The C Terminus of Apolipoprotein A-V Modulates Lipid-binding Activity*

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Human apolipoprotein A-V (apoA-V) is a potent modulator of plasma triacylglycerol (TG) levels. To probe different regions of this 343-amino-acid protein, four single Trp apoA-V variants were prepared. The variant with a Trp at position 325, distal to the tetraproline sequence at residues 293–296, displayed an 11-nm blue shift in wavelength of maximum fluorescence emission upon lipid association. To evaluate the structural and functional role of this C-terminal segment, a truncated apoA-V comprising amino acids 1–292 was generated. Far UV circular dichroism spectra of full-length apoA-V and apoA-V-(1–292) were similar, with ~50% α-helix content. In guanidine HCl denaturation experiments, both full-length and truncated apoA-V yielded biphasic profiles consistent with the presence of two structural domains. The denaturation profile of the lower stability component (but not the higher stability component) was affected by truncation. Truncated apoA-V displayed an attenuated ability to solubilize L-α-dimyristoylphosphatidylcholine phospholipid vesicles compared with full-length apoA-V, whereas a peptide corresponding to the deleted C-terminal segment displayed markedly enhanced kinetics. The data support the concept that the C-terminal region is not required for apoA-V to adopt a folded protein structure, yet functions to modulate apoA-V lipid-binding activity; therefore, this concept may be relevant to the mechanism whereby apoA-V influences plasma TG levels.

In 2001, a new member of the exchangeable apolipoprotein family was independently discovered by comparative genomics (1) and as an mRNA that is up-regulated in rat liver following partial hepatectomy (2). In humans, the mature protein, termed apolipoprotein (apo)A-V, comprises 343 amino acids (3).

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2 The abbreviations used are: apo, apolipoprotein; ANS, 8-anilino-1-naphthalene-sulfonic acid; CD, circular dichroism; DMPC, L-α-dimyristoylphosphatidylcholine; HSPG, heparan sulfate proteoglycan; TG, triacylglycerol; VLDL, very low density lipoprotein; WT, wild type.

Northern blot analysis of various tissues revealed that apoA-V mRNA expression is restricted to hepatocytes (1). To evaluate its function, Pennacchio et al. (1) generated transgenic mice that overexpressed apoA-V as well as gene-disrupted mice that lacked apoA-V. The transgenic mice displayed a 3-fold lower plasma triacylglycerol (TG) level compared with control littermates. By contrast, apoA-V gene knock-out mice revealed a 4-fold higher plasma TG content compared with controls. Levels of very low density lipoprotein (VLDL) particles were increased in homozygous knock-out mice and decreased in transgenic mice compared with controls. Van der Vliet et al. (4) confirmed the effect of apoA-V on plasma TG levels using an adenovirus construct to overexpress apoA-V in mice. ApoA-V-overexpressing mice displayed markedly decreased plasma TG levels that were the result of lower VLDL levels. Interestingly, changes in plasma TG concentration were directly opposite of those reported for apoC-III knock-out and transgenic mice (5, 6). Although apoA-V knock-out mice displayed a 4-fold increase in plasma TG, apoC-III gene-disrupted animals showed a 30% decrease. The mechanism whereby apoA-V influences plasma TG levels is unknown but may be related to an ability to influence TG-rich lipoprotein biogenesis, secretion, or metabolism. Three distinct hypotheses have been proposed to explain the action of apoA-V on plasma TG levels, (a) an intracellular mode of action wherein apoA-V modulates TG-rich lipoprotein biogenesis and/or secretion (7), (b) a direct activation of lipoprotein lipase activity (8, 9), or (c) an indirect effect on TG-rich lipoprotein metabolism or clearance from plasma (10–12).

Previous studies (7) have revealed that apoA-V possesses strong lipid-binding activity, whereas a naturally occurring C-terminal truncation variant associated with hypertriglyceridemia has been largely recovered in the lipoprotein-free fraction of plasma (13). Interestingly, apoA-V contains four consecutive proline residues (Pro293–Pro296) near the C terminus of the protein. We postulated that the 51-residue segment beyond this site constitutes a discrete structural element in apoA-V that may function to modulate its lipid affinity. To test this hypothesis and to further define the structural and functional role of this region of apoA-V, we have examined the spectroscopic, lipid-binding, and dynamic interfacial characteristics of a series of single tryptophan mutants and a truncated variant lacking the terminal 51 amino acids as well as a peptide corre-
sponding to the deleted C-terminal segment. Data from these studies establish that the C-terminal segment modulates the lipid-binding activity of apoA-V in a manner that may directly impact its ability to lower plasma TG.

