Impact of Self-association on Function of Apolipoprotein A-I*S

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Background: Self-association is an intrinsic property of exchangeable apolipoproteins but an under-explored feature of the major protein of good cholesterol, apolipoprotein A-I.

Result: Different degrees of apolipoprotein A-I self-association exhibit distinct in vitro lipid remodeling and cellular lipid release efficiencies.

Conclusion: Self-association of apolipoprotein A-I modulates the biogenesis of high density lipoprotein.

Significance: This is the first study to demonstrate that self-association of apolipoprotein A-I attunes key steps in reverse cholesterol transport.

Self-association is an inherent property of the lipid-free forms of several exchangeable apolipoproteins, including apolipoprotein A-I (apoA-I), the main protein component of high density lipoproteins (HDL) and an established antiatherogenic factor. Monomeric lipid-free apoA-I is believed to be the biologically active species, but abnormal conditions, such as specific natural mutations or oxidation, produce an altered state of self-association that may contribute to apoA-I dysfunction. Replacement of the tryptophans of apoA-I with phenylalanines (ΔW-apoA-I) leads to unusually large and stable self-associated species. We took advantage of this unique solution property of ΔW-apoA-I to analyze the role of self-association in determining the structure and lipid-binding properties of apoA-I as well as ATP-binding cassette A1 (ABCA1)-mediated cellular lipid release, a relevant pathway in atherosclerosis. Monomeric ΔW-apoA-I and wild-type apoA-I activated ABCA1-mediated cellular lipid release with similar efficiencies, whereas the efficiency of high order self-associated species was reduced to less than 50%. Analysis of specific self-associated subclasses revealed that different factors influence the rate of HDL formation in vitro and ABCA1-mediated lipid release efficiency. The α-helix-forming ability of apoA-I is the main determinant of in vitro lipid solubilization rates, whereas loss of cellular lipid release efficiency is mainly caused by reduced structural flexibility by formation of stable quaternary interactions. Thus, stabilization of self-associated species impairs apoA-I biological activity through an ABCA1-mediated mechanism. These results afford mechanistic insights into the ABCA1 reaction and suggest self-association as a functional feature of apoA-I. Physiologic mechanisms may alter the native self-association state and contribute to apoA-I dysfunction.

Exchangeable apolipoproteins play a central role in lipoprotein assembly, lipid transport, and lipid metabolism by mediating interactions with receptors, enzymes, and lipid-transport proteins. At the secondary structure level, amphiphatic α-helices are the common lipid-binding element of apolipoproteins (1, 2), but a structural landscape more complex than a simple amphiphatic α-helix is required to regulate apolipoprotein lipid binding (3, 4). Structural elements implicated in apolipoprotein function may involve tertiary and quaternary interactions, such as the four-helix bundle, a structural motif that is essential for function in apolipoprotein (apo) E (5–7) and apoA-I (8–10). At the quaternary structure level, propensity for self-association is a widespread feature of apolipoproteins that may also affect function (apoE (11–13), apoA-I (14, 15), apoA-II (14)), apoA-IV (16), and apoCs (17)). In the case of apoE, for instance, wherein the apoE4 allele associates with higher incidence of Alzheimer disease (18), the isoform-specific distribution of self-associated species (tetramers, dimers, and monomers) has been shown to contribute to the different lipid-binding efficiencies of the two major isoforms (apoE3 and apoE4) and has been proposed to participate in the mechanism determining the dysfunctional phenotype associated with apoE4 (19–21).

Although the effect of self-association on apoE structure-function is under scrutiny, much less is known about the influence of self-association on apoA-I function. Increasing our knowledge of this fundamental property of apoA-I has clinical significance because apoA-I plasma levels uniquely predict risk of cardiovascular disease (22–24). One pathway through which apoA-I exerts its beneficial lipid trafficking function involves the plasma membrane transporter ATP-
binding cassette A1 (ABCA1) \(^3\) (25–27). In particular, cholesterol-overloaded macrophages, one of the foremost players in atherosclerosis progression (28, 29), primarily efflux excess cholesterol through an ABCA1-dependent mechanism (30, 31). The main extracellular recipient of ABCA1-mediated cholesterol release is lipid-free apoA-I (32, 33), and minimal lipidation of apoA-I greatly reduces its ABCA1-mediated cholesterol release efficiency (34–37).

Early studies indicated that lipid-free apoA-I self-associates to form dimers, tetramers, and octamers in a concentration-dependent manner and that simple dilution to \(\leq 0.1\) mg/ml disrupts the self-associated species to monomeric apoA-I (15, 38, 39). However, the actual distribution of apoA-I self-associated species in solution is not known, and their presence has been reported even at 0.1 mg/ml (40). In humans, the plasma concentration of apoA-I is \(-1\) mg/ml, where \(-90--95\%\) is HDL-associated and \(-5--10\%\) lipid-free/lipid-poor apoA-I (41, 42). Based on this rough estimation, the general notion is that plasma concentration of lipid-free apoA-I is lower than 0.1 mg/ml. Furthermore, binding of as few as two lipid molecules is sufficient to disrupt apoA-I self-association in favor of monomeric lipid-poor species (39, 43). Thus, based on a probable lipid-free apoA-I concentration below 0.1 mg/ml and the abundance of lipids, monomers have been hypothesized to be the predominant form of lipid-free apoA-I in plasma. However, the true physiological self-association state of lipid-free apoA-I in the subendothelial artery space, wherein apoA-I exerts its main antiatherogenic function, remains elusive.

