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Deletion of the N- or C-terminal helix of apolipoprotein III to create a four-helix bundle protein

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Abstract

Apolipoprotein III (apoLp-III) is an exchangeable apolipoprotein found in insects and plays an important function in lipid transport. The protein has an unusual five-helix bundle architecture, deviating from the common four-helix bundle motif. To understand the role of the additional helix in apoLp-III, the N-terminal or C-terminal helix was deleted to create a putative four-helix bundle protein. While the protein lacking helix-1 could be expressed in bacteria albeit at reduced yields, apoLp-III lacking helix-5 could not be produced. Mutational analysis by truncating helix-5 showed that a minimum segment of approximately one third of the C-terminal helix is required for protein expression. The variant lacking helix-5 was produced by inserting a methionine residue between helix-4 and -5; subsequent cyanogenbromide cleavage generated the four-helix variant. Both N- and C-terminal helix deletion variants displayed significantly reduced helical content, protein stability, and tertiary structure. Despite the significantly altered structure, the variants were still fully functional. The rate of dimyristoylphosphatidylcholine vesicle solubilization was enhanced 4- to 5-fold compared to the wild-type protein, and the deletion variants were effective in binding to lipolyzed low density lipoprotein thereby preventing lipoprotein aggregation. These results show that the additional helix of apoLp-III is not essential for lipid binding, but is required for proper folding to keep the protein into a stable conformation.

Graphical abstract

Deletion of the N- or C-terminal helix of apolipoprotein III to create a four-helix bundle protein
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Apolipoprotein III (apoLp-III) is a well-studied exchangeable apolipoprotein found in insects, and known for its role in lipoprotein mediated lipid transport.¹ The protein exists in

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lipid-bound and a lipid-free states, for the latter X-ray and NMR solution structures have been resolved for apoLp-III from *Locusta migratoria* and *Manduca sexta*.²⁻⁴ Recently, backbone assignments have been completed for *Galleria mellonella* apoLp-III towards elucidating the NMR structure of this apoLp-III, which allows for structure-guided studies to better understand the function in innate immunity.⁵ Lipid-free apoLp-III is composed of a bundle of five amphipathic α -helices folded in an up-and-down topology. The protein interior is predominantly made of hydrophobic residues, which are sequestered in the amphipathic α -helices forming a continuous non-polar face directed towards the protein interior. When apoLp-III associates with lipoproteins, the non-polar face of the amphipathic α -helices is presented to the hydrophobic lipoprotein surface. This requires reorientation of the amphipathic α -helices and it has been hypothesized that opening of the helix bundle exposes the protein interior. In vivo, lipoprotein association is triggered by massive diacylglycerol (DG) loading on existing lipoprotein particles in response to flight stimuli.⁶⁻⁷ This creates suitable binding sites for apoLp-III, and in *L. migratoria* up to 14 apoLp-IIIs bind to the lipoprotein surface.⁸ The diameter of the lipoprotein increases two-fold due to the increased amount of DG and protein, creating a lipoprotein with a significantly lower density: low density lipophorin. The term lipophorin, or apolipophorin for the protein part, is used to distinguish insect from vertebrate lipoproteins.⁹ Despite some differences, animal (apo)lipoproteins such as human apolipoprotein A-I and apolipoprotein E share remarkable similarities in structure and function with their insect counterparts.¹⁰

ApoLp-III is a relatively unstable protein, and loosely packed helices presumably facilitate opening of the helix bundle, allowing the switch from a lipid-free to a lipid-bound state.¹¹ While no high-resolution structure of lipid-bound apoLp-III has been resolved to date, there is ample evidence for a distinct conformation compared to the lipid-free protein. A combination of circular dichroism, fluorescence spectroscopy, disulfide-bond engineering and FTIR analysis of discoidal lipoprotein particles made of apoLp-III and phosphatidylcholine suggest an extended helical conformation, supporting the helix bundle opening hypothesis.¹²⁻¹⁶

The five-helix bundle motif of apoLp-III is rarely found in other proteins; a few other examples include vinculin tail and a fragment of Talin, although their helix topology is clearly distinct from apoLp-III.¹⁷ Several other protein domains contain five-helix bundles made of short α -helices that form a more compact globular fold such as FK506 binding proteins and HectD1, and aciniform spidroin 1.¹⁸⁻¹⁹ In contrast, the elongated four-helix bundle motif is found in many proteins with a wide variety of functions.²⁰⁻²² To gain insight in the unique character of the five-helix bundle, complete helical segments of the protein termini were removed from *L. migratoria* apoLp-III by site-directed mutagenesis to create a putative four-helix bundle. These and several other truncation variants were analyzed for protein structure and their ability to bind to lipid and lipoprotein surfaces.

