Reconstituted High-Density Lipoproteins with a Disulfide-Linked Apolipoprotein A-I Dimer: Evidence for Restricted Particle Size Heterogeneity

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ABSTRACT: The apolipoprotein A-I_{Milano} (apoA-I_M) is a molecular variant of apoA-I characterized by the Arg^{173}→Cys substitution, resulting in the formation of homodimers (A-I_M/A-I_M) and heterodimers with apoA-II. In order to examine the effects of the introduction of an interchain disulfide bridge on the lipid-binding properties of apoA-I, the present studies compare the kinetics of association of A-I_M/A-I_M and apoA-I with dimyristoylphosphatidylcholine (DMPC), and the structure and properties of reconstituted HDL containing palmitoyloleoylphosphatidylcholine (POPC) and either A-I_M/A-I_M or apoA-I. The results show that apoA-I dimerization does not affect the rate of association with DMPC. Apolipoprotein−POPC complexes instead, when analyzed by nondenaturing gradient gel electrophoresis, demonstrate that, differently from apoA-I, A-I_M/A-I_M forms only two species of rHDL particles despite a wide range of initial lipid to protein ratios. These two rHDL species contain one or two A-I_M/A-I_M molecules and have a diameter of 7.8 nm and 12.5 nm. Investigations of the A-I_M/A-I_M structure in these two rHDL, by circular dichroism, fluorescence, and second-derivative UV spectroscopy, suggest that the secondary and tertiary structures of A-I_M/A-I_M are remarkably similar in both small and large particles. These results suggest that the introduction of an interchain disulfide bridge does not affect the association of apoA-I with lipids but restricts HDL particle size heterogeneity, thus possibly affecting HDL function in lipid metabolism and atherosclerosis protection.

The plasma high-density lipoproteins (HDL)[1] consist of heterogeneous particles differing in size, density, lipid, and apolipoprotein composition. The major physiological function of HDL is to facilitate reverse cholesterol transport, the process by which excess cholesterol is removed from peripheral tissues and transported to the liver for final elimination (*1*). This mechanism is believed to explain the protective role of HDL against the development of atherosclerosis, that has been observed in a large number of epidemiological studies (*2*). Apolipoprotein A-I (apoA-I), the major protein component of HDL, actively modulates the function of HDL in reverse cholesterol transport, by being a very effective acceptor of cell cholesterol (*3*), by being the best activator of the lecithin:cholesterol acyltransferase (LCAT) enzyme (*4*), and by facilitating the direct transport of LCAT-derived cholesteryl esters to the liver, possibly through receptor-mediated recognition (*5*).

ApoA-I is a single polypeptide chain, composed of 243 residues (*6*). The most striking feature of the apoA-I sequence is the presence of internal repeat units of 11 or 22 amino acids, believed to arrange into antiparallel amphipathic α-helices, interrupted by *β*-turns occurring at proline and glycine residues (*7*). In the presence of phospholipids, apoA-I generates discoidal particles with the size of nascent HDL, also known as reconstituted HDL (rHDL) (*8*). Jonas and collaborators have developed a general model for apoA-I structure in rHDL, in which the amphipathic helices run from side to side of the disk (*9*−*11*), with charged residues facing the aqueous phase and hydrophobic residues facing the acyl chains of the phospholipid bilayer (*12*). The calculated number of α-helices which can be accommodated around the periphery of the disk varies with the size and the composition of the rHDL (*13*). Well-defined, discretely sized rHDL made with synthetic phosphatidylcholines are characterized by a precise stoichiometry of two, three, or four molecules of apoA-I per particle, and can accommodate six, seven, or eight α-helices for each apoA-I molecule (*9*).

Apolipoprotein A-I_{Milano} (A-I_M) is a molecular variant of apoA-I characterized by the Arg^{173}→Cys substitution (*14*), resulting in the formation of a disulfide-linked homodimer (A-I_M/A-I_M). A-I_M/A-I_M possesses molecular properties that are unique compared to those of normal apoA-I. The introduction of the disulfide bridge in A-I_M/A-I_M results in facilitated interhelix interactions, with an increased secondary structure, and a more folded tertiary structure (*15*). The occurrence of a disulfide bridge in the middle of one of the amphipathic helices of apoA-I (*7*) would drastically affect the ability of the apolipoprotein to readily convert from a lipid-free to a lipid-bound state, as well as the physical properties of stable lipid−protein complexes. In the present study, we compared the lipid-binding properties of a recombinant A-I_M/A-I_M (*15*) vs those of normal apoA-I. We

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[1] Abbreviations: HDL, high-density lipoprotein(s); apoA-I, apolipoprotein A-I; LCAT, lecithin:cholesterol acyltransferase; A-I_M, A-I_{Milano}; A-I_M/A-I_M, A-I_M homodimer; rHDL, reconstituted HDL; LDL, low-density lipoprotein(s); POPC, palmitoyloleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; GGE, gradient gel electrophoresis; DMS, dimethyl suberimidate; CD, circular dichroism.