Importantly, increasing evidence suggest that dysfunctional apoA-1s, either by natural mutations (44, 45) or by oxidative modifications (46), correspond to an altered self-association state that may contribute to the mechanism underlying protein malfunctioning. Recently, the heterozygous apoA-I S36A mutation has been linked to severe reduction in plasma pre-malfunctioning. Recently, the heterozygous apoA-I S36A modification (46), correspond to an altered self-association antiatherogenic function, remains elusive. The above cases suggest that increased or stabilized self-association can be a negative determinant of apoA-I lipid binding efficiency, whereas reduced native self-association may affect the stability of apoA-I structure in solution. The two opposite alterations both have dysfunctional consequences, and it can be hypothesized that maintenance of the native self-association state of lipid-free apoA-I is essential to preserving different aspects of apoA-I functionality.

Here we compared the structural and functional properties of wild-type apoA-I (WT-apoA-I) and a Trp to Phe variant (ΔW-apoA-I) with enhanced propensity to self-association. Characterization of ΔW-apoA-I structure-function showed that modifications of specific apoA-I residues can cause global structural rearrangements that propagate to the quaternary structure level with striking functional consequences. Interestingly, ΔW-apoA-I retains wild-type-like lipid-binding and ABCA1-mediated cellular lipid release efficiencies when monomeric or in its low self-association state but displays severely reduced activity when in a high self-association state. These results demonstrate for the first time the effect of self-association on the efficiency of apoA-I as a cellular lipid recipient. Furthermore, structure-function data for specific ΔW-apoA-I self-associated subclasses permit novel conclusions about the structural requirements underlying ABCA1-mediated cellular lipid release.

**EXPERIMENTAL PROCEDURES**

*Generation, Expression, and Purification of Protein Variants—*

Primer-directed PCR mutagenesis was used to introduce single amino acid mutations within human apoA-I cDNA as described previously (50). Multiple Trp to Phe mutations were generated one by one using the mutated DNA as template for the following primer-directed PCRs. DNA sequences were confirmed by automated sequencing and subcloned into the pET-20b bacterial expression vector (Novagen). ApoA-I proteins were expressed in BL21 *E. coli* (Invitrogen) and purified by nickel affinity chromatography as described previously (51). Protein masses were verified by SDS-PAGE and MALDI-TOF mass spectrometry (29,798 and 29,642 Da for WT- and ΔW-apoA-I, respectively). Further details about the protein sequence are reported in the supplemental material.

*Protein Stock Solution Preparation—*

Stock solutions of WT- and ΔW-apoA-I were prepared as follows. In WT[High] and ΔW[High], WT- and ΔW-apoA-I, respectively, were refolded at high concentration (\(-3.0\) mg/ml) by dialysis versus sodium phosphate buffer (20 mM, pH 7.4) (PB) just after affinity column purification. WT[Low] and ΔW[Low] were derived from “High” samples by overnight incubation at 37 °C in the presence of 3 M guanidine HCl (GdnHCl) as denaturant (52). The denatured samples were refolded at \(-0.1\) mg/ml by dialysis versus PB with three buffer changes. All stock solutions were stored at 4 °C. The concentration of each preparation were stored “High” and

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\(^3\) The abbreviations used are: ABCA1, ATP-binding cassette A1; PB, sodium phosphate buffer; GdnHCl, guanidine HCl; NDGGE, non-denaturing gradient gel electrophoresis; SEC, size exclusion chromatography; DSC, differential scanning calorimetry; MLV, multilamellar vesicles(s); DMPC, 1,2-dimyristoyl-sn-glycerol-3-phosphocholine; PL, choline-phospho-olipid(s); FC, free cholesterol; MPO, myeloperoxidase.
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“Low” stock solutions was determined by amino acid analysis with an accuracy of ±2% (Molecular Structure Facility at Genome Center, University of California, Davis, CA) and confirmed by the BCA assay (Pierce). After storage at 4 °C and prior to each experiment, concentration of the stock solutions and protein integrity were checked by BCA and SDS-PAGE, respectively. Protein stock solutions were prepared in alternative buffer systems: Tris-buffered saline (TBS; 8.2 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0) and sodium phosphate-buffered saline (PBS; 20 mM phosphate, 500 mM NaCl, pH 7.4). No differences in protein stabilities and self-association properties were detected in these buffers.

Non-denaturing Gradient Gel Electrophoresis (NDGGE)—Novex™ 4–20% Tris-glycine gels (Invitrogen) were loaded (53). Staining by GelCode Blue under non-denaturing conditions in Tris-glycine buffer (250 mM Tris base, 2.5 mM glycine, pH 8.35). Staining by GelCode Blue (Pierce) and imaging were performed as described previously (53).

Size Exclusion Chromatography (SEC)—Protein analysis and isolation of self-associated species were performed on a Superdex 200 prep grade XK 16/100 column and/or a Superose 10/300 GL column (GE Healthcare) controlled by an AKTA UPC 10 FPLC system (GE Healthcare). Elution by PB was performed at flow rates of 1.5 and 0.5 ml/min for Superdex 200 and Superose 6 columns, respectively.

Identity of Lipid-free ΔW-apoA-I Self-association Sub-classes—Initial estimation of the identity of individual self-associated species was performed by chemical cross-linking by bis(sulfosuccinimidyl) suberate (BS3) and SDS-PAGE analysis, as described previously (40, 54). An increase in the molecular weight of cross-linked species was observed upon cross-linking of higher order self-association species (data not shown). However, interpretation of chemical cross-linking results, specifically for lipid-free apolipoproteins, is not straightforward, because the amount and nature of cross-linked species depend on reagent concentrations and reaction conditions (stoichiometry, temperature, and incubation time). At high cross-linker/protein ratios, inter-oligomer cross-linking may produce apparent high molecular weight species that are not present in the native mixture (55). At low cross-linker/protein ratios, non-exhaustive cross-linking may not bind all of the protein units present in the higher molecular weight species. These two possibilities cannot be completely ruled out in any experimental conditions; thus, chemical cross-linking is a weak technique for secure identification of self-associated species of lipid-free apolipoproteins. To identify the self-associated species, we rather relied on the following three independent techniques: SEC, with both Superose 6 and Superdex 200 columns; NDGGE with native protein standards; and dynamic light scattering. In SEC experiments, the apparent mass of protein samples was determined by comparing their distribution coefficients (Kav) with those of globular proteins of known mass as described in the supplemental material. Wherever applicable, dynamic light scattering (see supplemental material) was used to confirm the molecular weight of the self-associated species.

