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Helix 1 tryptophan variants in *Galleria mellonella* apolipoprotein III

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Abstract

Apolipoprotein III (apoLp-III) from *Galleria mellonella* is a critical apolipoprotein aiding in lipid transport and has gained considerable interest for a role in innate immunity. Both functions are likely related and form the rationale to gain a more detailed understanding of the lipid binding properties of this insect apolipoprotein. Tryptophan residues were introduced at positions 16, 20 or 24, all in helix 1 as it may play a critical role in the initial steps of lipid binding. Steady-state fluorescence analysis showed that each tryptophan displayed unique properties, indicating different environments both in lipid-free as in lipid-bound states, and demonstrating potential for use in lipid binding analysis. While α -helical contents of wild-type and the tryptophan variant proteins were similar, W20- and W24-apoLp-III displayed increased protein stability. These variants were significantly slower in their ability to convert phosphatidylcholine vesicles into discoidal lipoproteins, which was employed as a measure for lipid binding. In contrast, W16-apoLp-III displayed decreased protein stability but an order of magnitude higher rate of discoidal lipoprotein formation. This demonstrates an inverse correlation between protein stability and the ability to convert vesicles in discoidal lipoproteins. The most stable W20-apoLp-III variant displayed compromised LDL binding capabilities, indicating a partial loss of function. Thus, there is a delicate balance between helix bundle stability and the ability to bind lipids, and helix 1 may play a critical role in this process.

Graphical Abstract

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Keywords

Apolipoprotein; apolipophorin; tryptophan fluorescence; circular dichroism; protein-lipid interaction

1. Introduction

Apolipophorin III (apoLp-III) is a small protein that plays an essential role in lipid transport processes in several insect orders [1]. The protein is well characterized in locusts and sphinx moth for which high resolution structures are available [2–4]. The one-domain organization, availability of high-resolution structure, and monomeric nature make this an excellent model apolipoprotein and insights derived from this protein have significantly contributed towards a better understanding of apolipoprotein function in other animal systems [5]. The lipid-free protein is made of a bundle of five amphipathic α -helices, the structural elements responsible for lipid binding [6]. The helix bundle undergoes a dramatic conformational change to allow binding to hydrophobic surfaces [7–10]. ApoLp-III is a marginally stable protein, and this feature likely facilitates opening of the helix bundle allowing binding to lipid surfaces [3, 11]. It has been suggested that repositioning of helix-1 and helix-5 are the first steps of lipid binding, after which the protein unfurls on the hydrophobic surface [12]. Indeed, helix tethering studies by introduction of cysteine residues in the N- and C-terminal α -helices renders the protein ineffective for vesicle solubilization, which has been used to assess lipid binding properties [10, 12–13]. ApoLp-III from the greater wax moth, *Galleria mellonella*, is often used to investigate the role in innate immunity [14–17]. However, lipid binding properties of *G. mellonella* apoLp-III are not well studied despite that this is likely related to the function in innate immunity [18]. For example, when binding of apoLp-III to lipopolysaccharides (LPS) was characterized, a stronger binding interaction was observed if the lipid A portion was present [19], implying the importance of hydrophobic interactions in the LPS binding interaction. In the current study we focused on the first helix of *G. mellonella* apoLp-III, and engineered three variants in which a hydrophobic residue was changed into tryptophan at a different position in helix-1. These newly introduced tryptophan residues can then be employed as intrinsic fluorescent markers to gain insight in the interaction of the protein with hydrophobic surfaces. While these mutations were considered conservative and similar in nature, substantial differences were observed in the tryptophan fluorescence properties and protein stability.

