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DETERMINING THE FOLDING DOMAIN OF LIPID FREE APOA-I IN SOLUTION BY MUTATION†

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Objectives Heart disease remains the leading cause of death for both women and men in the USA. High blood pressure and elevated plasma cholesterol are two main risk factors for heart disease and lead to atherosclerosis. Plasma levels of high-density lipoproteins, HDL, are negatively correlated with the incidence of atherosclerosis and the mechanisms of the anti-atherogenic effects of HDL are mainly related to its involvement in the pathways of reverse cholesterol transport (RCT). As the major protein component of HDL, apolipoprotein A-I (apoA-I) plays an important structural and functional role in RCT. In order to examine the folding domains of apoA-I and, the N and C terminal functions, WT and two truncated apoA-I forms D (185–243), D (1–59, 185–243) were expressed in *E. coli*, purified and studied by Circular Dichroism, fluorescence spectroscopy and DMPC binding kinetics in the lipid free state.

Methods Generation of Expression Plasmids, Expression and Purification of apoA-I WT and Mutant Proteins. Gateway recombination cloning was used to facilitate the construction of the fusion protein expression vector. PCR products were recombined by Gateway cloning into the donor vector to yield entry clones, then into destination vector to generate fusion expression vectors. The wild type and mutant proteins were overexpressed in *E. coli* BL21 (DE3) cells. All of the purified proteins and plasma apoA-I

used for the experiments were redissolved into 6 M guanidine hydrochloride (GdnHCl) followed by extensive dialysis against 5 mM sodium phosphate, 0.01% EDTA and 0.02% NaN3, pH=7.4 (PB). Protein concentrations in samples were determined by modified Lowry protein assay.

Circular Dichroism Spectroscopy. CD spectra were measured with an Aviv 62DS or Aviv 215 spectropolarimeter equipped with thermoelectric temperature control (Aviv Associates, Lakewood, NJ). Far-UV (250–185 nm) spectra were recorded at 1nm bandwidth and 1 nm step size with 5s accumulation time for each data point at 25°C (a-Helical Content in Lipid Free State. Normalised far-UV spectra of the plasma, WT and apoA-I variants were used to estimate the a-helical content in the secondary structure of the recombinant proteins. Helical residue numbers were estimated from the a-helical content for each recombinant protein. There was 1% not statistically significant difference in a-helical content between plasma and WT apoA-I demonstrating that the expressed WT apoA-I is well folded and has similar secondary structure to plasma apoA-I in the lipid-free state. The C terminal deletion, D (185–243), caused 8% statistically significant increase in a-helical content. Although 59 residues were deleted, only ~15 helical residues are lost from secondary structure suggesting that the majority of the C terminus of apoA-I (185–243) lacks helical structure in the lipid free state and deletion of the C terminus has little effect on N terminus of apoA-I. This result is consistent with our previous studies. Double deletion of N and C termini D (1–59, 185–243) caused 2% statistically significant increase in a-helical content again consistent with our previous studies. The deletion of 118 residues caused the protein to lose ~58 helical residues. Considering that there are only ~15 helical residues lost on deletion of the C terminus alone, there are ~43 additional helical residues lost from N terminus (1–59). This suggests that either there is very high helical content in N terminal region (~73%) or the N terminus is vital to maintain the whole protein secondary structure. Our previous studies of the (1–44) apoA-I peptide have demonstrated that it is unfolded in aqueous solution confirming that the N terminus is vital to stabilise apoA-I lipid free structure.

Thermal and Chemical Unfolding. Both the plasma and WT apoA-I have Tm=62°C further substantiating that the WT apoA-I was well folded and had similar structure to plasma apoA-I. C terminal deletion D (185–243) caused a small (4°C) reduction in Tm compared to WT suggesting a slightly destabilising effect after the deletion of the C terminus. However the DHv increased 15.7 kcal compared to WT suggesting a higher cooperativity during the thermal unfolding perhaps due to deletion of the less structured C terminus. Double deletion of N and C terminal region D (1–59, 185–243) caused a large (24°C) reduction in Tm and DHv (14 kcal) compared to WT suggesting that the N terminus was essential to maintain the structure of apoA-I and deletion of this region decreased the cooperativity during the thermal unfolding, while the C terminus had little effect on the whole structure.