Far UV Circular Dichroism (CD) Spectroscopy—CD data were recorded using AVIV 400 or AVIV 62DS spectropolarimeters with thermoelectric temperature control. Unless mentioned otherwise, far-UV CD spectra (185–250 nm), and melting data were recorded from 20 µg/ml protein solutions in PB.

The CD data were normalized to protein concentrations and reported as molar residue ellipticity, [Θ]. Protein α-helical content was estimated from the molar residue ellipticity at 222 nm ([Θ]222) using the equation, % α-helix = \((\frac{\Theta_{222} - \Theta_{3000}}{\Theta_{3000}}) \times 100\) (56). In melting experiments, CD data [Θ]222 (T) were recorded upon sample heating from 5 to 98 °C at a constant rate of 80 K/h. The reversibility of thermal unfolding was checked by cooling the protein to 5 °C and repeating the temperature scan for the same sample at the same scan rate.

Differential Scanning Calorimetry (DSC)—Excess heat capacity, C\(\text{p}\)(T), was measured using a VP-DSC Microcal differential scanning microcalorimeter (Northampton, MA). WT[High] and ΔW[High] stock solutions where diluted to 0.5 mg/ml in PB and rapidly degassed just before DSC analysis. Data were recorded during sample heating from 5 to 98 °C at a rate of 90 °C/h, corrected for buffer base line, and normalized to protein concentration. No protein precipitation was visually observed after temperature scanning. However, to test for potential protein loss by precipitation from solution, post-DSC samples were centrifuged at 10,000 × g, and protein concentration was measured by BCA analysis. Sample protein concentrations before and after DSC were within the experimental error, suggesting that exposure of proteins to high temperature did not lead to any significant protein loss from solution.

Phospholipid Clearance Assay—ApoA-I-induced clearance of multilamellar vesicles (MLV) of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was monitored at 25 °C by turbidity using a Varian Cary-300 UV-visible spectrophotometer equipped with thermoelectric temperature control. DMPC-MLV were produced as described (57). The DMPC-MLV suspension (1 ml) was placed in a 1-cm path length quartz cell and equilibrated at 25 °C for 5–10 min, during which the turbidity was recorded at 325 nm with an averaging time of 5 s as described (45). No significant changes in turbidity were detected during equilibration. The clearance experiment was initiated by briefly stopping the data recording and adding a small volume of the stock protein solution to final lipid and protein concentrations of 80 and 20 µg/ml, respectively. The sample was mixed gently, and the turbidity measurements were immediately resumed and continued for 1 h.

Reconstitution of DMPC-HDL Particles—DMPC-HDL particles were reconstituted by incubating ΔW[High], ΔW[High]60, and ΔW[High]90C with DMPC-MLV (1:4, w/w) overnight at 25 °C. After incubation, the samples were spun down to pellet any unbound lipid (not soluble at the incubation condition used), and total lipid and protein contents were quantified in the supernatant by Bartlett and BCA assays, respectively. The final protein:lipid content was similar (~1:100 molar ratio) for all reconstituted HDL samples, irrespective of the self-associated state of apoA-I. Furthermore, lipid-free monomeric apoA-I was not detected in the samples by NDGGE and SEC analysis after 24 h of incubation (data not shown). Taken together, these observations suggest that the amount of lipidated protein was similar in the three samples.
Electron Microscopy (EM)—DMPC–particles were analyzed by photophototryptic negative staining EM under low dose conditions in a CM12 transmission electron microscope (Philips Electron Optics) as described (58). 200–250 particles/micrograph were assigned to five different diameter classes (7.2, 9.6, 12.0, 14.4, and >14.4 nm) by determining their diameters with an accuracy of ± 1.2 nm.

Preparation of Lipid-free ΔW-apoA-I Self-association Subclasses—Distinct self-association ΔW-apoA-I subclasses were prepared either by thermal treatment of ΔW[High] (tetramers and monomers) or isolation from ΔW[High] by SEC (hexadecamers and octamers) as described in the supplemental material.

ABCA1-mediated Cellular Lipid Release—HEK293 cells stably expressing human ABCA1-EGFP fusion protein (293/2c) (59) were maintained in a 1:1 (v/v) mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (DF medium) supplemented with 10% (v/v) fetal calf serum (FCS). Cells were subcultured in a 6-well tray in 10% FCS-DF medium at 1.2 mrn/hr with 10% (v/v) fetal calf serum (FCS). Cells were bly expressing human ABCA1-EGFP fusion protein (293/2c)

Table 1: Superdex 200 SEC distribution coefficients (Kav) of the main peaks of WT-apoA-I and ΔW-apoA-I samples

| Protein          | SEC distribution coefficient (Kav) | α-Helical content | [α]222/[α]356° |
|------------------|-----------------------------------|-------------------|-----------------|
| WT[High]         | 0.23                              | 0.32*             | 0.35*           |
| WT[High] → 0.1 mg/ml |                                   |                   | 0.45*           |
| WT[Low]          | 0.46                              | 0.32              | 0.47            |
| WT[High]60C      | 0.32*                             |                   | 0.39*           |
| WT[High]90C      | 0.32                              |                   | 0.42            |
| ΔW[High]         | 0.12                              | 0.21              | 0.13            |
| ΔW[High] → 0.1 mg/ml |                                   |                   | 0.12            |
| ΔW[Low]          | 0.22                              | 0.32              | (0.32)          |
| ΔW[High]60C      | 0.21                              | 0.32              | (0.45)          |
| ΔW[High]90C      | 0.21                              | 0.32              | 0.43            |
| X16              | 0.21                              |                   |                 |
| X8               |                                   |                   |                 |
| X4               |                                   |                   |                 |
| X3               |                                   |                   |                 |
| X2               |                                   |                   |                 |

α-Helical content was calculated from the molar ellipticity at 222 nm with 5% accuracy, which includes variability from different samples (at least three independent sample preparations) and instrument variability.