**EXPERIMENTAL PROCEDURES**

**Apolipoproteins**—Recombinant human apoA-V was prepared as described by Beckstead et al. (14). Site-directed mutagenesis was performed using the QuikChange II XL kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. All mutations were verified by DNA sequencing. A 48-amino-acid peptide corresponding to apoA-V residues 296–343 was prepared by solid phase peptide synthesis, isolated by reversed phase high performance liquid chromatography, and characterized by mass spectrometry on an Applied Biosystems Voyager System 1054. The observed mass (5,319.7) was in good agreement with the expected molecular mass based on the amino acid sequence (5,318.8).

**Analytical Procedures**—Protein concentrations were determined using the bicinchoninic acid assay (Pierce) using bovine serum albumin as the standard. C-terminal peptide concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient $\varepsilon = 5500 \text{ M}^{-1} \text{ cm}^{-1}$. SDS-PAGE was performed on 4–20% acrylamide slab gels run at a constant 30 mA for 1.5 h. The gels were stained with Gel Code Blue (Pierce).

**Far UV Circular Dichroism Spectroscopy**—Far UV circular dichroism (CD) measurements were performed on a Jasco 810 spectropolarimeter. Scans were repeated five times to obtain the average value using a 0.02-cm path length and a protein concentration of 0.6 mg/ml. Protein was dissolved in 50 mM sodium citrate, pH 3.0, 150 mM NaCl. For guanidine HCl denaturation experiments, apoA-V samples (0.2 mg/ml) were incubated overnight at a given denaturant concentration to attain equilibrium, and ellipticity was measured at 222 nm (0.1 cm path length). Ellipticity values were converted into molar ellipticity (millidegrees cm$^2$ dmol$^{-1}$) using a mean residue weight value of 113.4 for full-length apoA-V and 114.3 for apoA-V(1–292). Protein secondary structure content was calculated using the self-consisted method with Dicroprot, version 2.6 software (15).

**Fluorescence Spectroscopy**—Fluorescence spectra were obtained on a Horiba Jobin Yvon FluoroMax-3 luminescence spectrometer. Protein (40 $\mu$g/ml) was dissolved in 50 mM sodium citrate, pH 3.0, 150 mM NaCl. $\alpha$-Dinonyristophosphatidylcholine (DMPC)-bound apoA-V samples were prepared at a 5:1 DMPC:apoA-V ratio (w/w). Samples were excited at 295 nm and emission collected from 300 to 450 nm (slit width 2.0 nm). Spectra of 8-anilino-1-naphthalene sulfonic acid (ANS) solutions (250 mM) were obtained in buffer alone and in the presence of apoA-V at 50 mg/ml on a PerkinElmer Life Sciences LS50B luminescence spectrometer. Samples were excited at 395 nm with emission monitored from 400 to 600 nm (3.0 nm slit width). Because ANS fluorescence in buffer is negligible (16), spectra were recorded in the presence of a minimum 100-fold excess of ANS with respect to protein (mol/mol).

**DMPC Solubilization Studies**—Small unilamellar vesicles of DMPC were prepared by extrusion through a 200-nm filter as described by Weers et al. (17). Protein and lipid vesicles were prepared in 50 mM sodium citrate, pH 3.0. DMPC (250 $\mu$g) was incubated in the absence or presence of 100 $\mu$g of apolipoprotein, maintaining the sample temperature at 24 °C with a Peltier controlled cell holder. The change in sample light scatter intensity was measured with a Shimadzu spectrophotometer at 325 nm.

**Studies of apoA-V at the Triolein/Water Interface**—The interfacial behavior of apoA-V at the oil/water interface was analyzed using a Tracker® automatic tensiometer (IT Concept, Parc de Chancolan, Longessaigne, France) (7, 18). Triolein drops (10 $\mu$l) were rapidly formed into a cuvette containing 25 $\mu$g/ml full-length apoA-V or apoA-V (1–292) in 10 mM citrate buffer, pH 3.0, and the adsorption of protein to the triolein/water interface was recorded as the decrease in interfacial tension ($\gamma$) over time. Exponential adsorption rate constants were calculated by log transformation of the initial segment of the $\gamma$ versus time curves. Interfacial elasticity ($\psi$) was determined by sinusoidal oscillation of the drop volume and analyzing the phase angle between the changes in surface area and tension (19).

