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Apolipophorin III: A Unique Insect Protein

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Abstract

Insect lipoprotein termed as apolipophorin III (apoLp-III), has a unique five-helix bundle protein structure. The protein is used by insects to supply high supply of energy to their flight muscles (wings) during unstopped long-distance migration. However, it is not clear why compared to most prominent four-helix bundle protein structure found in nature, apoLp-III has five helix bundles. This review provides a concise structural and functional comparative analysis of apoLp-III with respect to other prominent four and five helix bundle proteins.

Keywords: *Apolipophorin III; Insect Lipoprotein; M. sexta*

Introduction

Lipid and cholesterol belong to lipid biomolecules and they do not dissolve in aqueous solutions due to their hydrophobic nature. This insolubility causes difficulty in the transport of lipid and cholesterol in the blood stream. However, lipid and many other hydrophobic metabolites need to be transported to various tissues, for example triglycerides to adipose tissue for storage. These vital functions of water insoluble lipid biomolecules are very important and therefore an efficient lipid transport system is critical in all biological systems. Biologically, the transport of lipids is made possible by lipoproteins. Lipoproteins are made up of lipids and proteins [1,2]. Lipoprotein's structure is made up of a spherical particle consisting of an outer layer and core region. A monolayer of phospholipids, unesterified cholesterol, and proteins makes outer layer [1,2]. The core region contains neutral lipids, mainly cholesteryl esters and triglycerides [1-3]. Despite similarity in the overall structure, lipoproteins differ significantly in relative proportion of lipid and protein contents. Based on density, there are five major classes of lipoproteins [4-6] (Table 1). These major classes are chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high-density lipoproteins (HDL) [6,7].

Lipoproteins	Diameter (nm)	Density	Major Lipids	Apolipoproteins
Chylomicrons	75-1200	0.93	TG	B48, E, C, A-I, A-II, -IV
Very low density	30-80	0.93-1.006	TG	B100, E, C
Intermediate	25-35	1.006-1.019	TG	B100, E, C
Density				
Low Density	18-25	1.019-1.063	CE	B100
High Density	5-12	1.063-1.210	Cholesterol, CE,	B100, A-I, A-Phospholipid II, C

Table 1: *Lipoproteins: Classification and Composition.*

**TG: Triglyceride; CE: Cholesteryl ester (5, 7).*

The protein components of a lipoprotein particle are called apolipoproteins. Most apolipoproteins are amphipathic in nature, containing both polar (water soluble) and nonpolar (water insoluble) regions. The amphipathic apolipoproteins consist of α-helical segments termed amphipathic α-helices [1,2], a structural motif required for lipid binding. These amphipathic regions act as protein detergents and play an important role in the size determination and stabilization of the lipoprotein particles [2]. Apolipoproteins play significant roles in metabolism as mediators, either as cofactors for lipoprotein metabolism or as cellular receptor ligands [1,2,5].

Apolipoproteins are grouped into two classes, non-exchangeable apolipoproteins (apoB100 and apoB48) and exchangeable apolipoproteins (apoA-I, A-II, A-IV, C-I, C-II, C-III, and E) (2) (Table 2). Non-exchangeable apolipoproteins are components of chylomicrons, VLDL, IDLs, and LDLs. Due to their higher insolubility in aqueous solutions and larger size, non-exchangeable apolipoproteins remain with the lipoprotein particle at all times.

Apolipoproteins	Molecular Mass (Da)	Function
ApoB100	550,000	Structural: ligand for LDL*
ApoB48	264,000	Structural*
ApoE	33,000	Ligand for various receptors*
ApoC-I	6,630	Hepatic receptor inhibition*
ApoC-II	8,900	Lipoprotein lipase activation*
ApoC-III	8,800	Lipoprotein lipase inhibition*
ApoA-I	28,000	LCAT activation*
ApoA-II	17,400	Structural*
ApoA-IV	44,500	Surface activity*

Table 2: *Major Apolipoproteins: Molecular Mass and Functions.*

**(2, 7)*

The exchangeable apolipoproteins are soluble in aqueous environment and relatively smaller in size. The number of exchangeable apolipoproteins per lipoprotein particle varies in contrast to the non-exchangeable apolipoproteins.

