275
Sulfur	2	4	5	2	3	2
Total number of atoms	2956	2922	2918	2888	2930	2691
Instability index	48.57	47.51	40.98	37.99	45.89	36.72
Aliphatic index	87.81	84.73	68.31	77.85	72.58	94.06
Grand average of hydropathicity	0.564	0.600	0.602	0.600	0.746	0.308

of interactions between exchangeable apolipoproteins and lipoproteins. According to this hypothesis, a two-step sequential mechanism for binding of apoLp-III to lipoprotein surfaces has been proposed. The first step involves a reconsisting process (through exposed hydrophobic amino acids) consisting of the adsorption of apoLp-III to a nascent hydrophobic defect in the phospholipid bilayer casued by the presence of diacylglycerol. This is followed by a conformational opening to expose the protein interior. In *L. migratoria* and *M. sexta* apoLp-III, conserved leucine located in the loops between helices are responsible for the initial association process. An alternative proposal is that helix 3' may play a role in the initiation of stable lipoprotein binding. Helix 3' contains five residues (Asp, Val, Glu, Lys and Glu), four of which possess charged hydrophilic side chains. The suggestion that charge-charge interaction between the phospholipid head groups and helix 3' provide a relatively long-range attraction, which localize apoLp-III in close proximity to the lipoprotein surface, is supported by studies with model phospholipids. Wang *et al.* (1997) have observed > 300 interhelical long range NOEs in the structure of apolipoprotein-III from *M. sexta*. Those interhelical contacts are mainly from hydrophobic residues such as leucine, valine, isoleucine, phenylalanine and alanine. These hydrophobic interactions are the major force contributing to stabilization of the helix bundle structure. In the multiple alignments of the sequences of various apolipoproteins, the hydrophobic residues have been highlighted quite, interestingly. These residues are well conserved across all the sequences from the different species. Hence it is envisaged that a common mechanism may underlie in the folding and function of apolipoproteins.

3D structure and homology modeling of apoLp-III in *Hyphantria cunea*

The homology of the protein with a known 3D structure using PSI-BLAST was identified (PDB template of *M. sexta* structure). Structural analysis reveals that *H. cunea*

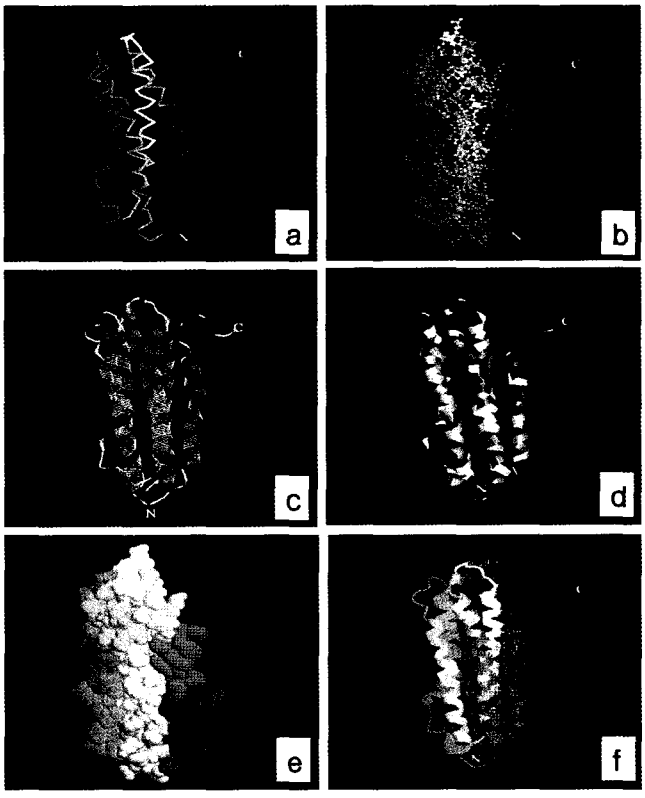

Fig. 2. Different 3D structures of apoLp-III in *Hyphantria cunea*. a) Backbone structure, b) Ball and stick structure, c) Strand structure, d) Ribbon structure, e) Space filling structure, and f) Cartoons and label structure.