Materials and Methods

Generation of truncated apoLp-III variants

All constructs were generated from wild-type *L. migratoria* apoLp-III. The apoLp-III H₂-H₅ variant was produced by amplifying the corresponding region by PCR and included the MscI

site at the 3' end, and the EcoRI site at the 5' end to facilitate ligating the PCR product into the pET-22b (+) vector. Forward and reverse primers were 5'-CCGGCCTG CCCACC CCCGACGAG-3' and 5'-GCTCGAATTCAGTTGGCGGGCTTCTG-3'. Fifty µg template DNA plus 125 ng of each primer, dNTP mix, and 2.5 U of PfuUltra HF DNA polymerase. The PCR product and vector were digested with EcoRI and MscI and purified using agarose electrophoresis using the Qiagen gel-extraction clean-up kit. One unit of T4 ligase was used to ligate the digested PCR product into the linearized vector. The other mutations were generated by introducing a stop codon in the apoLp-III coding region at the desired location using the QuikChange site-directed mutagenesis kit II (Agilent Technologies, Santa Clara, CA). The following primers were synthesized by MWG Biotech (High Point, NC, complementary primers not shown): apoLp-III_{H1-H4} 5'-GGGCTCCGGTGTAGAGCGCGCTG-3'; apoLp-III₁₃₆₋₁₆₂ 5'-AGAGCGCGCTGTAGGAGGCCGCC-3'; apoLp-III₁₄₀₋₁₆₂ 5'-GCAGGAGGCCGCTAGAAGACCAAGGA-3'; apoLp-III₁₄₄₋₁₆₂ 5'-CCGAGAAGACCAAGTAGGCAGCGCCAAC-3'; apoLp-III₁₅₀₋₁₆₂ 5'-CAGCGGCCAACCTGTAGAACTCCATCCAG-3'; apoLp-III₁₅₄₋₁₆₂ 5'-TGCAGAACTCCATCTAGTCGGCCGTCCAG-3'; apoLp-III_{V131M} 5'-CGTGGGCTCCGATGCAGAGCGCG-3'. Template DNA (25 ng) and 125 µg of forward and reverse primers were used to generate the mutation in a thermocycler (PTC 200 Thermocycler, MJ Research, Inc.). The PCR product was incubated with 1 µL of DpnI restriction enzyme (10 units/µL) for 1 h at 37°C to cleave parental DNA, which was then used to transform XL 1-Blue supercompetent cells. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). To verify that the correct constructs of apoLp-III were obtained, plasmids were analyzed by DNA sequencing at the CSUPERB Microchemical Core Facility DNA Lab at California State University, San Diego.

Protein expression and purification

BL 21 (DE3) *E. coli* cells were transformed with wild-type and mutant *L. migratoria* apoLp-III constructs. The wild-type protein and helix-5 deletion mutants lack the N-terminal Arg-Pro segment, and thus resembles the apoLp-IIIb isoform (162 residues total) and amino acid numbering starts with the N-terminal aspartate. In addition, while the native protein is glycosylated, the recombinant protein lacks carbohydrate but this has no effect on the structure of the protein. A 25 mL overnight culture (with 50 µg/mL ampicillin) was prepared and used to seed 2 L minimal medium (47.75 mM Na₂PO₄, 22 mM KH₂PO₄, 8.65 mM NaCl, 18.7 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, and 13.3 mM glucose at pH 7.4) with 50 µg/mL ampicillin. The culture was grown for 2-3 h at 37°C in a shaking incubator at 220 rpm until the optical density at 600 nm reached a value of 0.6 (measured in a Shimadzu UV-2401PC UV-Vis spectrophotometer) after which over-expression was induced by 2 mM isopropyl-β-D-thiogalactopyranoside. The culture was grown for an additional 4 to 5 h, and cells were removed by centrifugation at 6,000 rpm for 15 min at 4°C using a Sorvall RC 5C Plus centrifuge with a FiberLite F14 rotor. Since the protein escapes the bacteria during over-expression, the supernatant was further processed and concentrated to 10 mL using a concentrator with a 10k membrane (Pall Corp.). The concentrated protein solution was purified by size-exclusion chromatography using a Sephadex G-75 column equilibrated in phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, pH 7.4). The

flow rate was set at 1 mL/min and elution was monitored by the absorbance at 280 nm. The apoLp-III peak was collected and further purified by reversed-phase HPLC (System Gold, Beckman Coulter) using a semi-preparative column (C8 Zorbax, Agilent, Santa Clara, CA). Proteins were eluted with a linear gradient of water and acetonitrile with 0.05% trifluoroacetic acid. The apoLp-III peak was collected and dried for 2 days using a Labconco freeze dryer, and stored at -20°C until further use. The purity of the protein preparation was verified with sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using Novex Tris-glycine 4-20% gradient gels (Invitrogen Co., Carlsbad, CA). For experimentation, proteins were dissolved in the appropriate buffer and the concentration determined by the bicinchoninic acid assay (Pierce Co., Rockland, IL) or the absorbance at 280 nm. The mass of the apoLp-III truncation variants was determined by matrix assisted laser desorption/ionization-time of flight spectroscopy (MALDI-TOF) using a sinapinic matrix (IIRMES core facility at CSU Long Beach).