The number of exchangeable apolipoproteins per lipoprotein particle depends on the lipid content of the lipoprotein particle. Since apolipoproteins help in the stabilization and solubility of lipoproteins, the insoluble nature of the lipid is compensated by the association of apolipoproteins. Upon removal of the lipid from lipoproteins, apolipoproteins may not be needed and can detach from the lipoprotein surface.

The Amphipathic α-Helix

The amphipathic α-helix is a α-helix with both polar and nonpolar segments. It is a common secondary structure motif found in proteins. The amphipathic α-helix has been studied in lipid binding proteins, such as hormones, venoms, antibiotics, complex transmembrane proteins and the human immunodeficiency virus glycoprotein [8,9]. Amphipathic α-helices show unique internal 11 residues long polar and nonpolar amino acid repeats as the most striking feature [8]. This amino acid repetition results in a 22-residue amphipathic α-helix between the faces of the two identical 11 residue halves consist of the polar and nonpolar faces which has been observed in apoA-I, A-IV, and E [1,8]. Since the common 11 residue/22 residue tandem repeats are found in exchangeable apolipoproteins, it has been hypothesized that a single gene has evolved and gave rise to the current multigene family of apolipoproteins [8,10,11]. Based on de novo designed amphipathic peptides, it has been concluded that an ability to interact with phospholipids is directly dependent upon the amphipathic nature of α-helical segments [8,10]. Furthermore, lipid interaction increases with hydrophobicity of the nonpolar face of the amphipathic peptides [1,12,13].

All exchangeable apolipoproteins contain amphipathic α-helices, which is a signature feature for lipid binding and required for proteins to act as the plasma detergent. The presence of multiple amphipathic α-helices per protein directly augments the lipid binding ability of individual exchangeable apolipoprotein. Thus, lipid binding affinity not only depends on the amphipathicity of the individual helix but also on the number of the amphipathic α-helices per protein. Based on their physical-chemical and structural properties, amphipathic α-helices are divided into seven distinct classes (A, H, L, G, K, C, and M) [1,8]. The distinctive feature of class A amphipathic α- helix is a unique clustering of positively charged amino acid residues at the polar-nonpolar interface and negatively charged residues at the center of the polar face, and lipid binding have been shown by class A amphipathic α-helical domain [11]. The lipid binding affinity decreases from a well-defined class A to non-class A amphipathic α-helical domain. The properties of class A amphipathic helices lead to the classification of exchangeable apolipoproteins into three separate groups: apoA-II, C-I, C-II, and C-III with well-defined class A amphipathic helices, apoA-IV as an atypical class A, and apoA-I, E and insect apolipophorin III (apoLp-III) with typical but less well-defined class A amphipathic helical domain [1]. Class L and H are structurally similar with highly positively charged amphipathic α-helices. Class L peptides disrupt phospholipid bilayer due to their hemolytic nature, however class H exhibits mostly hormonal activities e.g., β-endorphins and secretin. Class M belongs to transmembrane proteins with polar groups in the hydrophobic membrane. Class G displays similar properties to class A from apolipoproteins, however due to lack of marked clustering of positive and negative residues as shown in class A, class G peptides do not exhibit lipid binding feature [1,8].

Insect Lipoproteins and Apolipophorins

Two well characterized insect models for lipoprotein metabolism are the locust, *Locusta migratoria*, and the tobacco hornworm, *Manduca sexta* [14]. Insects carry only one major type of lipoprotein, called lipophorin. The term lipophorin was first introduced by Chino., *et al* [15]. The density of a lipophorin particle ranges from 1.03-1.24 g/mL, which overlaps with the density range of human high-density lipoprotein (HDL) (Table 1). The molecular mass of high density lipophorin (HDLp) has been recorded in the range of 600 kDa [16]. HDLp is synthesized in the locust fat body [3,17]. HDLp particles usually consist of 50 - 60% protein (in the form of two non-exchangeable apolipoproteins) and 40 - 50 % lipid. In contrast to human HDL, insects HDLp contain diacylglycerol (DG) as a major lipid, and phosphatidylethanolamine (PE) as the major phospholipid component. Lipophorin particles contain two non-exchangeable apolipoproteins, apolipophorin I (apoLp-I) and -II (apoLp-II) [3,16,18]. ApoLp-I and -II are similar to mammalian apoB100 and 48. ApoLp-I is the largest of the apolipophorins with a molecular mass of ~240 kDa and structurally essential for the formation of all lipophorin particles [3,16,19]. ApoLp-II is also an integral apolipophorin component with a molecular weight of ~78 kDa [19]. The two non-exchangeable apolipophorins are produced from a posttranslational cleavage of a precursor protein, apolipophorin II/I (apoLp-II/I) during lipoprotein synthesis [20,21].