2. Experimental procedures

2.1. Protein expression, purification and mutagenesis

ApoLp-III was expressed and purified in a well-established bacterial recombinant protein expression system as described in detail previously [20]. This system routinely produces more than 50 mg pure protein per L culture medium. Recombinant apoLp-III escapes the bacteria and is purified from the minimal medium used to culture the bacteria by size-exclusion chromatography and reversed-phase HPLC. The purity of each protein preparation was verified by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and native PAGE using 4–20 % precast gels (Life Technologies, Grand island, NY); freeze-

dried proteins were stored at -20°C . Prior to experimental analysis, proteins were dissolved into the appropriate buffer, after which the protein concentration was determined by the absorbance at 280 nm and/or the Pierce bicinconinic acid assay. Site-directed mutagenesis was employed to introduce tryptophan residues in helix-1. Complementary primer pairs were designed (Eurofins Genomics, Huntsville, AL) and the desired mutation was introduced in the apoLp-III pET-22b plasmid using the Quick-change site-directed mutagenesis kit (Agilent Technologies, Santa Clara CA). The following primers were used to generate the tryptophan mutant proteins; the tryptophan codon is shown in bold. F16W 5'-GGAGAAGCACGCCGCCGAGT**GGC**AGAAGACGTTTCAGTGAGC-3' (forward) and 3'-CCTCTTCGTGCGGCGGCTC**ACCGT**CTTCTGCAAGTCACTCG-5' (reverse); F20W 5'-CGAGTTCCAGAGACGT**GGAGT**GAGCAGCTGAACG-3' (forward) and 3'-GCTCAAGGTCTTCTGC**ACCTC**ACTCGTCACTTGC-5' (reverse); L24W 5'-CGTTTCAGTGAGCAGT**GGAACG**CTTCACCAACTCG (forward) and 3'-GCAAGTCACTCGTCA**CCCTT**GCGCAAGTGGTTGAGC-5' (reverse). The DNA sequence of each mutated apoLp-III construct was verified using T7 promoter and termination primers (Genewiz, San Diego, CA).

2.2. Circular dichroism

Protein samples in 20 mM sodium phosphate buffer (pH 7.2) were recorded from 185 to 260 nm in a Jasco 810 polarimeter using a concentration of 0.20 mg/mL in a circular cuvette with a 1 mm pathlength. The ellipticity was converted into molar ellipticity as reported earlier [21]. To measure the protein stability, protein samples (0.20 mg/mL) were denatured in a range of 0 to 1.2 M guanidine HCl, incubated for 24 h at room temperature, after which the ellipticity was recorded at 222 nm. Ellipticity values were converted to % change assuming 100% unfolding of apoLp-III at a 1.2 M guanidine HCl concentration. To calculate the stability of the protein, the free energy of unfolding ($\Delta G_D^{\text{H}_2\text{O}}$) was determined according to Pace by plotting the guanidine-HCl concentration against the free energy of unfolding in the transition phase and extrapolating to 0 M guanidine-HCl [22].

2.3. Fluorescence

Tryptophan fluorescence was measured in a Perkin-Elmer LS 55B spectrofluorometer. Proteins were dissolved in phosphate buffered saline (PBS); 150 mM NaCl, 10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , pH 7.4. Samples containing 50 μg protein in 1 mL were excited at 280 nm, and the emission was measured between 300 and 450 nm using a 3 nm slit width. For tryptophan quenching with KI, a 4 M KI stock solution was prepared with 1 mM $\text{Na}_2\text{S}_2\text{O}_3$ to prevent formation of free iodine, and was titrated into a 50 $\mu\text{g}/\text{mL}$ apoLp-III solution in PBS. The emission intensity was corrected for dilution, and analyzed by use of the Stern-Volmer equation $F_0/F = 1 + K_{SV} [Q]$, in which F_0 and F represent emission intensities in the absence or presence of quencher Q , respectively. The Stern-Volmer constant K_{SV} was obtained from the slope of F_0/F versus quencher concentration as described [23].