The ratio [α]222/[α]356° was estimated within an error of ± 0.02 from 10 different spectra per experimental condition.

RESULTS

Unique Self-association Properties of ΔW-apoA-I—Self-association of WT-apoA-I and over 300 full-length apoA-I variants incorporating single or multiple Trp → Phe, Xaa → Trp, Xaa → Cys, Met → Leu, and Tyr → Phe were compared by NDGGE and SEC analyses. In particular, altered states of self-association, such as a different size or more resolved self-associated species, were screened. Among this large sample of point mutations, only substitution of the four native tryptophans (Trp-8, Trp-50, Trp-72, and Trp-108) with slightly more hydrophobic phenylalanines (ΔW-apoA-I) significantly altered the self-association behavior of the protein variant (Table 1 and Fig. 1). In agreement with previous studies (43), at high concentration, WT-apoA-I (WT[High] ~ 3.0 mg/ml) ran as a diffused band encompassing native globular markers with sizes of 8.2 and 7.1 nm in diameter (Fig. 1A, lane 1). Similarly, a majority of WT[High] eluted by SEC as a broad peak with three main unresolved components at Kav = 0.32, 0.35, and 0.45 (Fig. 1B, black line). By data regression analysis using globular protein markers as calibration standards, we identified these species as tetramers, trimers, and monomers, respectively, of WT-apoA-I. In contrast, ΔW-apoA-I self-associated species focused by NDGGE in two main bands running at about 12.2- and 9.5-nm apparent globular diameters (Fig. 1A, lane 4) and eluted by SEC as two sharp and well resolved peaks at Kav = 0.12 and 0.21 (Fig. 1C, black line), corresponding to hexadecamers and octamers. To our knowledge, such a high order of self-association has not been reported for any other full-length apoA-I variants.

Effect of Concentration on Self-association of ApoA-I Variants—In WT-apoA-I, self-association was readily disrupted by dilution to ~0.1 mg/ml (Fig. 1B), in accordance with the largely accepted notion that self-association of apoA-I is concentration-dependent (15, 38). In fact, the single narrow peak at Kav = 0.47 ml in the SEC chromatogram of diluted WT[High] (Fig. 1B, gray line, and Table 1) represents WT-apoA-I monomers. Strikingly, the NDGGE and SEC pro-
files of ΔW[High] were virtually unchanged by dilution to ~0.1 mg/ml in the same conditions (Fig. 1, A (lanes 4 and 5) and C (black versus gray lines) and Table 1). Upon denaturation by GdnHCl and refolding at 0.1 mg/ml, WT-apoA-I (WT[Low]) exists predominantly as monomer (Fig. 1, A (lane 3) and B (light gray line) and Table 1). Interestingly, in ΔW[Low], high molecular weight oligomers reassembled rapidly. By NDGGE and SEC analysis, five species were clearly visible in ΔW[Low] stored at 4 °C for 48 h (Fig. 1, A (lane 6) and C (light gray line) and Table 1), with predominance of octamers. Taken together, SEC and NDGGE analysis revealed the propensity of ΔW-apoA-I to self-associate in large oligomeric species even at low protein concentration conditions.

Secondary Structure Analysis of ApoA-I Variants—To determine the effect of self-association on the secondary structure of apoA-I, we performed a systematic far-UV CD analysis. In the CD spectra of both WT- and ΔW-apoA-I, a double negative minimum at 208 and 222 nm and a positive maximum at 190 nm indicate the presence of an α-helical conformation (Fig. 2A). The estimated α-helical content of WT[High] (~60%) was similar to previously reported values under the same conditions (15, 61), whereas the ~82% α-helical content of ΔW[High] was significantly higher and comparable with the α-helicity of apoA-I on reconstituted discoidal HDL (15, 61, 62).

Upon dilution in the 1.00–0.01 mg/ml range, a small but significant loss of α-helical content was measured in WT[High] (Fig. 2B), consistent with a previous report of Massey et al. (63), wherein a reduction in α-helical content was observed consequent to disruption of plasma apoA-I self-association. Loss of α-helical content (Fig. 2B) alongside self-association (Fig. 1, A and B) upon dilution of WT[High] suggests a relationship between these two properties of apoA-I. Interestingly, dilution did not change either self-association (Fig. 1, A and C) or α-helical content (Fig. 2B) of ΔW[High], confirming an interplay between self-association and α-helicity.

Thermal Stability of ApoA-I Variants—To assess the combined effects of high self-association and α-helicity on protein thermal stability, we analyzed ΔW-apoA-I and WT-apoA-I by two independent methods: CD spectroscopy (Fig. 3) and calorimetry (Fig. 4 and supplemental Fig. S2A).