apoLp-III molecules consist of five, long amphipathic alpha helical bundles with short loops connecting the helices helix 1 (N-terminal helix), helix 2, 3 and 5 (C-terminal) to the center of helix 4 (Figs. 2 and 3) with their hydrophobic faces oriented towards the center of the bundle, so their hydrophilic faces interact with the solvent. This molecular architecture resembles that of the 22 KDa N-terminal domain of human apolipoprotein E, the crystal structure of which reveals a 4-helix bundle (Wilson *et al.*, 1991). The prediction diagram clearly demonstrates a five-helix bundle structure for lipid-free *H. cunea* apoLp-III that is similar to the X-ray crystallographic structure of

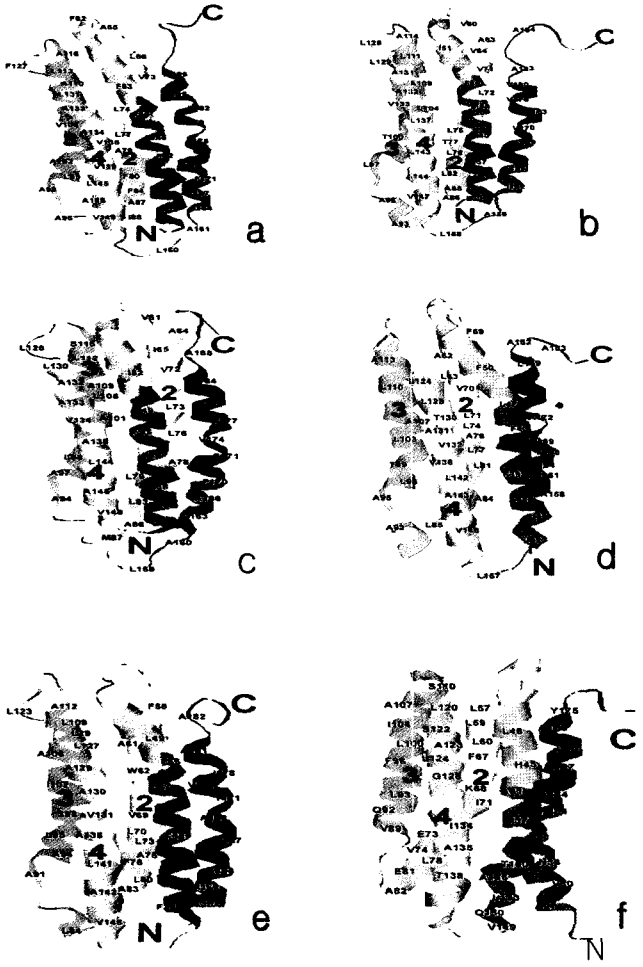

Fig. 3. Ribbon diagram of the molecular fold of *Hyphantria cunea* apoLp-III compared with other insects. The model building is based on EXPASY tool available at WWW.expasy.ac.in. The figures are generated in geno3D-PDB viewer *i.e*, ribbon display and is based on the template of *Manduca sexta* PDB structure of apoLp-III. The hydrophobic residues which are likely to occur in the amphipathic helixes and form inter helical contacts, are labeled. a) *Hyphantria cunea*, b) *Spodoptera litura*, c) *Manduca sexta*, d) *Galleria mellonella*, e) *Bombyx mori* N4, and f) *Locusta migratoria*.

M. sexta and *L. migratoria* apoLp-III (Breiter *et al.*, 1991; Weers *et al.*, 1999). Table 3 reveals that the presentation of hydrophilic residues to the aqueous environment facilitates the existence of apoLp-III as a water soluble, monomeric protein in the lipid free state. This protein shows structural and functional homology to other lepidopteran insects as well as *L. migratoria* apoLp-III (Fig. 3), but is non-glycosylated and shares only 29% sequence identity (Kanost *et al.*, 1988).