2.4. Phospholipid vesicle solubilization

To prepare unilamellar vesicles, 10 mg of dimyristoylphosphatidylcholine (DMPC), purchased from Avanti Polar Lipids (Alabaster, AL), was dissolved in chloroform methanol (1:3 ratio by volume). The solvent was evaporated under a gentle stream of N₂ followed by 3 h incubation under vacuum. The dried thin lipid film was rehydrated in 1 mL PBS at 37 °C under vigorous vortexing. The sample was then extruded >15 times in a mini extruder (Avanti Polar Lipids, Alabaster, AL) at 37 °C using 200 nm membranes. The freshly prepared large unilamellar vesicles (LUV) were used the same day to measure the rate of protein-induced conversion into discoidal lipid-protein complexes. To 500 µg phospholipid, 500 µg apoLp-III was added in a total volume of 1 mL, and the light scatter intensity was recorded in a Beckman spectrophotometer. The incubation temperature was maintained at 24 °C using a Peltier unit and the absorbance wavelength was set at 325 nm and monitored for 60 min. All samples were equilibrated at the incubation temperature before mixing.

2.5. Low density lipoprotein aggregation assay

Low density lipoprotein (LDL) was used to assess the binding of apoLp-III to lipoprotein surfaces [24]. Fifty µg LDL protein (Sigma-Aldrich, St. Louis, MO) was incubated at 37°C with 2 mU of phospholipase C (Sigma-Aldrich, St. Louis, MO) in 20 mM Tris-HCl, 2 mM CaCl₂, 150 mM NaCl, pH 7.4, in a total volume of 200 µL. Incubations were carried out in the presence or absence of 25 or 50 µg apoLp-III. The change in sample turbidity as a result of phospholipase-C induced LDL aggregation was monitored by the absorbance at 340 nm in a microplate reader (Thermo Labsystems, Helsinki, Finland) for 60 min.

3. Results

3.1. ApoLp-III tryptophan mutant engineering

G. mellonella apoLp-III is a 163 amino acid residue protein that does not contain tryptophan. A single highly quenched tyrosine residue is present in helix 5 (Tyr-142); the fluorescence properties of this unique residue have been exploited to measure binding to lipid and LPS. Upon binding interaction the fluorescence intensity dramatically increases due to relocation of tyrosine into a new microenvironment [19, 20]. Protein conformational changes likely facilitate this change as has been shown for the highly similar *Manduca sexta* apoLp-III when binding to phosphatidylcholine vesicles [7, 25]. Nevertheless, tyrosine fluorescence emission intensity is relatively weak, making it challenging for use as an intrinsic probe especially when only small amounts of protein are used, i.e. to reduce light scatter. Tryptophan fluorescence intensity is much stronger and predictions of the polarity of the tryptophan environment can be directly made from the wavelength of maximum emission (λ_{max}) [26]. To introduce tryptophan in the protein, three hydrophobic amino acid residues in helix-1 were selected, each positioned four residues apart: Phe-16, Phe-20, and Leu-24. These residues are part of a helical sequence (₁₅EFQKTFSEQLN₂₅) almost identical to the corresponding sequence in *M. sexta* apoLp-III (₁₈EFQKTFSEQFN₂₈). The only exception is Leu-24, which is phenylalanine in *M. sexta* apoLp-III. The position of the three target amino acids are shown in Figure 1 using the NMR solution structure of *M. sexta* apoLp-III (PDB ID 1EQ1, no high resolution structure is currently available for *G. mellonella* apoLp-III) [3]. The *M. sexta* apoLp-III structure indicates that Phe-20 and Phe-24

are buried, while Phe-16 is more exposed. The three mutant proteins, W16-, W20-, and W24-apoLp-III were generated by site-directed mutagenesis, expressed in *E. coli*, and purified to homogeneity by size-exclusion chromatography and reversed-phase HPLC.

3.2. Tryptophan fluorescence

Tryptophan fluorescence emission scans of the three single tryptophan mutants showed large differences in intensity and λ_{max} values (Figure 2). Emission intensities of W20- and W24-apoLp-III were an order of magnitude higher compared to W16-apoLp-III. The λ_{max} of 327 nm of W20-apoLp-III indicates a location in the hydrophobic environment of the protein interior (Table 1). In contrast, emission spectra of both W16- and W24-apoLp-III were red-shifted with λ_{max} values of 348 nm, indicating exposure to the aqueous environment. The accessibility of tryptophan to the KI quencher was also determined. Stern-Volmer plots yielded relatively low Stern-Volmer constants for all variants in the order of ~ 1 . Nevertheless, W16-apoLp-III was the most accessible, followed by W24- and W20-apoLp-III (Table 1). When SDS was added to the protein samples to mimic a lipid environment, this induced significant changes in λ_{max} values and emission intensity. The emission intensity of W16-apoLp-III increased 2.6 times, with a large 21 nm blue-shift in λ_{max} . No significant change in λ_{max} was found for W20-apoLp-III, however, the emission intensity was reduced 2.3 fold. The reduction in emission fluorescence intensity of W24-apoLp-III was 8-fold, and the λ_{max} could not be determined.