Heat-induced changes in protein secondary structure were measured by CD spectroscopy at 222 nm. Before starting the melting experiments, protein stock solutions were diluted to 20 μg/ml, wherein WT[High] is monomeric (Fig. 1). Thermal denaturation of WT-apoA-I monomers was reversible and with apparent melting temperature of ~63 °C (Fig. 3, A and B, black lines), which is similar to previously published data (56). The melting curve and the apparent melting temperature of a second temperature scan were not significantly different from the first melting results (Fig. 3, A and B, black lines), indicating that WT-apoA-I monomers denatured and refolded within the time scale of the consecutive melting experiments. Although the apparent melting temperature of ΔW[High] (~63 °C; Fig.
3D, black line) was similar to that of WT[High], nearly 30% of the α-helical content was lost upon cooling to 25 °C. The irreversible nature of the heat-induced transition is probably due to disruption of high order self-associated species. Consistent with this interpretation, the melting curve of a second temperature scan was completely reversible (Fig. 3C, light gray line), confirming that the self-associated species were disrupted by the high temperatures at the end of the first heating cycle and did not reform in the time scale of the consecutive melting. It is notable that the apparent melting temperature in the second scan of ΔW[High] (~58 °C, Fig. 3D, light gray line) was ~5 °C lower than the first melting temperature (Fig. 3D, black line). Similar to the increased reversibility, the lower apparent melting temperature in the second scan suggests that the self-associated species are disrupted upon completion of the first heating cycle, and the second melting curve represents the reversible unfolding of less stable ΔW-apoA-I monomers.

To compare the thermodynamic stability of ΔW-apoA-I and WT-apoA-I self-associated species, we analyzed ΔW[High] and WT[High] by DSC at higher protein concentrations (0.5 mg/ml). In DSC of WT[High], endothermic transitions occurred at ~63 °C (main peak) and ~51 °C (shoulder) (Fig. 4A, black line). To investigate the nature of these transitions, we performed a second temperature scan on the same sample, wherein self-association had probably been disrupted by the high temperature at the end of the first heating cycle. In the second DSC scan, the absence of a shoulder at ~51 °C demonstrates that this transition is related to dissociation of self-associated species and that the DSC peak at ~63 °C represents melting of the α-helices of WT-apoA-I monomers (Fig. 4A, light gray line).

In contrast to WT[High], only one peak with a maximum at ~63 °C was observed in the DSC of ΔW[High] (Fig. 4B, black line). The absence of transitions at lower temperatures indicates that self-associated species in ΔW-apoA-I are more stable than in WT-apoA-I. In a second DSC scan of ΔW[High], the peak was broader and shifted to lower temperature with a maximum around ~56 °C (Fig. 4B, light gray line). This temperature change suggests that during the first DSC scan, both α-helix melting and dissociation of self-associated species contribute to the observed endothermic transition at ~63 °C, whereas in the second DSC scan, only melting of the α-helices of monomers is observed. Interestingly, the different melting temperatures of the second CD and DSC scans of ΔW[High] and WT[High] (~56–58 °C and ~63 °C, respectively; Figs. 3 and 4) suggest that the α-helical structure in ΔW-apoA-I monomers is less stable than in WT-apoA-I monomers.

When measured by pressure perturbation calorimetry, the volume changes accompanying protein unfolding were remarkably higher for ΔW[High] than for WT[High] (ΔV/V = ~0.95 and ~0.3%, respectively) (supplemental Fig. S3), and other globular proteins (64). In agreement with the DSC results, these unusually large volume changes in ΔW[High] may be due to the combined effect of secondary structure unfolding and self-association disruption, which contribute to increasing the solvent exposure of charged groups otherwise restricted in a solvent-excluded environment within self-associated species. Consistent with this view, a significant reduction in the magnitude of volume change was observed in the second consecutive pressure perturbation calorimetry scan of ΔW[High] (data not shown).

Taken together, the thermal stability measurements indicate that the Trp to Phe mutations partially destabilize the α-helical structure of apoA-I monomers and prompt formation of self-associated species that are more stable than those in WT-apoA-I.

Isolation and Analysis of Low Order ΔW-apoA-I Self-Associated Species—The thermal stability studies (Figs. 3 and 4) suggested that heating of ΔW[High] above the transition temperature disrupts high order self-associated species. To systematically investigate the effect of heating on self-associated species, we treated ΔW[High] and WT[High] at different temperatures for 1 h, followed by NDGGE and SEC analysis (Fig. 5). Upon thermal treatment, no protein degradation was detectable by SDS-PAGE analysis (data not shown).

Heating at 37 °C did not significantly change the self-association pattern of ΔW[High] and WT[High] (Fig. 5A). In con-
contrast, heating WT[High] at 60 °C, which is above the WT-apoA-I self-associated species transition temperature, led to a significant increase in low order self-association species (in the monomer to tetramer range), as shown by NDGGE and SEC analyses (Fig. 5, A and B, gray line). Unlike WT[High], 60 °C treatment of ΔW[High] (ΔW[High]60C) disrupted octamers and hexadecamers toward only one species with an apparent globular protein size of 7.5 nm (Fig. 5A) and SEC $K_{av}$ of 0.32 (Fig. 5C, gray line), corresponding to a tetramer of ΔW-apoA-I. The presence of tetrarmers in ΔW[High]60C was further confirmed by dynamic light scattering measures (supplemental Table S1). Although we observed no significant amount of monomeric species in ΔW[High]60C, heating at 90 °C resulted in largely monomeric forms of both WT- and ΔW-apoA-I ($K_{av}$ = 0.42 and 0.43, respectively; Table 1 and Fig. 5, B and C, light gray lines). These results suggest thermal treatment as a simple tool to produce individual low order self-associated subclasses from otherwise stable high order ΔW-apoA-I self-associated species.