GdnHCl unfolding curves were used to determine the DG^0_D, $D_{1/2}$ and m values. Similar to the thermal unfolding experiments, plasma and WT apoA-I have similar DG^0_D, $D_{1/2}$ and m values due to their similar structure. C terminal deletion D (185–243) led to increase in DG^0_D (0.9 kcal/mol) compared to WT apoA-I again suggesting a stabilising effect after deletion of the C terminus, while the $D_{1/2}$ showed no significant change. Double deletion of N and C termini D (1–59, 185–243) led to significant reduction in DG^0_D (3.46 kcal/mol) compared to WT apoA-I and also in D1/2, ~0.7 M. Again this suggests that the N terminus of apoA-I is vital to maintain the whole structure while the C terminus seems to have little effect.

Near-UV Spectra. The major contribution to the near-UV spectra comes from the aromatic side chains. WT and plasma apoA-I contain four Trp (W8, 50, 72, 108). Plasma and WT exhibited similar near-UV spectra after normalisation to protein concentration and showed a large negative peak at 292 nm that corresponded to the Trp and a smaller peak at 285 nm that corresponded to the Trp and Tyr, suggesting that the WT and plasma apoA-I had similar tertiary structure. The C terminal deletion D (185–243) mutant still processed all four Trp, and showed similar near-UV spectra after normalisation to protein concentration to WT and plasma apoA-I but with deeper peaks at 292 nm and 285 nm. Since the four Trp are located within the first 184 amino acids, deletion of the C terminus did not change the Trp packing environment. Rather it increased the Trp signal in the near-UV spectra suggesting a more rigid environment of the Trp. This suggests that deletion of C terminus did not change the N terminal tertiary structure but made it more compact and that the N terminal structure might represent an independent folding domain. Double deletion of N and C termini D (1–59, 185–243) changed the near-UV spectra significantly, maybe due to loss of two Trp (W8, 50) and/or lack of the defined tertiary structure after the stabilising N terminal has been deleted.

ANS and Trp Fluorescence. Fluorescence of ANS in the presence of the lipid-free WT and variant forms of apoA-I was measured to determine if the mutations affect the exposure of hydrophobic surfaces or cavities of apoA-I. The intrinsic fluorescence of ANS has been shown to be enhanced and blue shifted upon binding to hydrophobic surfaces or cavities, while the water-phase dye does not contribute to the emission. In phosphate buffer, ANS fluorescence has a very low intensity and an emission maximum at 517 nm. In the presence of bovine serum albumin that functions as a fatty acid transporter and has multiple hydrophobic binding pockets, there is a significant (41 nm) blue shift and almost 11 fold enhancement in ANS fluorescence compared to the ANS in phosphate buffer alone. In the presence of plasma or WT apoA-I, ANS emission shows a 40 nm blue shift and ~4.7 fold increase in the intensity compared to the ANS in the buffer alone. In the presence of C terminal deletion D (185–243) apoA-I, ANS emission shows only 27 nm blue shift and ~2.2 fold increase in the intensity compared to the ANS in the buffer alone. This suggests the C terminus of apoA-I has exposed hydrophobic surface and probably lacks defined secondary structure in lipid-free state. In contrast to this region, the N terminus is well packed and the hydrophobic surface is well shielded consistent with the CD data. Double deletion of N and C termini, D (1–59, 185–243) apoA-I, results in ANS emissions that show 41 nm blue shift and ~5.8 fold increase in the intensity compared to the ANS in the buffer alone. Thus deletion of the N terminus of apoA-I exposed more hydrophobic surface than WT, again suggesting the N terminus of apoA-I is vital to main the N terminal domain compact structure and deletion of the N terminus will cause the compact structure to open and expose hydrophobic surface to the solution.