3.3. Secondary structure and protein stability

The Far-UV circular dichroism spectra of the tryptophan variants and wild-type apoLp-III displayed similar plots with distinct troughs at 208 and 222 nm typical for α -helical proteins (Figure 3). The α -helical contents were calculated and were between 65 and 70%; the differences between mutant and wild-type apoLp-III were relatively small and within 5% of the wild-type protein (Table 1). This indicates that the secondary structure of the mutant proteins was not affected upon introduction of tryptophan in helix-1. The stability of apoLp-III was determined by measuring the resistance to guanidine-HCl induced denaturation (Figure 4). The midpoint of denaturation, $[\text{Gdn-HCl}]_{1/2}$, was used as a measure of the stability which was 0.330 M for wild-type apoLp-III. A decrease in protein stability was observed for W16-apoLp-III as indicated by a $[\text{Gdn-HCl}]_{1/2}$ of 0.252 M. On the other hand, W20- and W24-apoLp-III displayed significantly higher $[\text{Gdn-HCl}]_{1/2}$ values of 0.457 and 0.441 M, respectively. Calculation of the free energy change of unfolding in the absence of guanidine-HCl provided a $\Delta G_{\text{D}}^{\text{H}_2\text{O}}$ value of 2.79 kcal/mol for wild-type apoLp-III. In good agreement with the $[\text{Gdn-HCl}]_{1/2}$ values, $\Delta G_{\text{D}}^{\text{H}_2\text{O}}$ of W16-apoLp-III was decreased to 2.03 kcal/mol, and higher values were found for W20-apoLp-III (3.81 kcal/mol), and W24-apoLp-III (3.35 kcal/mol). Thus, while α -helical contents did not change significantly, substitution of tryptophan for phenylalanine or leucine at position 20 or 24 resulted in a more stable protein, while a less stable protein was generated when tryptophan was substituted at position 16.

3.4. DMPC solubilization

To measure the interaction of the single tryptophan apolipoproteins with phospholipid membranes, LUVs of pure DMPC were employed. Upon incubation with apoLp-III, the turbid DMPC LUV suspension (with a diameter of ~200 nm) became optically clear, which is a result of apoLp-III-induced conversion into the much smaller discoidal complexes [27]. These small phospholipid-protein complexes have a diameter of ~15 nm, and their formation can be measured by changes in sample light scatter intensity (Figure 5). Conversion of LUVs into discoidal particles is apparent by the decrease in light scatter intensity when LUVs disappear, and is therefore also termed LUV solubilization [28]. This process is thought to resemble lipidation of human apolipoprotein A-I at the membrane surface [29]. Wild-type apoLp-III was able to solubilize the LUV suspension in ~600 s. The less stable W16-apoLp-III mutant was remarkably more effective and solubilized phospholipid vesicles in less than 100 s. On the other hand, the more stable W20- and W24-apoLp-III variants were significantly slower in their ability to convert LUVs into discoidal particles, and needed ~1500 s for complete solubilization. Initial rate constants of LUV solubilization, assuming first order kinetics, showed a similar pattern with low rate constants for W20- and W24-apoLp-III and a high rate constant for W16-apoLp-III (Table 1).