**RESULTS**

**Tryptophan Fluorescence of apoA-V**—Fluorescence spectra of wild type (WT) apoA-V revealed a wavelength of maximum Trp fluorescence emission of 340 nm in buffer that shifted to 339 nm when the protein was complexed with phospholipid (Table 1). At the same time, lipid association was accompanied by a near 3-fold enhancement in emission quantum yield (excitation 295 nm). WT apoA-V contains four Trp residues located at positions 5, 97, 147, and 325. Site-directed mutagenesis of apoA-V variants with Trp at positions 97, 147, or 325 underwent an 11-nm blue shift in Trp fluorescence emission of Trp97 or Trp147 apoA-V variants. The apoA-V variant with a Trp at position 325 underwent an 11-nm blue shift in Trp fluorescence emission of Trp325 apoA-V variant. Although fluorescence emission spectra of apoA-V variants with Trp at positions 97, 147, or 325 were excited at 295 nm and emission collected from 300 to 450 nm (slit width 2.0 nm). Spectra of 8-anilino-1-naphthalene sulfonic acid (ANS) solutions (250 mM) were obtained in buffer alone and in the presence of apoA-V at 50 mg/ml on a PerkinElmer Life Sciences LS50B luminescence spectrometer. Samples were excited at 295 nm and emission collected from 300 to 450 nm (slit width 2.0 nm). Spectra of 8-anilino-1-naphthalene sulfonic acid (ANS) solutions (250 mM) were obtained in buffer alone and in the presence of apoA-V at 50 mg/ml on a PerkinElmer Life Sciences LS50B luminescence spectrometer. Samples were excited at 295 nm and emission collected from 300 to 450 nm (slit width 2.0 nm). Spectra of 8-anilino-1-naphthalene sulfonic acid (ANS) solutions (250 mM) were obtained in buffer alone and in the presence of apoA-V at 50 mg/ml on a PerkinElmer Life Sciences LS50B luminescence spectrometer. Samples were excited at 295 nm and emission collected from 300 to 450 nm (slit width 2.0 nm). Spectra of 8-anilino-1-naphthalene sulfonic acid (ANS) solutions (250 mM) were obtained in buffer alone and in the presence of apoA-V at 50 mg/ml on a PerkinElmer Life Sciences LS50B luminescence spectrometer. Samples were excited at 295 nm and emission collected from 300 to 450 nm (slit width 2.0 nm).
played a 25% decrease in emission quantum yield upon lipid association.

C-terminal Truncation of apoA-V—Examination of the amino acid sequence of apoA-V revealed the presence of four consecutive proline residues at positions 293–296 of this 343-amino-acid protein. Because the cyclic structure of proline imposes geometric constraints that strongly influence the secondary structure of proteins, we hypothesized that the 51-amino-acid segment, including and beyond this polyproline sequence, constitutes a distinct structural element in apoA-V. Given that the C-terminal region of other members of the exchangeable apolipoprotein family is important in their lipid-binding activity (20) together with the observed lipid-binding-induced fluorescence blue shift for the apoA-V variant with a single Trp at position 325, we evaluated the effect of deleting this segment of the protein on its structural and interfacial properties. SDS-PAGE analysis of truncated apoA-V (Fig. 2) revealed a single band under reducing conditions that migrated faster than full-length apoA-V. On the other hand, under non-reducing conditions, evidence was obtained that an equivalent, although a minor fraction of apoA-V in both samples, exists as a disulfide-linked homodimer.

Characterization Studies—Far UV CD spectroscopy of full-length apoA-V and apoA-V-(1–292) yielded spectra with minima at 208 and 222 nm, consistent with the presence of α-helix (Fig. 3). Secondary structure calculations yielded an α-helix content of 50% for both full-length apoA-V and apoA-V-(1–292). The similarity between these values indicates that deletion of the 51-residue segment did not disrupt the helix-forming capacity of apoA-V. To evaluate the effect of truncation on the stability of apoA-V, guanidine HCl denaturation studies were performed (Fig. 4). In contrast to the apparent two-state, single transition observed with temperature-induced denaturation (14), the present results indicate the presence of independently folded structural elements in apoA-V. Full-length apoA-V gave rise to an initial transition with a midpoint at 1 M guanidine HCl and a second transition with a midpoint at \( \approx 2.6 \) M guanidine HCl. Interestingly, apoA-V-(1–292) yielded a nearly superimposable curve for the second transition component, whereas the first transition component was affected by the truncation. On the basis of these data, it may be concluded that the present C-terminal truncation variant behaves in a similar manner to full-length apoA-V and that the 51-residue C-terminal deletion exerts a minor influence on the unfolding behavior of apoA-V. In a separate study, the effect of Trp→Phe mutations in apoA-V on the secondary structure content of the protein was evaluated. Using the Trp\(^{225}\) apoA-V variant (in which three of the four Trp residues have been mutated to Phe) as a surrogate, the far UV CD spectrum was comparable with WT apoA-V (data not shown), indicating the Trp substitution mutations did not adversely affect the secondary structure content of the protein.