Chemical modification of apoLp-III

Cyanogen bromide (CNBr, Sigma-Aldrich) was used to cleave V131M-apoLp-III into two fragments. Ten mg of the methionine apoLp-III variant was dissolved in 500 μ L of 70 % formic acid (Fluka Analytical Inc.), after which 500 μ L formic acid containing 11 mg of CNBr was added (100-fold molar excess).²³ The reaction was carried out for 16 h at 24°C and terminated by addition of 5 mL of water. The sample was freeze-dried, and SDS-PAGE was used to verify completion of the CNBr digestion. Cleaved protein was dissolved in 20 mM sodium phosphate buffer (pH 7.4) and the apoLp-III variant comprised of helix-1 to -4 was isolated by reversed-phase HPLC as described earlier.

ApoLp-III was cross-linked with dimethyl suberimidate (DMS, Pierce Co, Rockland, IL) to assess self-association. Eight μ L of DMS (20 mg/mL) in 1 M triethanolamine (pH 9.7) was added to 15 μ g of apoLp-III and incubated at room temperature for 3 h.¹⁰ The reaction was terminated by addition of 25 μ L SDS loading buffer, and subjected to 4-20 % SDS-PAGE.

Secondary structure analysis and fluorescence

For circular dichroism analysis, protein samples were prepared at concentration of 1 mg/mL in 20 mM sodium phosphate, pH 7.2. The protein concentration was determined by the absorbance at 280 nm. Scans were carried out in a JascoJ-810 polarimeter with circular cuvettes with a path length of 0.1 or 0.02 cm. Spectra were recorded between 185 and 260 nm at room temperature. The molar ellipticity $[\theta]$ was determined by the following equation: $[\theta] = (MRW \cdot \theta) / (10 \cdot d \cdot c)$, in which θ = ellipticity (in degrees), d = pathlength (cm), c = protein concentration in g/L, and MRW = the mean residue weight.²⁴ The helical content was estimated using the molar ellipticity at 222 nm: $(-[\theta]_{222 \text{ nm}} + 3,000) / (39,000)$.²⁵ Guanidine-HCl denaturation analysis was carried by incubating protein samples of 0.20 mg/mL in 20 mM sodium phosphate, pH 7.2 with varying guanidine-HCl concentration from 0 to 1.5 M. After 16 h to reach full equilibrium, the ellipticity was measured at 222 nm. Denaturation plots were generated and the concentration of guanidine-HCl to cause 50% protein unfolding was determined by sigmoidal curve fitting using Sigma Plot graphing software.

For tryptophan fluorescence emission analysis, apoLp-III (20 µg/mL, in PBS) was excited at 280 nm in a fluorescence spectrophotometer (Perkin Elmer LS 55) using a quartz fluorometer cuvette cell (10 mm pathlength, Starna Cell, Inc). Emission scans were obtained over the range of 290 to 440 nm with a scan speed of 50 nm/s.

Phospholipid solubilization

To measure the rate of phospholipid vesicle solubilization, large unilamellar vesicles (LUV) were prepared by extrusion.²⁶ Five mg of dimyristoylphosphatidylcholine (DMPC, Avanti Polar Lipids, Birmingham, AL) was dissolved in 1 mL of 3:1 CHCl₃/CH₃OH (v/v), and dried under a stream of N₂ and placed under vacuum for at least 4 h. The lipid film was rehydrated in 1 mL of PBS, pH 7.0 and incubated for 1 min at 37°C following by vigorous vortexing for 1 min to form multilamellar vesicles. LUVs were made by passing the multilamellar vesicles 15 times through a 200 nm Whatman Nucleopore Track-Etch membranes in a mini-extruder (Avanti Polar Lipids, Alabaster, AL) equilibrated at 30 °C. To measure the rate of vesicle solubilization, protein and DMPC LUVs were mixed and incubated in a 1 mL cuvette at 24°C in a Shimadzu 2401 spectrophotometer. The temperature was maintained in a Peltier temperature-controlled cuvette holder (TCC-240A). The change in light scatter of the protein-lipid suspension was measured by following the absorbance at 325 nm. The first-order rate constants (k) were calculated using the equation: $A_t = A_0 e^{-kt}$, where A_t = absorbance at time t, A_0 = absorbance at t=0.