The arrangement of amphipathic helices in *H. cunea* is similar to those in *S. litura* (Fig. 3), *M. sexta*, *G. mellonella*, *B. mori* and *L. migratoria* apoLp-IIIs have a protein exterior containing polar residue and the interior has predominantly hydrophobic residues. On the basis of numerous interhelical nuclear overhauser enhancement contacts detected, it is postulated that hydrophobic interactions between paired helical segments stabilize the bundle conformation (Smith *et al.*, 1994; Sahoo *et al.*, 1998). This effect may be counter balanced by several polar residues located in the protein's hydrophobic interior, which may contribute to the overall low intrinsic stability of lipid-free apoLp-III. The distinct structural feature of the apoLp-III helix bundle is helix 3'. This short helix connects helix 3 and helix 4 and orients almost perpendicular to the helix bundle. In terms of the proposed helical repositioning upon lipid association (Breiter *et al.*, 1991), helix 3' may function in initiating the conformational opening of the protein. Sequence alignment of apoLp-IIIs (Fig. 1) from eight distinct insect species reveals the highest degree of sequence conservation at this region, suggesting an important role in the biological function of apoLp-III. A multiple sequence alignment and a hydrophobic plot (Fig. 4) reveal that despite the fact that helix 3' is short, its amino acid sequence is amphipathic, containing both hydrophobic (Val, Leu) and hydrophilic residues (Lys, Gly). A widely accepted concept proposed by Segrest *et al.* (2001) indicates that the amphipathic α-helix serves as a secondary structural motif for lipid binding (Fig. 3). In particular, two relatively conserved prolines and a glycine residue, are involved in breaks or turns between α-helices (Blaber *et al.*, 1993). It is also envisioned that the apoLp-III helix bundle will initiate lipid binding and contact with lipoprotein surface-binding sites (Narayanaswami *et al.*, 1999). By extension, it can be speculated that a partially folded molten globule-like state of apoLp-III may play an important physiological role in lipoprotein metabolism. Based on the information from this analysis, the observed conservation of structurally and functionally important residues, and the experimental evidence shows that *M. sexta* apoLp-III is functionally equivalent to *L. migratoria* apoLp-III (Van der Horst *et al.*, 1988), we propose that insect apoLp-IIIs share a common structural motif of five