Truncation of apoA-V had only minor effects on the heparin-binding activity of the protein. ApoA-V-(1–292)-DMPC com-
complexes eluted from a heparin-Sepharose column at 0.38 M NaCl, whereas full-length apoA-V-DMPC complexes eluted at 0.40 M NaCl. These data are consistent with previous studies that localized the heparin-binding domain of apoA-V between residues 186 and 227 (11).

Fluorescent Dye Binding—To evaluate the extent to which C-terminal truncation alters exposure of hydrophobic sites in apoA-V, the effect of full-length apoA-V and apoA-V-(1–292) on the fluorescence emission intensity of ANS was examined (Fig. 5). In the absence of protein, ANS has a low quantum yield. The enhancement in ANS fluorescence intensity induced by apoA-V was lower, suggesting the deleted CT segment possesses ANS binding sites and that removal of this segment did not introduce new ANS binding sites in the residual fragment. These data are similar to results seen upon C-terminal truncation of apoA-I (20).

Dynamic Interfacial Activity of apoA-V at the Oil/Water Interface—Full-length apoA-V and apoA-V-(1–292) bound to a triolein/water interface and rapidly decreased the surface tension from its base-line value of 35 millinewtons/m to ~15 millinewtons/m. Calculation of binding rate constants by log transformation of the initial linear portion of the time-tension curves yielded rates of 2.3 ± 0.2 ms⁻¹ for full-length apoA-V versus 3.3 ± 0.2 ms⁻¹ for apoA-V-(1–292) (Table 2). The interfacial elasticity of full-length apoA-V at the triolein/water interface was 23.0 ± 0.6 millinewtons/m. The corresponding value for apoA-V-(1–292) was significantly lower at 18.9 ± 0.6 millinewtons/m (p = 0.004), indicating this C-terminal deletion reduces the ability of apoA-V to adapt its conformation to changes in surface geometry.

Phospholipid Vesicle Solubilization Studies—The effect of C-terminal truncation of apoA-V on lipid-binding activity was further examined in phospholipid vesicle solubilization assays. Apolipoprotein-dependent alterations in DMPC small unilamellar vesicle light scatter intensity (measured spectrophotometrically at 325 nm) were monitored as a function of time (Fig. 6). In control incubations lacking apolipoprotein, DMPC vesicle turbidity did not change. The addition of full-length apoA-V to DMPC small unilamellar vesicles induced a rapid time-dependent reduction in turbidity. The initial rate constant (k) was 1.5 × 10⁻² s⁻¹, assuming first order kinetics, and the reaction

![Figure 4](Image 60x328 to 288x510)

**FIGURE 4.** Effect of guanidine HCl on the secondary structure content of apoA-V. Indicated amounts of guanidine HCl were added to WT apoA-V and apoA-V-(1–292) in buffer (50 mM sodium citrate, pH 3.0, 150 mM NaCl), and at each concentration, the ellipticity at 222 nm was determined. Filled circles, full-length apoA-V; open circles, apoA-V-(1–292).

![Figure 5](Image 217x26 to 244x38)

**FIGURE 5.** Effect of full-length and truncated apoA-V on ANS fluorescence intensity. ANS (1 mM) in 50 mM sodium citrate, pH 3.0, 150 mM NaCl was excited at 395 nm and emission monitored from 400 to 600 nm. Curve a, ANS in buffer at pH 7.0; curve b, ANS plus 20 μg of full-length apoA-V; curve c, ANS plus 20 μg of apoA-V-(1–292).

| ApoA-V variant | Final tension | Rate constant | Elasticity |
|---------------|--------------|--------------|------------|
| Full-length (1–343) | 14.5 ± 0.4 | 2.3 ± 0.2 | 23.0 ± 0.6 |
| Truncated (1–292) | 15.5 ± 0.2 | 3.3 ± 0.2 | 18.9 ± 0.6 |
| p value | 0.083 | 0.007 | 0.004 |

*Unit of measure mN/m is millinewtons/meter.