The presence of a third apolipoprotein, called apoLp-III with a molecular mass of 18 - 20 kDa, has also been observed. In contrast to apoLp-I and -II, apoLp-III associates with lipophorin particles in a reversible fashion. The function of apoLp-III is to stabilize DG-enriched lipophorin particles to aid in the transport of lipids from the fat body to flight muscles in hemolymph during prolonged flight. Long distance flight is one of the most energy demanding metabolic activities in nature. The limiting factor for this high energy requiring metabolic process is a quick availability of energy yielding substrates. Insects fulfill this demand by a quick supply of lipophorin-associated DG via hemolymph [16,21]. Insects mobilize triglycerides (TG) stores from the fat body as the energy source for long distance flight by secreting adipokinetic hormone (AKH) from Corpus Cardiacum [16,21]. Under the influence of AKH, a TG lipase is activated which converts fat body TG into DG. DG combines with preexisting circulating HDLp particles and results into the formation of low density lipophorin (LDLp) (Figure 1) [3]. The formation of the LDLp particle requires association of several molecules of apoLp-III; however, the number of apoLp-III per LDLp particle depends upon the lipid content of LDLp particle [16,21]. The increase in lipid content of LDLp induces the attachment of apoLp-III to cover its increased hydrophobic surface area and provide a hydrophilic coating of the expanding particle [3]. Due to the open circulatory system of insects, constant lipid supply to specific tissues (e.g. flight muscles) is not possible. This physiological disadvantage of lack of constant lipid supply is compensated by high plasma lipid concentration and availability of the lipid to the flight muscles [14,15].

Insects need a repetitive cycle of lipid transport from the fat body to flight muscles by recycling the components of the lipophorin particle, e.g. HDLp and apoLp-III. This repetitive cycle of lipid transport is called lipid transport shuttle [3,21,23-25] (Figure 1).

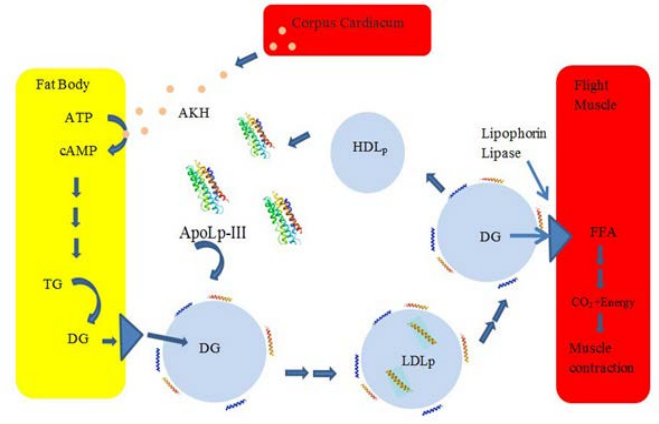

Figure 1: *Insect lipoprotein shuttle mechanism showing the effect of AKH on lipid transport of DG from the fat body to flight muscles in insects.*

Apolipophorin III

Several of lipoprotein metabolic functions have initially been discovered in the insect system and later observed in human. Simplicity of insect system compare to mammalian system makes it a good model system to study and gain insights into human exchangeable apolipoproteins [3,16,23,24]. The availability of high resolution structure of apoLp-III (*L. migratoria* and *M. sexta*) has the additional advantage for structure-functional analysis compared to other exchangeable apolipoproteins for which 3D structures have not been resolved yet [23,26]. ApoLp-III is mainly found in a lipid-free state in the hemolymph while most mammalian apolipoproteins are bound to lipoproteins [4,27].

Unlike vertebrate apolipoproteins, apoLp-III exists as a monomer in solution and this property has facilitated significant hydrodynamic and structural characterization of this protein [19]. The x-ray structure of locust apoLp-III was the first high resolution structure reported for a full length exchangeable apolipoprotein [28] (Figure 2). Locust apoLp-III consists of 164 amino acid residues with two tryptophans without methionine, tyrosine, or cysteine [28]. The first six residues of the N-terminal region and the last five C-terminal residues have been shown to be in an unstructured state in the x-ray structure.

