Deletion of Single Amino Acid E235 Affects the Structure and Lipid Interaction of Human Apolipoprotein A-I C-Terminal Peptides

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The C-terminal domain of apolipoprotein (apo) A-I plays an important role in lipid binding. ApoA-I Nichinan, a naturally occurring human apoA-I variant with a deletion of E235 located in the C-terminus, is associated with low high-density lipoprotein (HDL) cholesterolemia. In the present study, a series of variant peptides corresponding to residues 220—241 of human apoA-I were examined to clarify the influences of E235 deletion (ΔE235) on the structure and lipid interaction of the C-terminal region. NMR studies demonstrated that in trifluoroethanol, apoA-I 220—241/ΔE235 peptide forms the α-helical structure similar to wild-type (WT) peptide. Circular dichroism measurements revealed that the interaction with phospholipid vesicles induced structural changes from random coil to α-helix both in apoA-I 220—241 WT and E235A, a variant with a negative charge ablation, peptides. These peptides also showed abilities to form HDL-like particles through microsolubilization of phospholipid vesicles, indicating that the negative charge ablation in E235 has no effect on the lipid interaction. By contrast, neither lipid binding-induced α-helix formation nor microsolubilization of vesicles were observed in apoA-I 220—241/ΔE235 and L230P, a helix-breaking variant, peptides. In addition, fluorescence measurements showed that tryptophan fluorescence intensity of apoA-I 220—241/F225W greatly increased upon lipid binding, while only a little increase was observed for the corresponding ΔE235 variant. Taken together, these results suggest that the deletion of E235 causes defective lipid binding of apoA-I Nichinan because of the impaired helix-forming ability of the C-terminal residues.

Key words apolipoprotein A-I variant; helical structure; lipid binding; low high-density lipoprotein cholesterolemia

Human apolipoprotein (apo) A-I, a 243 amino acid protein, is the major protein component of high-density lipoprotein (HDL). ApoA-I plays an important role in lipoprotein metabolism, reverse cholesterol transport pathway, and protection against the development of atherosclerosis.[1)] In reverse cholesterol transport pathway, lipid-free or lipid-poor apoA-I molecules remove cholesterol from peripheral cells and transport it in the form of HDL back to the liver for excretion.[2)] Lipid-free apoA-I is folded into two domains, comprising an N-terminal domain forming a four-helix bundle and a discrete C-terminal domain, which is predominantly involved in lipid–protein interactions. These interactions are critical for HDL generation, including initial lipid binding and cholesterol efflux from plasma membrane.[3)] ApoA-I Nichinan, a naturally occurring human apoA-I variant with a deletion of E235 located in the C-terminus, is associated with low HDL cholesterolemia probably due to the impaired lipid binding and cellular interactions.[4,5)]

Computational analysis of the amino acid sequence of human apoA-I reveals that residues 44—243, encoded by exon 4, is composed of 11- or 22-amino acid tandem repeats, which can form amphipathic α-helices mostly punctuated by proline residues.[6)] Studies of synthetic peptides corresponding to each of 22-residue amphipathic segments of apoA-I have shown that the last repeated helix (residues 220—241) has greatest lipid affinity.[7,8)] In addition, our previous studies using deletion mutants of apoA-I demonstrated that deletion in the last helix leads to significant decreases in lipid-binding affinity compared to intact apoA-I.[9,10)]

A lack of E235 in apoA-I Nichinan variant is considered to bring about at least two possible structural influences such as 1) a negative charge ablation and 2) disturbance of amino acid distribution on the helix cross-section (*i.e.*, loss of amphipathicity) (Fig. 1). Previously, Panagotopulos *et al.* have revealed that the E235K mutation in full-length apoA-I does not affect lipid microsolubilization and cellular cholesterol efflux,[11)] suggesting the possibility that amphipathic nature of the C-terminal region in apoA-I Nichinan is influenced by the disturbance of amino acid distribution caused by E235 deletion. To examine the influences of E235 deletion on the α-helical structure and lipid interaction of the C-terminal region in apoA-I, we used a series of variant peptides corresponding to residues 220—241 of the apoA-I molecule in addition of ΔE235: E235A in which E235 was substituted with nonpolar alanine residue to examine the influence of a negative charge ablation, and L230P in which L230 was substituted with proline residue to disrupt the putative α-helical structure. We previously demonstrated that proline insertion instead of leucine residue at position 230 disrupts the C-ter-