**TABLE 2**

Recombinant ApoA-V studied by oil drop tensiometry

The data shown are the means ± S.E. of four separate experiments. The significance of the differences in interfacial parameters between full-length and truncated apoA-V was determined by unpaired Student’s t test.
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was near completion after ~300 s. In contrast, the initial rate constant was nearly 2-fold lower for apoA-V-(1–292) \((k = 7.4 \times 10^{-3} \text{s}^{-1})\). Increasing the protein concentration resulted in faster transformation rates and a lower final sample turbidity. Nevertheless, apoA-V-(1–292) turbidity remained at a higher level at all time points compared with full-length apoA-V, with a ~2-fold difference in solution clearance rate (results not shown). Thus, consistent with the observed decrease in ANS-accessible sites upon C-terminal truncation, this variant also displays a lower lipid-binding activity than full-length apoA-V.

Studies of the C-terminal Peptide—Based on the finding that deletion of the C-terminal 51 amino acids in apoA-V altered its lipid-binding properties, studies were performed to determine the intrinsic lipid-binding activity of the deleted segment. A peptide corresponding to apoA-V amino acid residues 296–343 was synthesized and isolated by reversed phase high performance liquid chromatography. This peptide contains a single Trp residue that corresponds to Trp325 in full-length apoA-V, and apoA-V-(1–292) decreased the interfacial tension of a triolein/water interface to the same extent, with apoA-V-(1–292) binding to the triolein interface at a faster rate. The differences observed between DMPC solubilization activity and triolein binding may be explained by intrinsic differences between the lipid substrates and the reaction end points measured. In DMPC solubilization assays, the apolipoprotein must not only bind to the phospholipid surface but also induce reorganization of the vesicles into smaller discoidal lipid-protein complexes (24). This requires apolipoprotein penetration, bilayer disruption, and a conformational change in the protein. If truncation affected any of these steps, the result would be manifested as a slower solubilization rate. In the case of the much more hydrophobic triolein/water interface, the 51-residue C-terminal deletion may have enhanced the global hydrophobicity of the residual protein such that its more rapid absorption to the interface was thermodynamically favored. Taken together, the data suggest that the C terminus of apoA-V functions in the lipid-binding activity of this protein. In this manner, the C terminus of apoA-V appears to resemble other members of the exchangeable apolipoprotein family (20, 25).

Confirmation of the role of the C-terminal segment of apoA-V in lipid-binding activity of this protein was obtained using an isolated peptide corresponding to this region. ApoA-V-(296–343) induced the formation of a discrete population of reconstituted HDL and showed markedly enhanced phospholipid solubilization kinetics.

By analogy, the C terminus of human apoA-I is recognized as the major lipid-binding element in this protein (26). Removal of C-terminal residues 193–243 from apoA-I induces a decrease in lipid-binding activity together with a loss of ANS binding sites. Recent x-ray crystallography evidence (27) suggests the C terminus of apoA-I exists as a distinct structural entity, whereas...
the main body of the protein adopts a four-helix-bundle molecular architecture, with the C terminus organized as two solvent-exposed helical segments. By the same token, the C-terminal domain of apoE comprises ~83 residues and is the major lipid-binding segment in this protein (28). In a manner similar to apoA-I, the N terminus of apoE is organized as a four-helix bundle.

Although further work is required to elucidate the precise domain structure in apoA-V, the present data indicate the existence of two independently folded structural elements, one of relatively high stability and a second of lower stability. Interestingly, the guanidine HCl denaturation profile observed in the present study is similar to that reported for apoE (29). The observation that the C-terminal truncation affected the guanidine HCl-induced unfolding of only the lower stability component of the curve suggests that, in a manner similar to apoE, the N-terminal region corresponds to the more stable structural entity. In contrast, a previous study employing temperature-induced denaturation of apoA-V revealed a single transition midpoint (14). It is worth mentioning that temperature-induced denaturation of apoE also revealed a single transition profile (30), whereas guanidine HCl denaturation studies revealed a two-domain structure (29). This is interesting, because the method used to unfold a protein gives insight into its structural interactions. For instance, comparing temperature and guanidine HCl denaturation studies, it has been shown that the former assesses the overall stability of a protein, whereas the latter assesses mainly the contributions of hydrophobic interactions on protein stability. This is because of the fact that guanidine HCl (a salt) can ionize in aqueous solution, masking the electrostatic interactions of the protein of interest (31). Taking into account the denaturation methods used and the data obtained from each, it can be concluded that the increased stability of the N-terminal region of apoA-V compared with the C-terminal region is due to hydrophobic or non-ionic interactions. Whether the N-terminal region of the protein adopts a helix bundle conformation similar to that seen for the N terminus of apoE (32), apoA-I (27), or apolipoporhin III (33, 34) remains to be determined. Studies are currently in progress to define the boundaries of the apparently distinct structural elements present in apoA-V and to determine the mechanism of its plasma TG modulation activity.

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