Analysis of the α-helical content of heated samples illustrates the correlation between protein self-association and secondary structure in individual self-association subclasses. Whereas the α-helical content of WT[High] converged to 52–53% after heating at 60 or 90 °C, the α-helicity of ΔW[High] was remarkably proportional to the degree of self-association: hexadecamers/octamers (ΔW[High]), 82%; tetramers (ΔW[High]60), 72%; monomers (ΔW[High]90), 54% (Fig. 5A). Thus, upon disruption of self-association, the secondary structures of monomeric ΔW-apoA-I and WT-apoA-I were similar. Furthermore, a large structure rearrangement occurs upon self-association in tetramers because these were 18% more α-helical than monomers, whereas only an extra 10% α-helicity was acquired upon formation of octamers/hexadecamers. The increased stability of higher order self-associated spe-
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Effect of Self-association on Lipid Binding—Massey et al. observed increased rates of DMPC clearance when plasma apoA-I self-association was disrupted by performing the DMPC clearance experiment in the presence of 0.5 M GdnHCl (63). Although no changes in secondary structure were reported at these denaturant concentrations, effects to the tertiary structure level with potential consequences on protein’s lipid-binding efficiency cannot be ruled out. In fact, small but significant differences in the near-UV CD spectra of WT-ApoA-I incubated for 1 h with and without 0.5 M GdnHCl are indicative of tertiary structure changes (data not shown). In the current study, we took advantage of the unique self-association properties of ΔW-apoA-I to test the dependence of lipid-binding kinetics on different degrees of apoA-I self-association in completely native conditions. Remarkably, DMPC clearance rates increased proportionally to the reduction in self-association levels (Fig. 6, A and B). Furthermore, lipid-binding kinetics of monomeric ΔW-apoA-I (ΔW[High]90C) and WT-apoA-I (WT[Low]) were comparable (Fig. 6, B and A, respectively). This result demonstrates that the introduction of four amino acid mutations in ΔW-apoA-I did not significantly change the lipid-binding efficiency of the protein in its monomeric form, making ΔW-apoA-I a reliable model for studying the effect of self-association on apoA-I function as a recipient of cellular lipid release.

Size and Morphology of DMPC-HDL Particles—By negative staining EM (Fig. 7), high order self-associated species in ΔW[High] (hexadecamers and octamers) produced particles heterogeneous in size but significantly larger (the most represented diameter class was 12.0 nm) than those generated by ΔW[High]60C and ΔW[High]90C. Surprisingly, monomeric ΔW[High]90C formed particles with the broadest distribution of diameters (7.2–14.4 nm) (Fig. 7C). This observation suggests that a variable number of monomers can associate on the MLV surface and produce particles of diverse protein-lipid stoichiometry.

By analysis of the amount of rouleau formation, higher order self-associated species (Fig. 7A) produced relatively fewer discoidal particles than tetrameric and monomeric apoA-I (Fig. 7, B and C, respectively). It is notable that tetramers (ΔW[High]60C) yielded a narrow particle size distribution centered at the 9.6-nm diameter class. Based on size and morphology, these particles probably contain two apoA-I molecules per particle (53).

Because the amount of lipidated protein was similar in the three samples (see “Experimental Procedures”), the observed differences in particle morphology and size are probably due to the different self-association states of the initial protein component.

Evaluation of the ratio of molar residue ellipticity at 222 and 208 nm ([θ]222/ [θ]208) yields useful information on the nature of these tertiary and quaternary interactions. The [θ]222/ [θ]208 ratio provides an estimate of the level of coiled-coil inter-α-helical contacts because the ratio is <1.00 for non-interacting α-helices and >1.00 in the presence of two-stranded coiled-coils (65, 66). The [θ]222/ [θ]208 ratio was within the 0.81–0.88 range for all WT-apoA-I samples at any protein concentration tested (Table 1 and Fig. 2B), suggesting that in WT-apoA-I, no coiled-coil structure is formed at any degree of self-association. Likewise, the ratio was 0.83–0.85 for monomeric or self-associated ΔW-apoA-I up to tetramers. Strikingly, the [θ]222/ [θ]208 ratio was 1.03 for ΔW[High], which is composed of octamers and hexadecamers and 1.01 for ΔW[Low], which mainly contains octamers and hexadecamers together with a small amount of lower order self-associated species. Thus, in contrast with the mechanism of self-association of apoE, where coiled-coil interactions contribute to the formation of low order self-associated species, such as dimers (13), in ΔW-apoA-I, coiled-coil interactions participate in the stabilization of octamers/hexadecamers but are absent in lower order self-associated species.

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Because the amount of lipidated protein was similar in the three samples (see “Experimental Procedures”), the observed differences in particle morphology and size are probably due to the different self-association states of the initial protein component.
Impact of Self-association on ApoA-I-mediated Cellular Lipid Release—Lipid-free apoA-I is known to activate cellular lipid release through an ABCA1-dependent pathway (30, 34). To study the effect of self-association on the efficiency of apoA-I as the recipient of ABCA1-dependent cellular lipid release, four distinct self-association subclasses of ΔW-apoA-I (namely hexadecamers, octamers, tetramers, and monomers) were prepared as described under “Experimental Procedures” (Fig. 8 and supplemental Fig. S1). We measured the cellular lipid release efficiencies of the four apoA-I self-association subclasses by incubating 293/2c cells expressing high levels of ABCA1 in the presence of increasing concentrations of apolipoproteins.

Under lipid release subsaturation conditions (apoA-I ≤15 μg/ml), both cell culture medium PL and FC levels were reduced to ≤50% in cells treated with ΔW[High] or hexadecameric ΔW-apoA-I (ΔW[High] SEC F1) when compared with WT[High], which is predominantly monomeric at these incubation conditions (Fig. 9). Lipid release levels of octameric ΔW-apoA-I (ΔW[High] SEC F3) were higher than for ΔW[High] or hexadecameric ΔW-apoA-I but still 50–70% of monomeric WT-apoA-I. Remarkably, tetrameric (ΔW[High]60C) and monomeric (ΔW[High]90C) ΔW-apoA-I did not vary significantly from monomeric WT-apoA-I in activating PL and FC release.