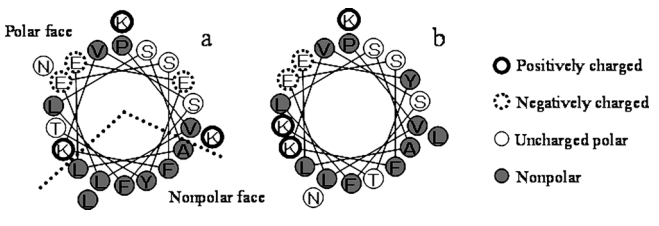

Fig. 1. Helical Wheel Representations of the C-Terminal Functional Region (Residues 220—241) in ApoA-I WT (a) and Nichinan (b)

E235 residue is deleted in apoA-I Nichinan. Bold circles, positively charged; dotted circles, negatively charged; open circles, uncharged polar; gray circles, nonpolar amino acids. Dotted lines indicate polar-nonpolar interface, which is disappeared by a single amino acid E235 deletion in apoA-I Nichinan.

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minal α-helical structure and reduces the lipid-binding affinity of apoA-I.[12] In addition, we substituted phenylalanine with tryptophan to monitor the lipid binding of peptides. The present results show that a lack of E235 directly affects the dysfunction of apoA-I Nichinan through the impaired ability to form α-helical structure upon binding to lipid.

Experimental

Materials Fmoc amino acid derivatives were obtained from Peptide Institute, Inc., (Minoh, Japan), and were used without further purification. Egg phosphatidylcholine (PC) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dimyristoylphosphatidylcholine (DMPC) was from NOF (Tokyo, Japan). All other reagents were special grade.

Peptide Synthesis Peptide synthesis and purification were performed by the methods described.[13] Primary sequences of the synthetic peptides used in the present study are listed in Table 1. The N- and the C-termini were capped with an acetyl group and an amide group, respectively. Peptides were cleaved from the resin using standard trifluoroacetic acid methods and purified by HPLC on an Atlantis Prep T3 column (Waters), as assessed by matrix-assisted laser desorption ionization mass spectrometry (Voyager-DE PRO). In all experiments, peptides were freshly dialyzed from 6 M guanidine hydrochloride or 8 M urea solution into the appropriate buffer before use. Peptide concentrations were determined by Lowry procedure using bovine serum albumin (Bio-Rad) as a standard.

Preparation of Lipid Vesicles A film of egg PC or DMPC on the wall of a glass tube was dried under vacuum overnight and hydrated with buffer. Egg PC small unilamellar vesicles (SUV) with a diameter of approximately 25 nm, were prepared by sonication and then ultracentrifuged in a Beckman TLA110 rotor (51000 rpm) for 2 h to remove larger particles and titanium debris as described.[9] The PC concentration was determined using an enzymatic assay kit for choline from Wako (Osaka, Japan).

NMR Spectroscopy All one- and two-dimensional ^{1}H-NMR measurements were performed on a Varian VNMRS-500 spectrometer (^{1}H: 499.8 MHz) with a 5 mm probe at room temperature. Peptide samples were dissolved at 200 μg/ml in 80% (v/v) 2,2,2-trifluoroethanol (TFE)-d_3/H_2O mixture. The large H_2O signal and the residual proton signals of TFE-d_3 were suppressed with the WET pulse sequence.[14] Mixing times of 60-ms and 300-ms were used in total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY), respectively. Chemical shifts were referenced with respect to the N-terminal acetyl methyl proton of each sample set at 2.225 ppm.

Circular Dichroism Spectroscopy Circular dichroism (CD) spectra of apoA-I 220—241 peptides were obtained from 195 nm to 255 nm at 25 °C using an Aviv 62ADS spectropolarimeter. Peptide samples were diluted to 50 μg/ml either in 10 mM sodium phosphate buffer (pH 7.4) or in 80% (v/v) TFE/buffer. Molar ellipticity ([θ]) was calculated from the equation: $[\theta]=(\mathrm{MRW})\ \theta/10lc$, where θ is a measured ellipticity in degrees, l is the cuvette path length (0.2 cm), and c is the peptide concentration in g/ml, and the mean residue weight (MRW) obtained from the molecular weight and the number of amino acids. The α-helical contents were calculated from the equation using [θ] at 222 nm: % α-helix$=[(-[\theta]_{222}+3000)/(36000+3000)]\times100$.[12] For lipid-binding experiments, apoA-I peptide was incubated with egg PC SUV (lipid/peptide$=$100/1 (w/w)) for 1 h prior to the measurement.