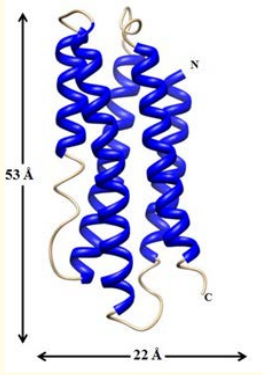

Figure 2: *X-ray of locust apoLp-III (PDB code 1AEP) with a dimension of 54 x 22 Å. This image was generated using CHIMERA UCSF, an interactive visualization and analysis tool to analyze molecular structure of biomolecules.*

ApoLp-III has been shown to consist of five helices connected by short loops (Figure 3). Helix 1 is comprised of residues 7-32, helix 2, 35-66; helix 3, 70-86; helix 4, 95-121; and helix 5, 129-156 [28]. It is a glycosylated protein with a carbohydrate mass of 2907 Da [23]. The NMR solution structure of locust apoLp-III showed the presence of a four-residue long helix 4' identified between Gln-127 and Pro-132 (128-AWAP-131) [31]. It has been proposed that helix 4' might act as a sensor to initiate lipid binding in locust apoLp-III [31]. This proposal was based on the higher flexibility of helix 4' compared to loop 1, 2 and 3 in locust apoLp-III. Several buried hydrophilic residues inside the hydrophobic core and many exposed hydrophobic residues have been observed in apoLp-III [26,28,29].

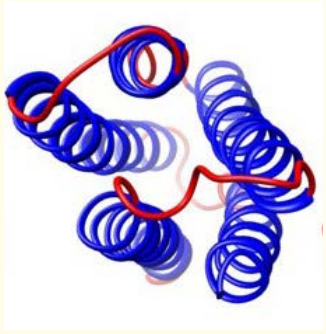

Figure 3: *Three-dimensional structures (end-on view from the top) of locust apoLp-III (PDB code 1AEP). This image was generated using CHIMERA UCSF, an interactive visualization and analysis tool to analyze molecular structure of biomolecules.*

There are hydrophilic residues has been observed inside the protein interior. One buried hydrophilic residue found between the contact area of helices 1 and 4, four between helices 1 and 5, and three between helices 2 and 3. However, most of the partially or fully buried hydrophilic residues have been observed between helices 2 and 5 and between helices 3 and 4, six between each pair (helices 2 - 5 and 3 - 4). Exchangeable apolipoproteins in the lipid-free form have a relatively low free energy of unfolding (ΔG) of < 4.5 kcal/mol [31]. In the absence of lipid, hydrophobic residues orient themselves in the protein interior and provide hydrophobic interhelical interactions to stabilize the helix bundle motif. Presence of hydrophilic residues in the interior of helix bundle destabilizes the overall protein stability. Furthermore, hydrophobic residues which are partially or fully exposed to the solvent also contribute to the destabilization of the protein. The effect caused by internal hydrophilic and externally exposed hydrophobic residues act together to make an overall marginally stable protein. It has been suggested that this marginal stability plays a significant role in the opening of the helix bundle [26,32]. It has been shown that the presence of a polar residue in the hydrophobic interior, Thr-31, lowers the overall stability and promotes lipid binding of the protein [32]. This study therefore supports the concept of the marginal stability of apolipoproteins needed for lipid binding. The hydrophobic helical interaction is replaced by helix-lipid interactions when the helix bundle opens and binds to lipids.

Like locust apoLp-III, *M. sexta* apoLp-III is also a bundle of five amphipathic α-helices consisting of 166 amino acids connected by short loops arranged in an up-and-down topology [23,30]. Despite the similarities in their three-dimensional structure, very little amino acid resemblance has been observed between locust and *M. sexta* apoLp-III. Furthermore, *M. sexta* apoLp-III is not glycosylated. There is a single tyrosine residue in *M. sexta* apoLp-III and tryptophan is absent. A short helix 3' connects helix 3 and helix 4 which has been implicated to play a similar role like helix 4' of locust apoLp-III [33]. Similar to locust apoLp-III, *M. sexta* apoLp-III also shows partially buried and completely buried hydrophilic amino acid residues in the protein hydrophobic interior. A total of 29 buried/partially buried hydrophilic residues have been observed in *M. sexta* apoLp-III. The presence of these 29 hydrophilic residues in the hydrophobic core contributes to an overall marginal stability of apoLp-III in a similar fashion as locust apoLp-III. Based on guanidine HCl (GdnHCl) induced denaturation studies, apoLp-III stability has relatively low midpoint of GdnHCl denaturation between 0.3 - 0.6 M for *M. sexta* and *L. migratoria* [34-36]. The lower stability of apoLp-III might be due to the presence of hydrophilic residues in a hydrophobic protein core.