Lipoprotein Binding

Human low density lipoprotein (LDL, 100 µg protein, Intracel, Frederick, MD) was incubated with 160 mU of phospholipase-C from *Bacillus cereus* (Sigma Co., St. Louis, MO) in the presence of 0, 15, or 100 µg of apoLp-III in 50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂ at pH 7.5.²⁷ The reaction was carried out at 37°C for 65 min in a 96 microwell plate with a total volume of 200 µL. LDL aggregation was monitored by measuring the absorbance at 340 nm in Varioscan plate reader (Thermo Electron Corporation, Milford, MA). The % LDL protection was calculated using the following equation: % LDL protection = (A_{340} in the presence of apoLp-III / A_{340} no apoLp-III) × 100.

Results

Design and production of truncation variants

Two terminal helix deletion variants were designed by deleting the N- or C- terminal helix in separate mutant proteins, see Fig 1A. To create the N-terminal deletion variant apoLp-III_{H2-H5}, the DNA coding region of helix-2 to -5 (amino acid residues 30-162) was amplified by PCR and inserted into the pET-22b(+) expression vector. Similarly, the apoLp-III_{H1-H4} construct was created by amplifying the DNA coding region of amino acid residues 1-131. DNA sequencing of the constructs showed that both helix deletion mutants were successfully engineered. Attempts were made to over-express the deletion mutants in a well-established bacterial expression system, in which the protein escapes the bacteria and accumulates into the culture medium.²⁸ SDS-PAGE analysis shown in Fig. 2 demonstrated that apoLp-III_{H2-H5} was produced in *E. coli* cultures with a yield of 10 to 12 mg/L, which is significantly below the typical yield of the wild-type protein (apoLp-III_{WT}, ~30 mg/mL).

The apoLp-III_{H1-H4} construct failed to produce detectable amounts of protein neither in the culture medium nor in the cell pellet, thus it was necessary to employ an alternative strategy to obtain the desired variant. Therefore, a methionine residue was inserted into the loop between helix-4 and -5 in place of valine 131. Since apoLp-III_{WT} is devoid of methionine, this provides a convenient target site for proteolytic cleavage. The apoLp-III_{V131M} variant was expressed and purified with a yield comparable to apoLp-III_{WT} (~25 mg/L). CNBr treatment of apoLp-III_{V131M} resulted into two fragments, apoLp-III_{H1-H4} and a small fragment comprised of helix-5 residues. The reaction was ~ 90 % complete, and apoLp-III_{H1-H4} was isolated by reversed-phase HPLC (Fig. 2). MALDI-TOF analysis showed that the masses of the purified truncation variants were within 25 Da of the theoretical mass based on the amino acid sequence and thus the desired truncation apolipoproteins were successfully produced.

The lack of apoLp-III_{H1-H4} in the culture medium indicated that the protein structure has been compromised because of helix-5 removal. To identify the critical residues in helix 5, five truncation variants were engineered by inserting stop codons at various positions in the DNA coding sequence of helix 5. All of the residues selected for stop codon replacement were hydrophilic (glutamine or glutamate) and facing outwards to minimize perturbation of the hydrophobic helix interior (Fig. 1B). The following deletion variants were generated: apoLp-III₁₃₆₋₁₆₂, ₁₄₀₋₁₆₂, ₁₄₄₋₁₆₂, ₁₅₀₋₁₆₂, and ₁₅₄₋₁₆₂. No measurable amounts of protein could be purified from expression cultures of apoLp-III₁₃₆₋₁₆₂ and apoLp-III₁₄₀₋₁₆₂. However, apoLp-III with shorter deletions could be successfully produced (apoLp-III₁₄₄₋₁₆₂, ₁₅₀₋₁₆₂, ₁₅₄₋₁₆₂) with yields of ~10 mg/L. Mass spectrometry verified the design and production of the correct variants.

Secondary structure analysis

The circular dichroism spectrum of apoLp-III is characteristic for α -helical proteins, with two prominent troughs at 208 and 222 nm and a positive peak at 192 nm. The circular dichroism plots of the helix truncation mutants apoLp-III_{H1-H4} and apoLp-III_{H2-H5} showed the proteins contained α -helical character but the decreased signal intensity compared to apoLp-III_{WT} indicated a significant loss of secondary structure (Fig 3). Calculations based on the ellipticity at 222 nm showed that the helical content for apoLp-III_{H1-H4} and apoLp-III_{H2-H5} was 32% and 44%, respectively. This is considerably lower than the 70% calculated for apoLp-III_{WT}. Helical contents of the variants with partial deletions of helix five showed decreased helical contents: 32% for apoLp-III₁₄₄₋₁₆₂, 36% for apoLp-III₁₅₀₋₁₆₂ and 41% for apoLp-III₁₅₄₋₁₆₂. As expected, the longer the helical segment of helix-5, the more helical character the protein retained. However, truncation at position 153 did not restore the helicity of the protein to values found in full-length apoLp-III. Thus, removal of 10 amino acids, most of them lying outside the helix 5 boundary, still caused a 30% reduction in α -helical content.