Fluorescence Spectroscopy Fluorescence measurements were carried out at 25 °C using a Hitachi F-7000 spectrophotometer. Peptide samples (25 μg/ml) were excited at 295 nm and emission spectra of tryptophan were recorded in a 4×4 mm cuvette from 300 to 420 nm with increasing concentrations of SUV. To avoid the light scattering caused by SUV, polarizers (excitation and emission polarization set to horizontal and vertical, respectively) were used as described.[15] Contributions from the buffer and SUV without peptide were subtracted from the experimental spectra. The wavelength of maximum fluorescence (WMF) was determined by the first derivation of the spectrum, at which the slope of the curve yields the value close to zero. Because the fluorescence intensity (FI) at a fixed wavelength is linearly related to the amount of bound peptide,[15] the changes in FI at 335 nm were compared among peptides. This wavelength is chosen because it is the WMF of peptides in the presence of excess amount of SUV.

DMPC Clearance Assay Interactions of the apoA-I peptide with DMPC vesicles were monitored by right-angle light scattering as described.[7,8,16] Peptides were mixed with DMPC vesicles (lipid/peptide$=$1/1.2 (w/w)) and turbidity clarification was followed by measuring the scattered light intensity using a Hitachi F-4500 spectrophotometer. Excitation and emission wavelengths were set at 600 nm. The measurements were initiated immediately after the addition of peptides or buffer. All measurements were carried out at 24.6 °C, the gel to liquid–crystalline phase transition temperature of DMPC vesicles.

Table 1. Primary Sequence and Molecular Mass of Synthetic ApoA-I 220—241 Peptides

Peptide	Sequence	Found: m/z (Calculated: M+H)
WT	PVLESFKVSFLSALEEYTKKLN	2585.8 (2583.4)
ΔE235	PVLESFKVSFLSALE_YTKKLN	2456.5 (2454.4)
E235A	PVLESFKVSFLSALE**A**YTKKLN	2525.6 (2525.4)
L230P	PVLESFKVSF**P**SALEEYTKKLN	2568.7 (2567.4)
F225W	PVLES**W**KVSFLSALEEYTKKLN	2622.3 (2622.4)
F225W/ΔE235	PVLES**W**KVSFLSALE_YTKKLN	2494.1 (2493.4)

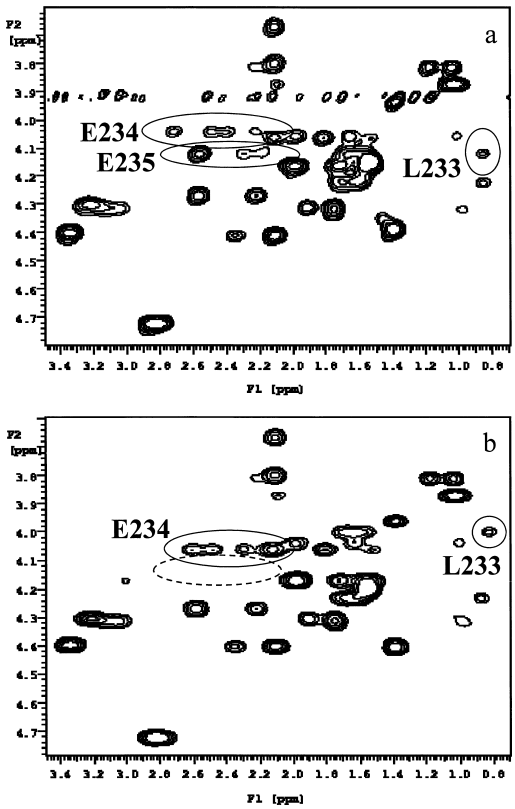

Fig. 2. TOCSY Spectra of C$^\alpha$H/Side-Chain Proton Region of ApoA-I 220—241 WT (a) and ΔE235 (b) Peptides in 80% (v/v) TFE-d_3/H_2O

Chemical shifts were referenced with respect to the N-terminal acetyl methyl proton of each sample set at 2.225 ppm.