3.5. LDL aggregation

An alternative method to evaluate the protein's interaction with lipid surfaces is their ability to associate with modified LDL. Incubation of LDL with phospholipase-C results in the removal of the choline headgroup from phosphatidylcholine, creating surface localized diacylglycerol [24]. The resulting increased surface hydrophobicity leads to a rapid aggregation of the LDL particles, evident by the increase in light scatter intensity measured by the absorbance at 340 nm (Figure 6). The generation of diacylglycerol on the LDL surface creates potential binding sites for exchangeable apolipoproteins, and upon binding, lipoprotein aggregation is prevented or delayed. In vivo, diacylglycerol is generated from fat body stores during insect flight and is transferred to high-density lipophorin to which apoLp-III associates allowing transport to flight muscles to provide energy [30]. Therefore, phospholipase-C treated LDL closely resembles the natural binding surface for apoLp-III. Inclusion of wild-type apoLp-III in the LDL phospholipase-C mixture delayed the onset of LDL aggregation for about 50 min after which the absorbance started to increase. Both W16- and W24-apoLp-III were effective to prevent the onset of LDL aggregation in a similar extent as wild-type apoLp-III. On the other hand, the mixture of LDL, phospholipase-C and W20-apoLp-III started to aggregate after 30 min and absorbance values increased steadily to values indistinguishable from LDL treated with phospholipase-C in the absence of apolipoprotein. Thus, W20-apoLp-III was significantly less effective in preventing LDL aggregation compared to wild-type and W16- and W24-apoLp-III. Thus, lipid binding was compromised in this more stable apoLp-III variant.

4. Discussion

The change of a phenylalanine into tryptophan residue is a standard operation when designing single tryptophan mutants and are considered conservative mutations as both residues are bulky and aromatic, decreasing the likelihood of unwanted structural changes in

the protein [26]. In good agreement, no difference in α -helical content was observed between the three apoLp-III tryptophan variants and the wild-type protein. However, the introduction of tryptophan at each of the three positions in helix 1 considerably altered the stability of the protein. This had a pronounced effect on the lipid binding activity of apoLp-III. The increase in protein stability of two variants resulted in a decreased ability to solubilize DMPC LUVs. The other tryptophan variant displayed lower protein stability and a marked increase in vesicle solubilization rate. Thus, the stability of the apoLp-III variants inversely correlated with the rate of conversion of LUVs into discoidal lipoproteins. This is in good agreement with previous data reported for *Locusta migratoria* apoLp-III, an apolipoprotein that shares less than 10% amino acid sequence identity with *G. mellonella* apoLp-III although both proteins share a similar three-dimensional fold [31]. When a single polar residue in helix 1 (Thr-31) was changed into a hydrophobic residue in *L. migratoria* apoLp-III, this led to an increase in protein stability but significantly decreased LUV solubilization rate [32]. Thus, this correlation has now been observed in apoLp-III from *G. mellonella* and from *L. migratoria*. It may be surprising that a single amino acid change causing a relatively small increase in apoLp-III stability resulted in a significant reduction in lipid binding. However, this region of the protein may be particularly sensitive to small changes in structure. The mutated residues are located at or near the interface of helix-1 and -5, and increased resistance to helix bundle opening likely impedes lipid binding. Moreover, the naturally occurring human apolipoprotein E (apoE) isoforms differ in two residues affecting protein stability, which also correlated inversely with DMPC vesicle solubilization rates [33, 34]. Both apoE and apoLp-III are bundles of α -helices, and share a common mechanism of interacting with phospholipid vesicles [5]. When binding to hydrophobic surfaces, the amphipathic α -helices rearrange to facilitate direct contact of the non-polar helix face with phospholipid acyl chains, a hydrophobic interaction that is likely the major driving force for lipid binding [21]. In support of the critical role the terminal helices of apoLp-III play in lipid binding, tethering helix-1 and -5 by engineering a disulfide bridge in apoLp-III in three different insect species severely hampered the ability to solubilize phospholipid vesicles [10, 12, 13].