Guanidine-HCl denaturation

The proteins were denaturated with guanidine-HCl to measure the effect of the truncations on protein stability. Protein samples were incubated with increasing denaturant concentrations after which the ellipticity at 222 nm was measured to reveal the denaturant-

induced decrease in α -helical structure. Plots of the change in secondary structure versus the guanidine-HCl concentration were generated, from which the midpoint of denaturation $[\text{Gdn-HCl}]_{1/2}$ was determined. This value indicates the resistance to denaturation, and provides a measure for protein stability. A significant decrease in the resistance to denaturation was observed for the two helix deletion variants, with $[\text{Gdn-HCl}]_{1/2}$ values of 0.29 and 0.19 M for apoLp-III_{H1-H4} and apoLp-III_{H2-H5}, respectively. This is significantly lower than the $[\text{Gdn-HCl}]_{1/2}$ of 0.50 M observed for apoLp-III_{WT} (Fig. 4). As expected, the partial C-terminal helix deletion variants also displayed less resistance to guanidine-induced denaturation, with $[\text{Gdn-HCl}]_{1/2}$ values in the range of 0.30 M (Table 1).

Tertiary structure and oligomerization

Tryptophan emission scans of the apoLp-III variants were obtained to determine the wavelength of emission maximum (λ_{max}), which provides information of tryptophan exposure to the aqueous environment. Two tryptophan residues reside in apoLp-III: one buried in helix 4 and a second more exposed residue in the loop linking helix 4 to 5 and is partially exposed.²⁹ Both tryptophan residues were retained upon generation of the truncation variants. Deletion of helix-1 led to a large redshift from 334 nm (apoLp-III_{WT}) to 349 nm, while the redshift was more pronounced upon removal of helix-5 (355 nm) (Fig 5). The λ_{max} from the partial helix deletion mutants depended on the extent of deletion, and ranged from 351 (apoLp-III₁₅₄₋₁₆₂) to 354 nm (apoLp-III₁₄₄₋₁₆₂). Thus, in all deletion mutants, tryptophan residues were increasingly more exposed to water, implying decreased tertiary contacts. Since the redshift was more pronounced in the helix-5 deletion variants, up to 21 nm, helix perturbation may have reached a critical threshold and prevented over-expression of apoLp-III_{H1-H4}.

Previous studies of apoLp-III from *Galleria mellonella* in which two helices were removed to generate three different “three-helix” proteins, resulted in variants with decreased helical content and protein stability.³⁰⁻³¹ These protein constructs displayed a high degree of dimers and trimers of this otherwise monomeric protein. Therefore, self-association of the *L. migratoria* apoLp-III variants was assessed using DMS cross-linker. However, only a marginal increase in the appearance of dimers was observed for the deletion variants (Fig. 6).

Phospholipid vesicle solubilization

The ability of apoLp-III to solubilize DMPC bilayer vesicles forming small discoidal particles was used as a measure for lipid binding. This transformation from vesicles into discs occurs mainly at the phosphatidylcholine gel/liquid/crystalline transition temperature (T_M), and practically limits this assay to myristylated phosphatidylcholine with a T_M of 24°C. The starting vesicle dispersion had a cloudy appearance because of the heavy light scatter caused by LUVs. Induced by addition of apolipoprotein, LUVs were converted into discoidal particles with a diameter of ~15 nm and thus light scatter intensity was greatly reduced, allowing to monitor the transformation using absorbance spectroscopy. Using a 1:1 ratio of apoLp-III and DMPC (w/w) the rate of transformation was fast for the helix deletion mutants clearing the vesicle dispersion in ~200 s, while apoLp-III_{WT} light scatter was reduced by only 20% at this time point (Fig. 7). Similar results were obtained for the partial

C-terminal helix truncated variants. Since the transformation rate was too fast to detect potential differences among the variants, the amount of protein was reduced to a protein to lipid ratio of 1:10 (w/w) at which the rate of conversion remained sufficiently high to accurately measure the transformation rate. ApoLp-III_{H1-H4} and apoLp-III_{H2-H5} displayed faster transformation rates, while the partial helix 5 truncation variants showed a somewhat decreased ability to transform the vesicles albeit still significantly better than the wild-type protein (see Table 1 for rate constants).