As a control, WT[High] was heated at 90 °C (WT[High]90C) at the same conditions as for ΔW[High]90C, and its cellular lipid release activity was measured at 2, 5, and 10 μg/ml. At most of the conditions tested, WT[High]90C was similar to WT[High], ΔW[High]60C, and ΔW[High]90C in activating cellular lipid release. We also evaluated non-ABCA1-mediated lipid release by HEK293 cells, which do not express ABCA1. In this cellular system, none of the apolipoproteins at any of the concentrations tested was able to activate PL or FC release at levels significantly different from control samples incubated in the absence of apolipoproteins (data not shown). Cellular lipid release results indicate that the ABCA1-mediated lipid release efficiency of apoA-I is severely impaired by formation of stable quaternary interactions in high order self-associated species, but disruption of self-association at or below the tetrameric level restores efficiency to levels similar to those of monomeric WT-apoA-I.

DISCUSSION

Tryptophans appeared in the apoA-I sequence relatively recently in evolution (67); one Trp (corresponding to human Trp-108) is conserved in the apoA-Is of terrestrial vertebrates, but all known fish apoA-Is have no tryptophans. Interestingly, the other three tryptophans of human apoA-I are specific to mammals and are extremely well conserved in this class (67, 68). Thus, we speculated that the acquisition of the four tryptophans samples were loaded on gel (3 μg/lane), and electrophoresis was performed overnight (≥16 h) (A). Molecular weight markers were the High Molecular Weight Calibration Kit (GE Healthcare). Shown is SEC analysis after thermal treatment of WT[High] (B) and ΔW[High] (C). Black lines, unheated samples; gray lines, samples within 24 h from 60 °C treatments; light gray lines, samples within 24 h from 90 °C treatments. α-Helical content was calculated as described under “Experimental Procedures” and the legend to Table 1. mAU, milliabsorbance (280 nm) unit.
The current work suggests that this property may involve the modulation of self-association. Monomeric lipid-free apoA-I comprises a largely unstructured C terminus (residues 187–243) and a N-terminal four-helix bundle (residues 1–186) (52, 69–72), in which tryptophans participate in the stabilization mechanism (62). Edmundson helical wheel projections of the three tryptophans predicted to be in α-helical regions (Trp-50, Trp-72, and Trp-108) (57, 73) locate the tryptophans within the non-polar face of amphipathic α-helices (Trp-50 and Trp-108) or close to the interface between the polar and non-polar faces (Trp-72) (supplemental Fig. S4) (67, 74). Consistent with this observation, solvent accessibility and Trp fluorescence measurements of monomeric lipid-free apoA-I demonstrate that the tertiary

FIGURE 6. Effect of disruption of apoA-I self-association by denaturation/dilution (A) or thermal treatment (B) on DMPC clearance rates. Results from at least three experiments with independent protein preparations were comparable. a.u., arbitrary units.

FIGURE 7. EM analysis of DMPC particles produced by different apoA-I self-associated species. Top panels, electron micrographs. Bottom panels, percentage of particles in each micrograph assigned to the different diameter classes. A, ∆W[High]/DMPC; B, ∆W[High]60C/DMPC; C, ∆W[High]90C/DMPC.
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structure folding provides a hydrophobic environment for all four tryptophans (62, 74, 75). This suggests that tryptophans are directly involved in interhelical interactions within the four-helix bundle. Although they share aromatic characteristics, Trp is considerably less hydrophobic than Phe (76). Thus, replacing tryptophans with phenylalanines may affect the interhelical arrangement of apoA-I. In fact, Trp to Phe substitutions reduced the stability of apoA-I monomers (62). In the work described here, mutation of all four native tryptophans to phenylalanines had a dramatic effect on the overall protein structure. In fact, lipid-free ΔW-apoA-I was composed exclusively of hexadecamers and octamers and about 82% α-helical, a marked 22% increase compared with WT-apoA-I at the same concentration and similar to the α-helicity of apoA-I in nascent HDL.

The N-terminal domain of monomeric lipid-free WT-apoA-I is predominantly α-helical (52, 70, 71), and thus the high α-helicity of self-associated ΔW-apoA-I (~200 of the 243 residues) must involve a propagation of the α-helical character to the largely unfolded C terminus (52, 72). This result is consistent with the notion that self-association of apoA-I is the structural response of an otherwise unstructured C-terminal domain, wherein hydrophobic residues seek protection from solvent exposure by establishing new intermolecular contacts (15, 77).

Furthermore, the only two self-associated species present in native ΔW-apoA-I are significantly larger and more stable than the self-associated species of WT-apoA-I. The increased stability of ΔW-apoA-I hexadecamers/octamers is illustrated by a dissociation temperature higher than the melting temperature of the residual α-helices in monomeric ΔW-apoA-I (Figs. 4 and 5), which suggests that their structure is stabilized through strong intermolecular and, potentially, new intramolecular (tertiary) contacts.

We speculate that the Trp to Phe substitutions destabilize the N-terminal four-helix bundle, exposing hydrophobic residues to solvent and potentially disrupting N terminus-C terminus interactions. This destabilization is compensated for by an overall structural rearrangement where C-terminal random coil regions fold in amphipathic α-helices, which participate in intermolecular hydrophobic interactions. Further supporting evidence is provided by our finding that formation of coiled-coil structures occurs exclusively in octamers/hexadecamers, indicating that interhelical interactions contribute to the stabilization mechanism in high order self-associated species.

Although we cannot exclude the possibility that specific structural features of the self-associated species generated by the Trp to Phe substitutions are unique to this variant, ΔW-apoA-I serves as a model for studying the gross effect of self-association on apoA-I biological function. In fact, the large self-association-dependent structural rearrangement had striking consequences for the functional activity of ΔW-apoA-I. The DMPC clearance and cellular ABCA1-mediated lipid release efficiencies of monomeric ΔW-apoA-I were not significantly different from the activity of monomeric WT-apoA-I, indicating that modification of the apoA-I primary sequence by Trp to Phe substitutions did not exert any direct influence on the main function of apoA-I. In contrast, DMPC clearance rates and ABCA1-dependent lipid release efficiencies of self-associated ΔW-apoA-I species were significantly reduced.