Lowering the protein stability greatly increased lipid binding of W16-apoLp-III and a *L. migratoria* apoLp-III variant that had a lysine residue introduced into the hydrophobic core [32]. While the increase in lipid binding activity may be seen as an advantage, the reduction in protein stability may have adverse effects. This may lead to an increase in susceptibility to proteases, or may affect the reversible nature of the apolipoprotein-lipid binding interaction, as the protein needs to switch back to a lipid-free state after delivery of the lipid cargo at the flight muscles [30]. Thus an intricate balance between helix bundle stability and lipid binding is required for proper function. Since the $\Delta G_D^{H_2O}$ of unfolding is significantly lower for apoLp-III compared to other helix bundle proteins, this balance may be more critical for apoLp-III [3]. In case of apoE4, the increase in protein stability of this naturally occurring isoform caused by one amino acid change made carriers of this gene more susceptible for Alzheimer's Disease [35]. This indicates that altering the apolipoprotein stability can have important consequences for protein function. In case of apoLp-III the most stable variant was not able to protect DG-enriched LDL to the same extent as the other variants and the wild-type protein, indicating a loss of in vivo function.

The fluorescence properties were distinctly different among the tryptophan variants. The $\lambda_{\max}$ value of 327 nm and inaccessibility to I^- quenching for F20W-apoLp-III predicted a location in the helix bundle. Both W16- and W24-apoLp-III displayed $\lambda_{\max}$ values of 348 nm, implying exposure to the aqueous environment; thus these side-chains are likely positioned on the protein surface. In support of this, the corresponding phenylalanine residue in the structure of *M. sexta* apoLp-III is facing outwards. However, Stern-Volmer constants were unexpectedly low for W16- and W24-apoLp-III, but charge repulsion of neighboring Glu-15 and Glu-16 may explain the poor accessibility of I^- . The strong fluorescence emission intensity of W20-apoLp-III makes it attractive for use in binding analysis to lipids, lipopolysaccharides and other hydrophobic surfaces. However, there was virtually no change in $\lambda_{\max}$ upon incubation with SDS. While the fluorescence intensity of W16-apoLp-III was relatively weak, SDS induced a strong increase in fluorescence intensity concomitant with a 21 nm redshift in $\lambda_{\max}$. Thus, in addition to a robust increase in fluorescence intensity upon binding, this variant can also be exploited for measuring the polarity of the tryptophan microenvironment. W24-apoLp-III showed a significant reduction in fluorescence emission intensity, but $\lambda_{\max}$ could not be determined because this residue became highly quenched.

Analysis of other exchangeable apolipoproteins showed that lipid-bound proteins often display $\lambda_{\max}$ values of 330–338 nm [23]. When $\lambda_{\max}$ values are used for lipid binding analysis, the single tryptophan variants should have a preferred $\lambda_{\max}$ outside of this range. This has been elegantly demonstrated for *L. migratoria* apoLp-III [23]. This protein contains two naturally occurring tryptophans, one in the helix bundle core (W115) and one in a loop connecting helix-4 and -5 (W130). Engineering single tryptophan mutants by replacing tryptophan with phenylalanine showed that W115 with a $\lambda_{\max}$ of 321 nm blue-shifted to 330 nm upon lipid binding. The $\lambda_{\max}$ of W130-apoLp-III shifted from 340 to 327 nm upon lipid binding.

In conclusion, the three tryptophan variants generated in *G. mellonella* apoLp-III are all suitable for binding analysis, with W16-apoLp-III having the advantage of the extensive redshift in $\lambda_{\max}$. Each of the three variants should be used with caution because of changes in protein stability, which altered their lipid binding properties. This may be an inherent problem when designing tryptophan variants in apolipoproteins, and apoLp-III may be particularly prone to structural changes because of the relatively low protein stability. In addition, helix-1 may be very sensitive because of its proposed role in the initial steps of helix bundle opening, and therefore it may be necessary to introduce tryptophan also in other parts of the protein.