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set out to evaluate the effects of the interchain disulfide bridge in A-I_M/A-I_M on the association of apoA-I with lipids and on the structure the protein adopts in well-defined rHDL.

EXPERIMENTAL PROCEDURES

Materials. A-I_M/A-I_M, expressed in *E. coli* and purified by conventional chromatographic procedures (*15*), was a generous gift of Pharmacia Therapeutics, Stockholm, Sweden. The A-I_M/A-I_M batch used in the present study contained ~95% A-I_M/A-I_M and ~5% A-I_M monomer, as determined by SDS−PAGE (not shown). Normal apoA-I was purified from human blood plasma, as previously described (*16*). Before use, lyophilized A-I_M/A-I_M and apoA-I were dissolved in 20 mM phosphate buffer, pH 7.4, containing 6 M Gdn-HCl and extensively dialyzed against 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM NaN_3, and 0.01% EDTA (reconstitution buffer). The protein concentration of the stock solutions was assayed by amino acid analysis, performed on a Beckman 6300 amino acid analyzer after acidic hydrolysis of samples in 6 M HCl for 45 min at 155 °C. Low-density lipoproteins (LDL) were isolated from blood plasma by salt density ultracentrifugation (*17*). L-α-palmitoyloleoylphosphatidylcholine (POPC), L-α-dimyristoylphosphatidylcholine (DMPC), and sodium cholate were purchased from Sigma.

DMPC-Binding Studies. The association of A-I_M/A-I_M and apoA-I to DMPC multilamellar liposomes was studied by a kinetic turbidimetric method (*18*). Weighed amounts of DMPC were dissolved in chloroform, and the solvent was evaporated under N_2; any remaining solvent was removed under vacuum for 2 h. Dispersions of DMPC in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, containing 8.5% KBr, were mixed with apoA-I or A-I_M/A-I_M at a 2.5:1 DMPC:apolipoprotein weight ratio (0.1 mg/mL of protein). The association of apolipoproteins with DMPC was followed by monitoring the rate of disappearance of liposomal turbidity at 325 nm in a Jasco UV-500 spectrophotometer, equipped with a thermostatically controlled cuvette holder, maintained at 24.0 ± 0.1 °C (*16*).

Preparation and Characterization of rHDL. Discoidal rHDL containing A-I_M/A-I_M, or apoA-I and POPC, were prepared by the cholate dialysis technique (*8*), using a sodium cholate:POPC molar ratio of 1:1 and 5 mg of apolipoprotein in each preparation. Initial POPC:apolipoprotein weight ratios, ranging between 1.09:1 and 4.34:1, were used for both A-I_M/A-I_M and apoA-I. Since the investigated apolipoproteins differ in molecular weight, weights rather than molar ratios were used to prepare comparable rHDL particles (*13*). These weight ratios correspond to POPC:apoA-I molar ratios ranging between 40:1 and 160:1. Sodium cholate was removed by extensive dialysis against reconstitution buffer, and the size and homogeneity of rHDL were estimated by nondenaturing gradient gel electrophoresis (GGE) (*19*), using the Pharmacia Phast System (Pharmacia Biotec). Precast 8−25% polyacrylamide gels were run at 400 V for 350 V-h, stained with Coomassie Blue R250, and scanned with a Pharmacia Ultroscan XL laser densitometer. The size of rHDL particles was calculated with the Pharmacia 2400 Gelscan XL software, using thyroglobulin (17.0 nm), apoferritin (12.2 nm), catalase (9.2 nm), lactate dehydrogenase (8.2 nm), and bovine serum albumin (7.1 nm) as calibration proteins.

The number of apolipoprotein molecules per rHDL particle was determined by cross-linking with dimethyl suberimidate (DMS) (*15*). Cross-linked samples were analyzed by SDS−PAGE on 4−10% acrylamide gradient slab gels, using the Tris−tricine buffer system of Schagger and Van Jagou (*20*) in a Mini Protean slab minigel apparatus (BioRad). The phospholipid content of rHDL was determined by an enzymatic method (*21*). Proteins were measured by the method of Lowry et al. (*22*), using bovine serum albumin as standard.