Interaction with BOG. BOG has been widely used as a mild lipid mimicking detergent to induce and stabilise the amphipathic a-helical structure during protein crystallisation. The CMC of BOG at 25°C is 20–25 mM. a-Helical content of both plasma and WT apoA-I increased from ~50% to ~60% as the BOG concentration increased from 0 mM to 50 mM and reached the maximum near the BOG CMC concentration 25 mM. This suggests that BOG can bind plasma and WT apoA-I and induce the a-helical structure by ~10%. This further demonstrates that the expressed WT apoA-I has similar structure to plasma apoA-I. a-Helical structure induction by BOG was not observed for the C-terminal deletion form of apoA-I, suggesting BOG maybe bind with the C terminus of apoA-I and induce the helical structure reaching maximum effect after BOG forms micelles. Most interestingly, D (185–243) lost ~5%

helical content at the CMC, suggesting that BOG may disrupt the more compact structure in the N-terminal domain by binding with the exposed non-structured region and affecting the structured region. Double deletion of N and C termini D (1–59, 185–243) may result in more easy to access by the BOG due to lack of defined tertiary structure and a helical content can be induced from ~50% to ~60% with no clear requirement for micelle formation.

DMPC Turbidity Clearance. Association of lipid free apoA-I with DMPC multilamellar vesicles causes a decrease in the turbidity at 325 nm. This process reflects the kinetics of formation of DMPC-apoA-I complexes. Compared to WT and plasma A-I, C terminal deletion D (185–243) decreased the initial rate of clearance of DMPC liposome turbidity dramatically and almost removed the DMPC binding ability completely. This is consistent with data from other laboratories suggesting that deletion the C terminal of apoA-I is vital to initiate the lipid binding process. The double deletion of N and C terminal D (1–59, 185–243) regained the ability to bind with DMPC at similar rate to WT apoA-I, suggesting that deletion of the N terminus causes exposure of lipid binding regions. Combining these results suggests that the C terminus is vital to initiate the DMPC binding and perhaps opens the N terminal folding domain. Deletion of the C terminus does not affect the N terminal domain compact structure or result in a more compact N terminal folding structure. Deletion of the N terminal 59 residues may cause the compact structure to re-open so that DMPC can bind to the hydrophobic surface. This result are consistent with our BOG binding and ANS florescent experiments.

Conclusions WT and plasma apoA-I exhibited similar secondary a helical conformation and thermodynamic characteristics suggesting that they have similar secondary and tertiary structure in lipid free solution. Deletion of the C terminal region, D (185–243), increased a-helical content by 8% compared to WT with increased unfolding cooperativity suggesting that the C terminus of apoA-I lacks defined structure and may interact with the N terminal region (1–184). Similar near UV spectral shape but increased intensity suggests that deletion of the C terminus of apoA-I has little effect on the tertiary structure of the N terminal region. Thus the N terminal region, 1–184, may form an independent folding domain with more compact structure. Significant decrease of ANS binding fluorescence and DMPC clearance further confirms the less exposed hydrophobic surface consistent with a more compact, helical structure of the folding domain. In contrast to the WT, BOG did not induce a-helical formation in D (185–243) suggesting that the hydrophobic regions are well packed in this domain and the C terminus of apoA-I is exposed to solution and lacks defined structure. Further deletion of the N terminal region, D (1–59, 185–243), caused significant decrease in thermal and chemical stability in far UV and significant change in near UV spectral shape suggesting that the N terminal region (1–59) is vital to maintain and stabilise the N terminal folding domain. ANS binding fluorescent, DMPC clearance and BOG helical induction suggest increased exposed hydrophobic surface after deletion of the N terminal 59 residues of apoA-I suggesting an opening of the N terminal domain. In summary, our experiments indicates lipid free apoA-I in solution adopting a more compact independent N terminal domain with buried hydrophobic surface and less structured C terminal region with exposed hydrophobic surface.