Lipoprotein binding

ApoLp-III does not normally bind to human LDL. However, when the LDL surface is incubated with phospholipase-C, surface phosphatidylcholine is converted to DG. The appearance of DG on LDL causes packing defects leading to lipoprotein aggregation. However, surface DG attracts apoLp-III, which main function is to bind and stabilize DG-enriched lipophorins.⁶ Upon apoLp-III binding, the LDL surface is protected and aggregation either prevented or delayed. The ability to prevent LDL aggregation was used to measure lipoprotein binding activity of apoLp-III.²⁷ As shown in Fig. 8, PL-C treated LDL rapidly aggregated evident by a steady increase in sample turbidity, which was monitored by the absorbance at 340 nm for 60 min. To test the ability of the helix deletion variants to delay or prevent the onset of LDL aggregation, LDL and phospholipase-C were simultaneously incubated with 100 µg of the apoLp-III variants. This showed that all proteins were able to provide full protection for the first 45 min, after which the absorbance started to increase. The absorbance values of all variants were similar, although apoLp-III_{H2-H5} appeared to have a somewhat lower activity. Since it was difficult to differentiate between the activities of the various mutant proteins, a limited amount of protein (15 µg) was used. The absorbance values were then used to calculate the % of protection at the 50 min time point. This showed that most variant proteins and apoLp-III_{WT} provided partial protection between 54 and 74 %. The protection of apoLp-III_{H2-H5} was lower compared to the wild-type protein (Table 1), confirming our initial results with 100 µg apoLp-III.

Discussion

All truncation variants engineered in the current study displayed significant loss of structure. Upon removal of the complete N- or C-terminal helix, the α -helical content decreased from 70% for apoLp-III WT, to 44% for apoLp-III_{H2-H5} and 32% for apoLp-III_{H1-H4}. In addition, the stability of these deletion variants reduced significantly, as indicated by the smaller amount of denaturant needed to unfold the protein. ApoLp-III is a marginally stable protein with a free energy of unfolding of ~2 kJ/mol, substantially less than most 4-helix bundle proteins, and may be quite sensitive to amino acid changes in particular when residues are involved in helix packing.^{3, 32-34} Fluorescence emission scans showed increased exposure of both tryptophans that reside in the protein, and the largest red-shift was observed when helix-5 was removed. This result suggests decreased helical contacts for both deletion variants, in particular for apoLp-III_{H1-H4}. Thus, while the stability of apoLp-III_{H1-H4} may be slightly higher, apoLp-III_{H2-H5} has a higher helical content and tertiary structure. While differences in biophysical characteristics between the two variants were observed, it is evident that removal of either helix caused major alterations in protein

structure. When designing the helix deletion variants, it was anticipated that the remaining helices would reorient themselves to allow formation of a stable 4-helix bundle. The five α -helices have similar hydrophobic moments, mean hydrophobicity, and the previously published helical wheel diagram indicate the feasibility to remove one terminal helix³⁵. However, helical content decreased dramatically and stability was significantly reduced, probably because of helix packing defects, suggesting that the protein did not adopt the classic 4-helix bundle fold.

Changes in secondary and tertiary structure likely affected the expression yields. While the apoLp-III_{H2-H5} variant was produced in reduced but adequate quantities, the apoLp-III_{H1-H4} variant could not be isolated from media of *E. coli* cultures. This variant was ultimately generated by introducing a unique methionine between helix-4 and-5, and upon expression, the purified protein was treated with CNBr to release the desired 4-helix protein. While the helix-5 truncation variants apoLp-III₁₃₆₋₁₆₂, ₁₄₀₋₁₆₂ could not be produced either, other variants with smaller truncations starting from amino acid position 144 were successfully expressed and purified from *E. coli* cultures. The lack of protein expression of apoLp-III_{H1-H4} indicates that helix-5 is critical for maintaining the structural integrity of the protein, and does not allow for much manipulation. In another study, variants lacking two successive N- or C-terminal helices of *G. mellonella* apoLp-III were produced in high quantities in an identical bacterial expression system.³⁰⁻³¹ While these variants also displayed a much lower helical content and protein stability, abundant formation of dimers and trimers may have compensated for the decrease in structure by sequestering hydrophobic residues. Therefore, self-association properties of the deletion variants in *L. migratoria* were analyzed, but showing no evidence of a substantial increase in oligomerization. Thus, exposure of unstructured sections of the *L. migratoria* apoLp-III variants produced in the current study may have contributed to lower expression yields or even in failure to produce any detectable amounts of recombinant protein.

Despite the decrease in secondary and tertiary structure, all truncation variant proteins were still able to maintain functionality. The variants were able to bind to the lipolyzed LDL surface and prevent lipoprotein aggregation. This indicates that the intrinsic ability to bind to the lipoprotein surface by the remaining amphipathic α -helical segments was not affected. Even more, removal of the N- or C-terminal helices resulted in a significant increase in binding to phospholipid vesicles. Rates of transformation of vesicles into discoidal particles were increased five-fold for apoLp-III_{H1-H4}, _{H2-H5}, and about four-fold for the helix-5 truncation variants. Similar results were reported for the two-helix truncation variants of *G. mellonella* apoLp-III.³⁰⁻³¹ The increase in phospholipid binding activity is in good agreement with previous studies where it was shown that helix bundle stability inversely correlates with phospholipid vesicle solubilization.³⁶⁻⁴⁰ Conceivably, the equilibrium between open and closed helix bundle states may have shifted to a more open conformation, leading to increased exposure of hydrophobic regions of the amphipathic α -helices, thereby promoting lipid binding. The helical content of the variants decreased substantially which should have led to some degree of disruption of the non-polar faces of the amphipathic α -helices. Nevertheless, other studies have shown that such unstructured regions still maintain the ability to adopt an α -helical conformation in the proper environment.³⁵