The initiation of lipid binding in exchangeable apolipoproteins is a multistep process which consists of recognition of surface hydrophobic defects, helix rearrangement and eventual formation of a lipid-protein complex [37]. A major conformational change is required when apoLp-III goes from a lipid free to lipid bound state. Initially it was proposed that helices 1, 4, and 5 move away from helices 2 and 3 [28]. This helical rearrangement leads to the exposure of hydrophobic interior to the lipid surface. The global change in the helix bundle topology has been supported by experiments like lipid binding study with lipoprotein particles upon apoLp-III addition with phospholipids and small discoidal vesicles formation with dimyristoyl phosphatidylcholine (DMPC) [37]. Furthermore, helix tethering experiments, circular dichroism, tryptophan and tyrosine fluorescence studies have been in support of the lipid induced conformational change [34,38-45]. The repositioning of helix 1 and helix 5 of apoLp-III has critical role in the lipid binding process [42]. In helix tethering experiments of helix 1 and helix 5 by a disulfide bond, lack of lipid binding was observed with locust and *M. sexta* apoLp-III [40,42,46]. Furthermore, the highest conformational flexibility has been observed for helix 1 and helix 5 [47]. Upon binding to DMPC vesicles, apoLp-III has shown a totally different conformation [34,37,43]. The conformation of the protein around the discoidal lipid particle has been shown as a "belt" model [48,49]. According to this model, the helix axis of apoLp-III orients perpendicular to the axis of the fatty acyl chains (i.e. parallel to the plane of the discoidal lipid particle) [43,48,49].

Four vs. Five Helix Bundle Motif

The four-helix bundle motif consists of four α-helices and is frequently found in proteins [50]. This four-helix bundle motif might be an assembly of separate polypeptide chains, e.g. bacterial luciferase, repressor of primer (ROP), tumor suppressor p53; as an isolated single polypeptide chain, e.g. human apoE-NT, granulocyte-macrophage colony-stimulating factor, human growth hormone, and human interleukin-4; or as an individual domain in larger proteins, e.g. T4 lysozyme and 3-isodehydrogenase [50,51] (Figure 5). The four helix bundle is further divided into two classes. The first class has all four helices in antiparallel orientation and the second class has a mixture of parallel and perpendicular orientation [50,52]. The up-down-up-down arrangement is the most frequently found in the four-helix bundle proteins. Four-helix bundles are functionally diverse in their biological activities. For example, ROP binds to RNA, cytochrome b562 is involved in electron transport chain, and T4 lysozyme cleaves polysaccharide chains [52]. Additionally, other important four helical bundle proteins are apolipoproteins, interleukin-2, interleukin-4, and granulocyte-macrophage colony-stimulating factor (Figure 5). Prion protein has also been modeled as a four-helix bundle protein. All four helix bundle proteins that have been characterized possess a common signature feature of an interior hydrophobic rich region and a surface region rich in polar residues. This partitioning of hydrophobic and hydrophilic residues could be explained in terms of the hydrophobic effect.

In contrast to the common four helix bundle motif, the five helix bundle motif has only been observed in apoLp-III and the tail domain of human vinculin [53]. Vinculin is a cytoskeletal protein with a molecular mass of 117 kDa, composed of 1066 amino acid residues. It is responsible for making contacts with neighboring cells [54-58]. This protein has two domains, a head domain responsible for binding with talin and α-actinin; while the tail domain binds to F-actin, paxillin, and lipids [53-58] (Figure 4).