It has been proposed that the membrane insertion of the loosely folded C terminus of monomeric apoA-I is a requirement for maintaining the HDL-forming activity of apoA-I through a structural rearrangement encompassing the whole molecule (52, 78). Within the process of HDL formation from PL vesicles in vitro, binding of lipid-free apoA-I to the vesicle surface is an essential step that is driven by a large enthalpic contribution provided by the transition of non-helical residues to an α-helical conformation (70, 79–82). The large number of α-helical residues in high order ΔW-apoA-I self-associated species (~200 of 243) indicates that their ability to form new α-helices is greatly reduced. Remarkably, the DMPC clearance rates of ΔW-apoA-I were proportional to the number of residues in non-α-helical conformation from hexadecamers/octamers (~18% non-α-helical) to tetromers (~28%) and monomers (~46%). However, in a cellular model where lipid release is ABCA1-dependent, apoA-I tetromers and monomers exhibited similar lipid release efficiencies, despite an 18% difference in α-helicity between the two species. In contrast, the cellular lipid release efficiency of octamers and hexadecamers was reduced to ≤50% of WT-apoA-I levels, despite only a 10% difference in α-helicity from tetromers. Thus, our observations suggest that the enthalpic gain provided by the formation of new α-helices determines the efficiency of HDL production from DMPC vesicles in vitro. However, in ABCA1-mediated conditions, where the cell membrane curvature is actively increased by the membrane transporter, lipid release efficiency is less dependent on the protein α-helix-forming ability and strongly affected by the presence of stable intermolecular interactions. The formation of stable quaternary structures reduces the cellular lipid release efficiency of apoA-I; we speculate that it does so by limiting protein flexibility at sites where flexibility is needed to promote the structural changes that follow initial...
lipid-binding and determine the efficiency of HDL formation in vivo.

Nascent HDL produced by lipidation of lipid-free apoA-I at the cell surface contains two or three molecules of apoA-I. Thus, in a scenario wherein lipid-free apoA-I is self-associated, two pathways are possible: (i) dissociation of self-associated apoA-I to monomers and reassembling of monomers at the cell membrane to generate nascent HDL and (ii) disruption of high order self-associated species to dimeric or trimeric apoA-I, which can act as minimal functional units directly interacting with lipids. Our results demonstrate that monomers and tetramers have similar cellular lipid release efficiencies, raising the interesting possibility that dimers generated from tetramers rather than monomers are the minimal functional unit of lipid-free apoA-I. Analysis of DMPC particles generated by different ΔW-apoA-I self-associated species revealed that tetramers preferentially form 9.6-nm discoidal particles, whereas the particles produced by monomeric apoA-I are largely heterogeneous in size and, potentially, number of apoA-I molecules per particle (Fig. 7). This evidence suggests that dimeric apoA-I dissociating from tetrameric species could be the species that most efficiently generates two-apoA-I-containing lipoproteins. However, cell culture conditions are very different from in vitro DMPC binding, and, although our in vitro studies (Fig. 8 and supplemental Fig. S2) did not detect monomer formation from high order self-associated ΔW-apoA-I during a 24-h incubation at 37 °C, we cannot exclude the possibility that

FIGURE 9. PL (A) and FC (B) release from 293/2c cells transfected with human ABCA1. PL and FC levels in culture medium were measured after treatment of cells with the indicated amount of apolipoproteins as described under “Experimental Procedures.” PL and FC concentration values are expressed per mg of cellular proteins. Averages and S.D. (error bars) are from 3–7 independent experiments with freshly prepared apoA-I self-association subclasses. In each experiment, protein concentration treatments were performed in triplicate. Probabilities that the reported average values are not significantly different from the efflux efficiency of WT[High] and ΔW[High] are indicated as follows: **, p ≤ 0.01 versus WT[High]; *, p ≤ 0.05 versus WT[High]; ●●, p ≤ 0.01 versus ΔW[High]; ●, p ≤ 0.05 versus ΔW[High]. Unlabeled histograms are values not significantly different (p > 0.05) from WT[High] and ΔW[High].
dissociation of tetramers to monomers occurs in cell culture. Thus, further experiments will be required to unequivocally establish which of the two reaction pathways happens in vivo.

According to the existing paradigm of structure-function relationship in apoA-I, the integrity of specific residues (i.e., primary structure) is necessary for preservation of the lipid acceptor function of apoA-I. This notion is derived from the dysfunctional consequences of naturally occurring point mutations and of in vivo oxidation of specific residues. For instance, in the atherosclerotic plaque, the macrophage-produced myeloperoxidase (MPO) catalyzes oxidation of several apoA-I residues and reduces the cellular cholesterol release efficiency of apoA-I (83–85). However, work from the Heinecke, Smith, and Hazen groups (86–88) did not release efficiency of apoA-I (83–85). However, work from the Heinecke, Smith, and Hazen groups (86–88) did not unequivocally identify the MPO-targeted residues (i.e., Tyr, Lys, and Met) that are directly implicated in the loss of the ABCA-1-mediated cholesterol release efficiency of apoA-I. To date, resistance to MPO-dependent loss of cholesterol release efficiency has been shown to occur only when tryptophans, which are only marginally modified by MPO (89), have been mutated to phenylalanines (90). We propose that this protection from MPO oxidation is mediated by the high acceptor function of apoA-I. This notion is derived from the fact that in vivo dissociation of tetramers to monomers occurs in cell culture. The large self-association-mediated structural changes may explain why at high concentrations (similar to the conditions used for protein crystallization) specific stable structural subspecies are selected, although these are not necessarily predominant at physiological concentrations. We hypothesize that in vivo the lipid acceptor function of apoA-I does not directly depend on the activity of specific residues but on a different and more general structural feature of the protein. Thus, protein self-association should be taken into consideration as one of the functional structure-determinant properties that can be targeted by in vivo protein modifications that render apoA-I dysfunctional.

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