It was intriguing to observe that the protein without helix-5 (ending at amino acid residue 131) could not be expressed in bacterial cell cultures. Many apoLp-III mutants have been engineered in our laboratory and by others, however, this was the first time we were not able to produce an apoLp-III variant. Partial truncation mutant analysis showed that helix-5 could be truncated until residue 144, however, the product was an unstable protein with properties similar to apoLp-III_{H1-H4}. This implies that the protein was able to tolerate removal of residues 144-162 with the remaining helix-5 residues providing sufficient structure for helix-helix contacts for protein folding. Nevertheless, helical content decreased and $\lambda_{\max}$ increased for removal of each consecutive helical segment until a critical value was reached after which the protein may have become too unstable and could not be produced.

Helix-2 and -5 are considered class A amphipathic α -helices³⁵, which are known for their high propensity to bind lipids.⁴¹⁻⁴² Removal of helix-5 did not result in decreased lipid binding, on the contrary, LDL binding remained similar to apoLp-III_{WT} while phospholipid vesicle solubilization increased. Helix-2 is another class A amphipathic α -helix which may have compensated for any lipid binding activity lost in the absence of helix-5. Helix-1 displayed characteristics of class G* α -helix: a high hydrophobic moment and hydrophobicity, a random distribution of charged residues, and lower or no lipid binding properties.³⁵ Thus, loss of this α -helix may not affect the lipid binding capabilities of apoLp-III, explaining, the results of the lipid binding analysis. While the characteristics of both terminal α -helices were different, neither of them are critical for lipid binding activity of apoLp-III.

When *L. migratoria* apoLp-III was cleaved into two approximately equal halves by proteolysis, both fragments were devoid of secondary structure.³⁵ The fragments lacked LDL binding and were not able to spontaneously transform phospholipid vesicles into discoidal particles, although co-sonication could force the protein into a lipid-bound conformation. In addition, the random-coiled protein could be converted into a highly α -helical protein when incubated in the presence trifluoroethanol. This implied the protein needs some level of secondary structure to form intramolecular helical contacts to be able to associate with lipid surfaces. In our current study, the deletion variants may have sufficient secondary and tertiary structure to preserve the ability to interact with lipid and lipoprotein surfaces. Since vesicle solubilization was much faster when one of the terminal helices was removed, this provides further support for the proposed critical role these terminal helices play in lipid binding.^{43, 44} Furthermore, amino acid changes in helix-1 in *G. mellonella* apoLp-III resulted in increased protein stability and lowered the ability to bind to lipid vesicles, in agreement with the critical role of helix-1 in initiation of lipid binding.⁴⁵ Other studies with apoLp-III from three different species in which the protein was tethered by introduction of a disulfide bond between helix-1 and -5 rendered the protein ineffective for phospholipid solubilization.^{12, 44, 46} Thus there is ample evidence that helix -1 and -5 are critical to keep the protein in a lipid-free closed conformation. The relatively weaker inter-helical contacts between helix-1 and -5 compared to other helical contacts facilitate separation of the terminal helices as a first step towards the open, lipid-bound conformation.^{43, 44} In the absence of one of the terminal helices, the protein is in a more loosely folded “active” conformation. This resembles the pH-induced molten globule-like state of apoLp-III, which displayed a much increased lipid binding activity.^{36, 47} The

loosely folded state of the helix deletion variants facilitate the more dramatic conformational change in the presence of a suitable lipid surface, see Fig 9. This involves major helical rearrangements, in which the protein switches to a fully open conformation allowing buried hydrophobic faces of all amphipathic α -helices making contact with the lipid surface.^{34, 48} This mechanism also provides a functional explanation of why removal of one of the terminal helices did not result in a stable 4-helix bundle protein as this conformation may not promote opening of the helix bundle to allow lipid binding. Thus, the terminal α -helices facilitate the conformational switch, promoting helix separation to allow reorientation to the new lipid surface. Similarly, N- and C-terminal truncation variants of human apoA-I adopt an extended helical conformation, presumably resembling a lipid bound state.^{49, 50}

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Abbreviations

apoLp-III

apolipophorin III

CNBr	cyanogen bromide
DG	diacylglycerol
DMS	dimethyl suberimide
DMPC	dimyristoylphosphatidylcholine
[Gdn-HCl]_{1/2}	midpoint of guanidine-HCl denaturation
λ_{max}	wavelength of emission maximum
LDL	low density lipoprotein
LUV	large unilamellar vesicles
MALDI-TOF	Matrix assisted laser desorption/ionization-time of flight spectroscopy
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T_M	transition temperature

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Figure 1.