Results and Discussion

Solution NMR Structure We determined the solution NMR structure of apoA-I 220—241 wild-type (WT) and ΔE235 peptides in 80% (v/v) TFE-d_3/H_2O mixture, a helix-inducible environment.[17,18] In water, it was not possible to determine the structure of these peptides in NMR condition due to peak broadening probably caused by self-association of peptides. Figure 2 shows TOCSY spectra in α-proton (C$^\alpha$H)/side-chain proton region of apoA-I 220—241 WT and ΔE235 peptides. No significant differences were observed in these spectra, except that the chemical shift values of E234 side-chain and L233 C$^\alpha$H proton in apoA-I 220—241/ΔE235

peptide were slightly shifted upfield. These results suggest that the overall structure of these peptides is similar. Chemical shift values of $C^{\alpha}H$ allow us to characterize secondary structure of peptide; for example, formation of α-helix and β-sheet structure leads to upfield and downfield shift from the random coil value, respectively.[19] Here, the chemical shift values of random coil structure were derived from the literature.[20] The chemical shift values of $C^{\alpha}H$ of residues 225—237 in both peptides were shifted upfield by 0.2—0.4 ppm. In NOESY spectra, NOE cross-peaks were observed between neighboring backbone amide proton of residues 228—237 in both peptides (data not shown), indicating formation of α-helical structure in this region, consistent with TOCSY results.[19] These results indicate that these peptides form nearly identical α-helical structure under the condition which compulsorily induces α-helical structure.

Secondary Structure Assessed by CD Secondary structure of peptides in different environments was determined by CD spectroscopy. Figures 3a and b show typical spectra of peptides in solution and membrane environment. α-Helical contents calculated from the molar ellipticity at 222 nm are listed in Table 2. ApoA-I 220—241 WT peptide exhibited structural changes from random coil (a single minimum around 200 nm) to α-helix (double minima at 208 and 222 nm) upon binding to SUV, whereas apoA-I 220—241/L230P did not (Table 2). ApoA-I 220—241/ΔE235 peptide appeared to form different structure from random coil probably because of self-association in solution, and did not show any structural changes upon binding to SUV (Fig. 3b). Given that α-helix formation is thought to be a driving force for lipid binding of apolipoproteins,[9] these results indicate that apoA-I 220—241/L230P and ΔE235 peptides are likely to have an impaired ability to bind to lipid membrane. In contrast, apoA-I 220—241/E235A peptide induced structural changes upon binding to SUV similarly to WT (Table 2). These results suggest that a negative charge ablation in E235 has only a minor influence on lipid binding of the C-terminal region of apoA-I.[11]

To examine the potential propensity to form α-helical structure of these peptides, CD measurements in 80% (v/v) TFE/phosphate buffer were also performed. All peptides formed similar α-helical structure except for apoA-I 220—241/L230P peptide (Table 2). Notably, the spectra of apoA-I 220—241 WT and ΔE235 peptides were almost superimposed (Fig. 3c), indicating that both peptides adopt similar helical structure, consistent with the NMR result (Fig. 2).

Lipid Binding Monitored by Tryptophan Fluorescence Binding of apoA-I 220—241 WT and ΔE235 peptides to SUV was also examined by monitoring tryptophan fluorescence. Since residues 220—241 in the human apoA-I sequence do not contain an intrinsic tryptophan residue, F225 in each of apoA-I 220—241 WT and ΔE235 peptides was substituted with tryptophan. Although this region contains two phenylalanine residues at positions 225 and 229, we chose F225 which is located more closely to polar-nonpolar interface of putative amphipathic α-helix (Fig. 1) because tryptophan residues in membrane proteins are known to be concentrated at the membrane-water interface.[21] CD measurements proved that there was no significant alteration in the lipid-binding property by incorporation of tryptophan residue at this position (data not shown). As shown in Fig. 4a, apoA-I 220—241/F225W peptide has WMF of around 350 nm in solution, characteristic of tryptophan residue in an aqueous environment,[22] consistent with the CD result showing that this peptide is unstructured in aqueous solution (Fig. 3a). In contrast, WMF of the lipid-free apoA-I 220—241/F225W/ΔE235 peptide is around 340 nm, indicating less solvent-exposed environment. Because the CD spectrum of ΔE235 peptide in the lipid-free state indicated different secondary structure from random coil (Fig. 3b), this peptide is likely to be self-associated in solution. Addition of SUV into apoA-I 220—241/F225W peptide caused a great increase in FI accompanied by a blue shift in the WMF (Figs. 4a, b). Such spectral changes are generally explained by the transfer of fluorophore into a less polar and/or motionally more restricted environment.[23] In contrast, there was a much smaller increase in FI for the apoA-I 220—241/F225W/ΔE235 peptide compared to apoA-I 220—241/F225W (Figs. 4a, b).