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Abbreviations

apoLp-III	apolipophorin III
DMPC	dimyristoylphosphatidylcholine

LDL	low density lipoprotein
LPS	lipopolysaccharides
LUV	large unilamellar vesicles
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
λ_{max}	wavelength of maximum emission
[Gdn-HCl]_{1/2}	midpoint of guanidine denaturation

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Highlights

- Three single tryptophan residues were engineered in helix 1 of apolipoprotein III
- All three variants are suitable for use as a reporter for local changes in the protein environment upon lipid binding interaction.
- Introduction of tryptophan 20 and 24 increased the protein stability, resulting in an increased resistance to solubilize phospholipid vesicles.
- Binding of the most stable W20-apoLp-III apoLp-III variant to modified LDL was compromised

Fig. 1. NMR solution structure of *M. sexta* apoLp-III (PDB ID 1EQ1). The protein is a bundle of five α -helices (labeled H1 to H5) and includes an additional mini-helix (H3'). The phenylalanine residues in helix 1 that were changed into tryptophan are shown in green. The N- and C-terminus of the protein are also indicated.

Fig. 2.

Tryptophan fluorescence emission scans. Proteins (50 μ g) were excited at 280 nm and the emission measured between 290 and 450 nm. Shown are W16-apoLp-III (-----), W20-apoLp-III ($\cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot$), and W24-apoLp-III ($-\cdot-\cdot-$).

Fig. 3.

Circular dichroism of wild-type and apoLp-III tryptophan variants. The ellipticity of the protein samples (0.2 mg/mL) was measured between 190 and 260 nm and converted into molar ellipticity. Shown are wild-type (—), W16-apoLp-III (-----), W20-apoLp-III (· · · · ·), and W24-apoLp-III (— · —).

Fig. 4. Guanidine denaturation of apoLp-III. Proteins were incubated with increased amounts of guanidine-HCl. The ellipticity at 222 nm was used to monitor unfolding of the protein. The change in ellipticity was expressed as % maximum change. The unfolding plots provide the $[Gdn-HCl]_{1/2}$, a measure of protein stability. Shown are apoLp-III wild-type ($\diamond$), W16-apoLp-III ($\bullet$), W20-apoLp-III (∇), and W24-apoLp-III ($\blacksquare$).

Fig. 5.

ApoLp-III-induced DMPC solubilization. LUVs of DMPC were incubated with wild-type or one of the three tryptophan apoLp-III variants. The LUVs were solubilized by apoLp-III and converted into small discoidal lipid-protein complexes which was followed by the decrease in light scatter measured by the absorbance at 325 nm. Shown are apoLp-III wild-type (—), W16-apoLp-III (-----), W20-apoLp-III (.....), and W24-apoLp-III (-.-.-).

Fig. 6.

ApoLp-III inhibition of LDL aggregation. Human LDL was incubated with phospholipase C, creating diacylglycerol on the lipoprotein surface leading to lipoprotein aggregation. Inclusion of apoLp-III in the reaction mixture prevented or delayed the onset of apolipoprotein aggregation. Shown are apoLp-III wild-type (◊), W16-apoLp-III (●), W20-apoLp-III (▽), W24-apoLp-III (■), and no protein (▲).

Table 1

Structural and functional properties of apoLp-III single tryptophan mutants

	Wild-type	W16	W20	W24
Trp λ_{max} (nm)	N/A	347.5 ± 0.5	327.0 ± 0.9	348.0 ± 0.5
Stern-Volmer constant	N/A	1.29 ± 0.09	0.85 ± 0.09	0.96 ± 0.02
% α-helix	66.3 ± 0.2	65.0 ± 0.2	69.7 ± 0.4	66.2 ± 0.1
[Gdn-HCl]$_{1/2}$ (M)	0.333 ± 0.005	0.253 ± 0.012	0.457 ± 0.003	0.441 ± 0.006
$G_D^{\text{H}_2\text{O}}$ (kcal mol^{-1})	2.79 ± 0.16	2.03 ± 0.09	3.81 ± 0.14	3.35 ± 0.17
$t_{1/2\text{SUV}}$ solubilization (s^{-1})	$11.4 \pm 0.1 \cdot 10^{-3}$	$85 \pm 7 \cdot 10^{-3}$	$4.8 \pm 0.1 \cdot 10^{-3}$	$5.0 \pm 0.2 \cdot 10^{-3}$

Values are the average of three independent determination $\pm$ standard deviation.