Panel A: X-ray structure of *L. migratoria* apoLp-III. The coding region of helix 1 (light blue) was removed to engineer apoLp-III_{H2-H5}, while removal of helix 5 (blue) of the intact protein created apoLp-III_{H1-H4}. Panel B: Close-up of helix-5 showing amino acid residues targeted for translation termination by introducing stops in the DNA coding region generating truncation variants with varying length to study the effect of helix-5 truncation.

Figure 2. SDS-PAGE of the isolation and purification of the helix deletion variants. Shown are apoLp-III_{H2-H5}, and apoLp-III_{H1-H4} which was generated by expressing and purification of apoLp-III_{V131M} followed by CNBr cleavage and HPLC purification.

Figure 3.

Secondary structure analysis of apoLp-III deletion variants. Ellipticity of apoLp-III was measured between 185 and 260 nm. Upon deletion of one of the terminal helices, a significant decrease in helical content was observed as shown in the Far-UV circular dichroism spectra of apoLp-III_{WT} (solid line), apoLp-III_{H1-H4} (dashed line) and apoLp-III_{H2-H5} (dotted line).

Figure 4.

Protein denaturation by guanidine-HCl. ApoLp-III deletion variants were incubated in the presence of increasing concentrations of guanidine-HCl followed by measurement of the ellipticity at 222 nm. The % change in ellipticity was plotted against the denaturant concentration. The denaturation profile of apoLp-III_{WT} (filled circles), apoLp-III_{H1-H4} (filled triangles) and apoLp-III_{H2-H5} (open circles) demonstrate that removal of one of the terminal helices significantly decreases the stability of the protein as it is less resistant to denaturant induced unfolding. Error bars represent the standard deviation (n=3).

Figure 5.

Trp fluorescence emission spectra. The protein samples were excited at 280 nm and emission monitored between 290 and 440 nm, showing apoLp-III WT (solid line), apoLp-III H1-H4 (dashed line), and apoLp-III H2-H5 (dotted line), showing different λ_{max} values for the deletion variants, implying increased exposure of tryptophan to the aqueous environment.

Figure 6. Crosslinking of apoLp-III. Proteins were cross-linked with DMS and separated by SDS-PAGE to determine the presence of oligomers. The wild-type protein showed a small amount of dimers, which increased slightly in the helix deletion variants.

Figure 7.

Apolipoprotein-induced vesicle solubilization. ApoLp-III was incubated with DMPC LUVs using a 1:1 lipid to protein weight ratio. The solubilization of vesicles thereby forming small discoidal complexes was followed by changes in the light scatter intensity measured by the absorbance at 325 nm. Shown are apoLp-III_{WT} (solid line), apoLp-III_{H1-H4} (dashed line) and apoLp-III_{H2-H5} (dotted line), revealing a much higher rate of discoidal complex formation for the helix deletion variants compared to wild type protein.

Figure 8.

ApoLp-III lipoprotein binding. LDL was treated with phospholipase-C to create apoLp-III binding sites on the LDL surface. Incubations in the absence of apoLp-III led to rapid aggregation, as seen by the increase in sample turbidity (closed squares). Inclusion of the apoLp-III deletion variants delayed the onset of aggregation to 50 min: apoLp-III_{WT} (filled circles), apoLp-III_{H1-H4} (filled triangles) and apoLp-III_{H2-H5} (open circles). Error bars represent the standard deviation (n=3).

Figure 9.

Model of apoLp-III helix bundle opening when binding to lipids. When helix-1 and -5 separate, increased exposure of the helix bundle interior allow for lipid-protein interactions, resulting in a major rearrangement of the amphipathic α -helices. Removal of one of the terminal helices creates a more loosely folded protein, facilitating lipid binding.

Table 1
Summary of biophysical and functional parameters of apoLp-III

ApoLp-III	[GdnHCl] _{1/2} (M)	α -helix (%)	Vesicle solubilization rate (10^{-3} s^{-1})	% LDL protection
WT	0.50 ± 0.00	70.1 ± 3.8	0.55 ± 0.12	64.5 ± 9.9
H1-H4	0.29 ± 0.04	32.3 ± 5.5	3.14 ± 0.60	57.6 ± 8.3
H2-H5	0.19 ± 0.02	44.3 ± 1.1	2.85 ± 0.04	32.3 ± 4.2
144-162	0.26 ± 0.08	32.1 ± 2.7	2.23 ± 0.08	53.8 ± 7.5
150-162	0.30 ± 0.06	36.4 ± 2.7	1.38 ± 0.07	62.5 ± 9.4
154-162	0.31 ± 0.03	41.2 ± 8.7	2.28 ± 0.51	76.6 ± 14.8

For determining the vesicle solubilization rate a protein to lipid ratio of 1:10 (w/w) was used, while 15 μg protein was used for the % LDL protection.