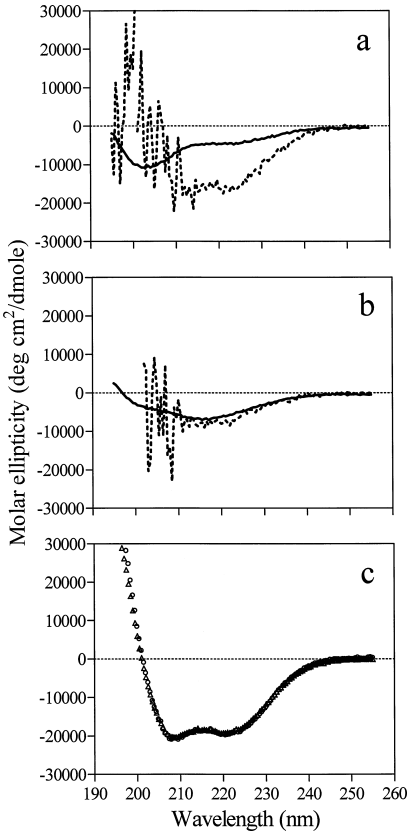

Fig. 3. Far-UV CD Spectra of ApoA-I 220—241 WT (a) and ΔE235 (b) Peptides, in the Lipid-Free State (Solid Lines) or Bound to SUV (Dotted Lines) and (c) CD Spectra of ApoA-I 220—241 WT (○) and ΔE235 (△) in TFE

Table 2. α-Helical Content of ApoA-I 220—241 Peptides[a)]

Peptide	α-Helical content (%)		
	Buffer	SUV	80% TFE/phosphate buffer
WT[b)]	20±2	49±4	57±4
ΔE235	Non-helical	Non-helical	56±4
E235A	16±1	42±3	60±2
L230P	15±2	13±1	44±1

a) Mean±S.D. from at least three independent experiments. *b*) Data are from ref. 13.

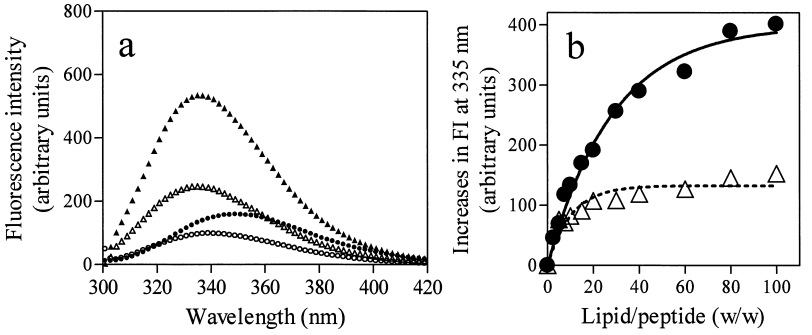

Fig. 4. (a) Fluorescence Emission Spectra

ApoA-I 220—241/F225W in the lipid-free state (●) and bound to SUV (▲); F225W/ΔE235 in the lipid-free state (○) and bound to SUV (△).
(b) Increases in the Fluorescence Intensity of Tryptophan upon Binding to SUV

ApoA-I 220—241/F225W (●), F225W/ΔE235 (△).

Taken together with the finding that apoA-I 220—241/ΔE235 peptide does not form α-helix in the presence of SUV (Fig. 3b), these results suggest that apoA-I 220—241/ΔE235 peptide binds to lipid in a manner distinct from that of apoA-I 220—241 WT peptide, possibly without any conformational changes.