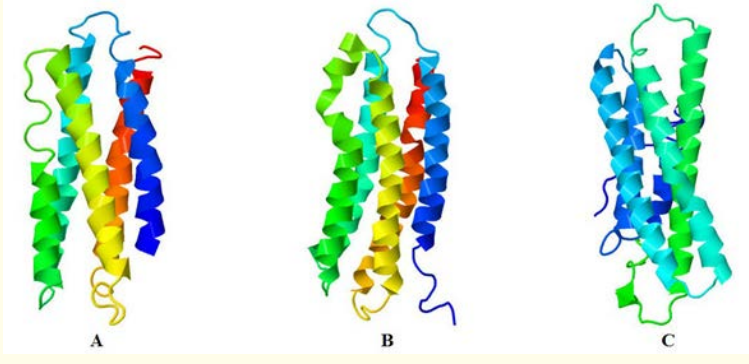

Figure 4: *A unique five helix bundle motif showing the up-and-down topology. (A) locust apoLp-III (PDB code 1AEP); (B) M. sexta apoLp-III (PDB code 1EQ1); (C) Tail domain of cytoskeletal protein, Vinculin (PDB code 1QKR). These images were generated using CHIMERA UCSF, an interactive visualization and analysis tool to analyze molecular structure of biomolecules.*

The lipid free helix bundle of apoLp-III needs to open to bind with lipid surface because of internally sequestered hydrophobic residues. Breiter., *et al*. (1991) proposed a conformational adaptation hypothesis for locust apoLp-III. According to this hypothesis, helix bundle opening occurs at one end of the hinge loop of the molecule. This hypothesis also corroborates the idea of reversible binding of exchangeable apolipoproteins [28,51,52]. Similar structure and function has been observed in the tail domain of vinculin.

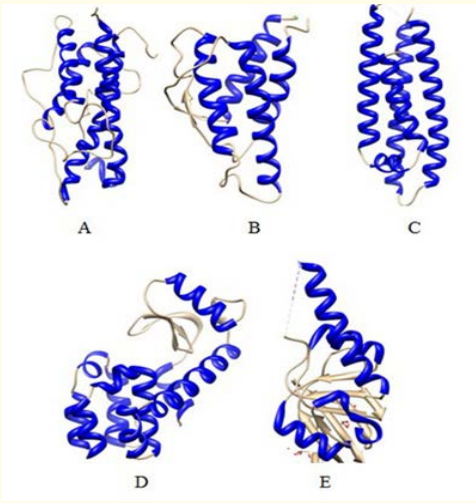

Figure 5: *High resolution structures of different four helical bundle proteins showing a common signature feature of the up-and-down helix bundle topology. (A) Human Growth Hormone (PDB code 1N1D); (B) Interleukin-4 (PDB code 1ITL); (C) Human apolipoprotein E-N terminal domain (PDB code1NFN); (D) T4 lysozyme (PDB code 1LYD); (E) Granulocyte-Macrophage Colony-Stimulating Factor (PDB code 1GML, A chain only). These images were generated using CHIMERA UCSF, an interactive visualization and analysis tool to analyze molecular structure of biomolecules.*

Apart from helix bundle opening mediated by hinge region of five helix-bundle, other possible bundle openings can not be excluded. It has been proposed that the conformational flexibility of helix 1 and 5 might be a key factor in the opening and lipid binding of locust apoLp-III [40,42,46]. The terminal helices of apoLp-III have been shown to possess the highest conformational flexibility in biophysical studies [39,44,47-50].

Conclusion

ApoLp-III has a unique five-helix bundle protein structure, which is found exclusively in some insects and few other human proteins. In insect class, this protein (apoLp-III) is used to supply high supply of energy to their flight muscles (wings) during unstopped long-distance migration. However, it is not clear why compared to most prominent four-helix bundle protein structure found in nature, apoLp-III has five helix bundle. The four-helix bundle motif consists of four α-helices and is frequently found in proteins. In contrast to the common four helix bundle motif, the five helix bundle motif has only been observed in apoLp-III and with few other proteins, e.g. tail domain of human vinculin. Recently, It has been proposed that the conformational flexibility of helix 1 and 5 of apoLp-III may be a key factor in the opening and efficient lipid binding of locust apoLp-III [40,42,46]. Based of previous studies, the terminal helices (helix 1 and helix 5) of apoLp-III have been shown to possess the highest conformational flexibility in biophysical studies [39,44,47-50]. Overall, apoLp-III is a unique protein based on its structure and functional aspects.

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