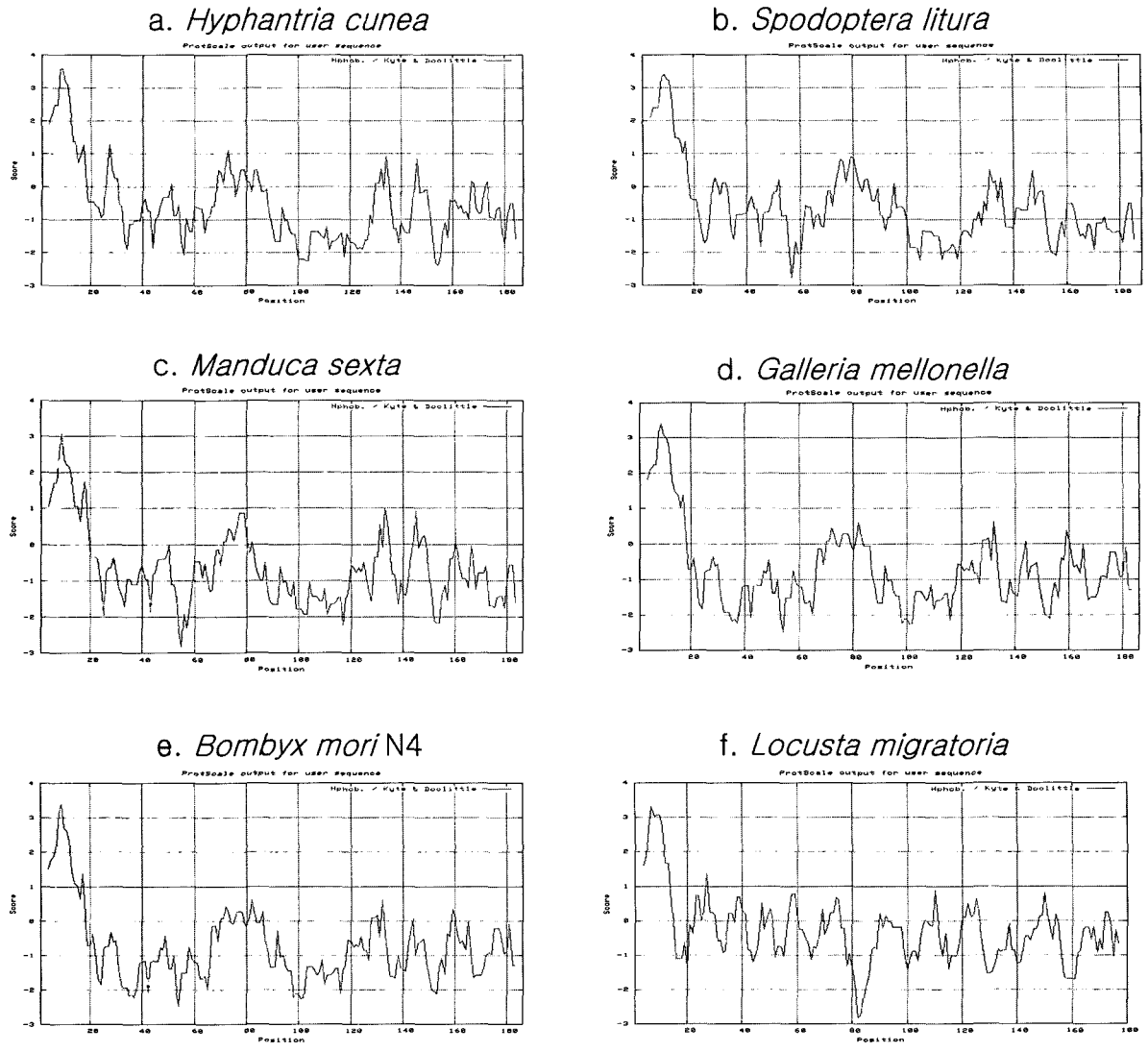

Fig. 4. Comparison of hydrophobicity plot of *H.cunea* apoLp-III with other insects. a) *Hyphantria cunea*, b) *Spodoptera litura*, c) *Manduca sexta*, d) *Galleria mellonella*, e) *Bombyx mori* N4, and f) *Locusta migratoria*.

amphipathic helices with properties similar to the class A amphipathic helical domains of vertebrate exchangeable apolipoproteins. In addition, precise amino acid identity appears less important than the distribution of polar and nonpolar residues.

Phylogenetic analysis

An unrooted tree shows the specifications of relationships but does not order them according to their history. The locust apoLp-III is distantly related to the lepidopteraon apoLp-III, but apoLp-III from the lepidopteran (*H. cunea*, *S. litura*, *M. sexta*, *G. mellonella*, *B. mori* etc.,) and orthopterans locust seem to be structurally and functionally equivalent in *in vitro* systems (Van der Horst *et al.*, 1988). It was very clear in paired sequence distance in *H. cunea* and *S. litura* show very close relationship as do *M. sexta* and *G. mellonella*, while others are not so closely related (Fig. 5). Despite the fact that the majority of non-identical residues represent conservative substitutions, this degree of sequence identity appears rather low, compared with that of other orthologous proteins, *Locusta migratoria* (0.849080). Boguski *et al.* (1986) reported similar results. The relationship between these proteins can be detected at the sequence level. From this we conclude that exchangeable apolipoproteins represent a critically important class of proteins that historically have been closely connected to lipoprotein metabolism. ApoLp-III plays an unexpected role in insect immune activation (Zakarian *et al.*, 2002; Kim *et al.*, 2004). Further study on apolipoprotein lipid-interaction, receptor binding and enzyme activation will

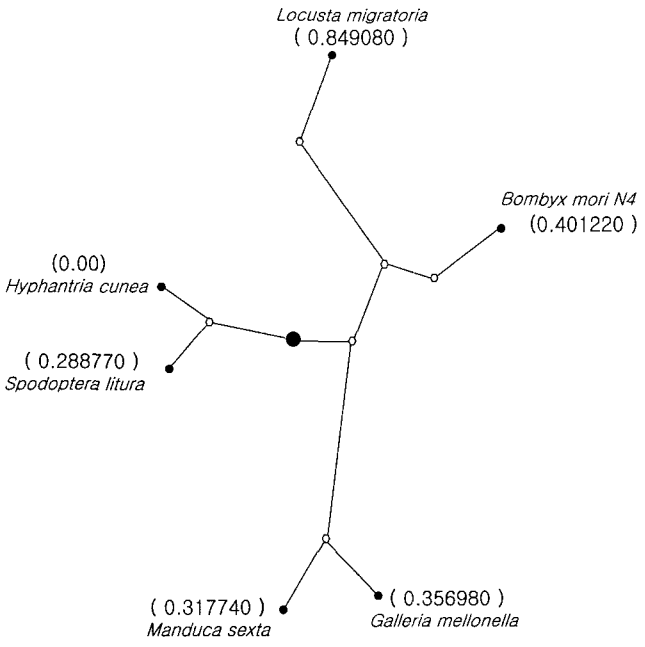

Fig. 5. Radial tree of phylogenic analysis from *Hyphantira cunea* apoLp-III and other insects. Lengths of branches along the axis are proportional to evolutionary distances calculated from the pairwise amino acid identity matrix.

provide important clues about how this protein functions in plasma lipoprotein metabolism. In the near future, we can look forward to the development of further methods and increased usage of all these techniques in the new era of proteomics and bioinformatics.

Acknowledgements

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