Lipid Microsolubilization Activity It has been suggested that apoA-I spontaneously generates discoidal HDL particles (*i.e.*, microsolubilization) from lipid domains on the cell surface.[24,25] Similarly, a peptide derived from apoA-I α-helical segments is shown to form small HDL-like particles.[7,26] Incubation of apoA-I peptide with phospholipid vesicles at the gel to liquid-crystalline phase transition temperature causes a clarification of the turbidity as a consequence of microsolubilization.[7,8] Thus, the scattered light intensity of DMPC vesicles with incubation of apoA-I peptides was monitored as a function of time (Fig. 5). In the absence of apoA-I peptides, there was no change in the scattered light intensity in 600 s. ApoA-I 220—241 WT and E235A peptides effectively solubilized DMPC vesicles, indicating that the presence of negative charge at a position of E235 is not essential for lipid microsolubilization.[11] In contrast, apoA-I 220—241/ΔE235 and L230P peptides caused no change in the scattered light intensity. It was reported previously that recombinant proapoA-I Nichinan solubilizes DMPC vesicles more slowly than normal proapoA-I.[5] Our present results suggest that the reduced rate of microsolubilization observed in proapoA-I Nichinan is caused by direct influences of the impaired lipid interaction of the C-terminal region. Hydrophobic acyl chains of phospholipid molecules at the edge of discoidal particles are covered with amphipathic α-helices of apoA-I to stabilize the discoidal HDL structure.[27] As shown by CD measurements, apoA-I 220—241/ΔE235 and L230P peptides cannot form the α-helical structure in the presence of SUV (Fig. 3, Table 2). Thus, decrease in helix-forming propensity appears to be related to the impaired lipid microsolubilization activity of these peptides. Microsolubilization process contains complexed, multiple-step interactions between peptides and lipids, including initial lipid binding, helix formation, and rearrangement of lipid molecules.[25,28] Some increases in tryptophan fluorescence upon SUV binding of apoA-I 220—241/F225W/ΔE235 peptide (Fig. 4b) suggest that the initial step, namely, binding to DMPC vesicles may occur even in the case of apoA-I 220—241/ΔE235 peptide without α-helix formation.[29]

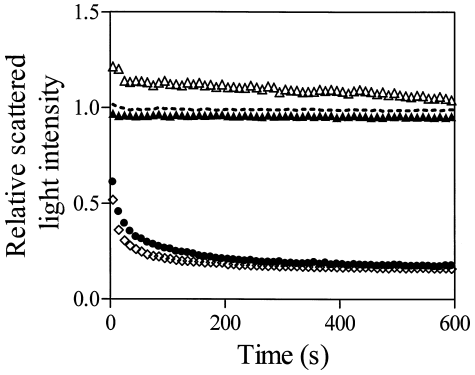

Fig. 5. Decreases in Scattered Light Intensity of DMPC Vesicles (50 μM) Measured at 90° after Addition of Peptides at 24.6 °C

Peptides were added to the vesicles (lipid : peptide = 1 : 1.2 (w/w)). Excitation and emission wavelengths were both set at 600 nm. ●, apoA-I 220—241 WT; △, ΔE235; ◇, E235A; ▲, L230P; dotted line, without peptide

Conclusion

Using synthetic peptides, we examined the influences of the E235 deletion on the α-helical structure and lipid interaction of apoA-I C-terminal region, residues 220—241. NMR and CD measurements revealed that apoA-I 220—241 peptides form similar α-helical structure irrespective of the presence of E235 in helix-inducible solvent, such as TFE. By contrast, E235 deleted variant, apoA-I 220—241/ΔE235 peptide, fails to form HDL-like particles. Lipid interaction of apoA-I 220—241 is unaffected by the negative charge ablation of E235. The self-association properties of apoA-I 220—241/ΔE235 peptide could influence the lipid interaction. Although self-association also occurs when apoA-I 220—241/E235A peptide is prepared at extremely high concentration, it still retained the lipid interaction (data not shown). Therefore, we concluded that the disturbance of amino acid distribution on the helix cross-section directly affects the dysfunction of apoA-I Nichinan, which is likely to be attributed to the impaired α-helix formation and rearrangement of lipid molecules during microsolubilization process. The inability of apoA-I 220—241/ΔE235 peptide to form α-helical structure might be caused by the lack of glutamic acid, which is favorable for α-helix formation.[30] The present reports may provide the underlying mechanism for

low HDL cholesterolemia caused by apoA-I Nichinan variant.

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