Spectroscopy. Circular dichroism (CD) spectra were recorded with a Jasco J500A spectropolarimeter at the constant temperature of 25 °C. Molar mean residue ellipticity, $[\theta]$, was expressed in degrees centimeter squared per decimole, and calculated as

$$[\theta] = \theta_{obs} 115/10lc$$

where θ_{obs} is the observed ellipticity in degrees, 115 is the mean residue molecular weight of the proteins, l is the optical path length in centimeters, and c is the protein concentration in grams per milliliter. All the spectra were base line corrected. The α-helical content was calculated by the method of Chang et al. (*23*).

The ultraviolet absorption spectra were measured with a Jasco UV-550 spectrophotometer equipped with an on-line digital data processor. Differential absorption spectra were calculated by subtraction of the base line spectrum stored in memory. Topographical location of tyrosyl residues was investigated by second-derivative analysis, according to Ragone et al. (*24*), as previously described (*15*). The "*a*" and "*b*" values were calculated as the peak to trough distances between the minima at 283 and 290.5 nm, and the maxima at 287 and 295 nm, respectively. The fractional tyrosine exposure was then expressed as $\alpha = (r - r_a)/(r_u - r_a)$, where r is the ratio a/b, measured from the second-derivative absorption spectra, r_u is the ratio for the protein dissolved in 6 M Gdn-HCl, and r_a is the ratio calculated for a model protein solution containing the same tyrosine:tryptophan ratio dissolved in ethylene glycol to simulate residues completely buried in the protein interior. For apoA-I, r_u and r_a were 1.20 and 0.35, respectively.

Uncorrected tryptophan fluorescence spectra were measured at room temperature with a Kontron SF-25 fluorescence spectrophotometer using a 280 nm excitation wavelength (*16*).

RESULTS

Lipid-Binding Properties of A-I_M/A-I_M. The association of A-I_M/A-I_M and apoA-I with DMPC was followed by measuring the clearance of liposomal turbidity as a function of time at 24 °C, i.e., the transition temperature of the phospholipid (*18*). As illustrated in Figure 1, the rates of turbidity decrease following mixing DMPC liposomes with A-I_M/A-I_M or apoA-I were similar; a complete clearance of liposomal turbidity was achieved with both apolipoproteins after 24 h incubation. Moreover, both A-I_M/A-I_M and apoA-I were almost completely (>95%) recovered into stable DMPC−apolipoprotein complexes upon fractionation of the reaction mixtures by either ultracentrifugation or gel filtration chromatography (not shown). The A_{325} values determined after 24 h of incubation were assigned as the values for scattering of the final product (A_∞); the data were then expressed as relative turbidity, calculated as $(A_t - A_\infty)/(A_0 - A_\infty)$, where A_0 and A_t are the A_{325} values at time zero and time t, respectively. A rate constant was defined as described

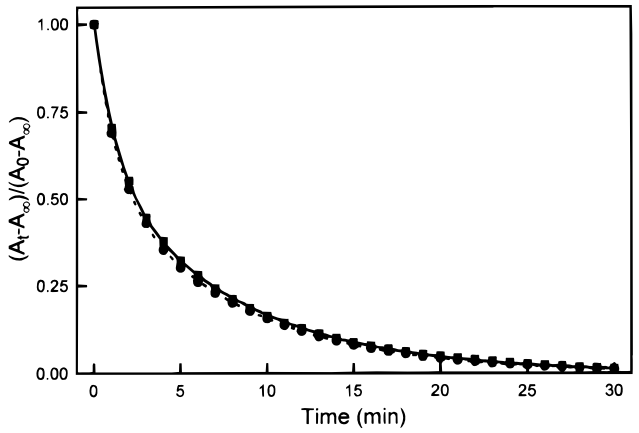

FIGURE 1: Solubilization of DMPC by apoA-I and A-I_M/A-I_M as monitored by turbidity change over time at 24 °C. Multilamellar DMPC liposomes (0.5 mg/mL) were mixed with apoA-I (●) or A-I_M/A-I_M (■) at a DMPC:apolipoprotein ratio of 2.5:1 (w/w), and the clearance of liposomal turbidity was continuously monitored at 325 nm. The initial and final A_{325} values of DMPC/protein mixtures were 1.179 ± 0.018 and 0.120 ± 0.019 for apoA-I, and 1.175 ± 0.013 and 0.117 ± 0.008 for A-I_M/A-I_M. Symbols are the means of five experiments.

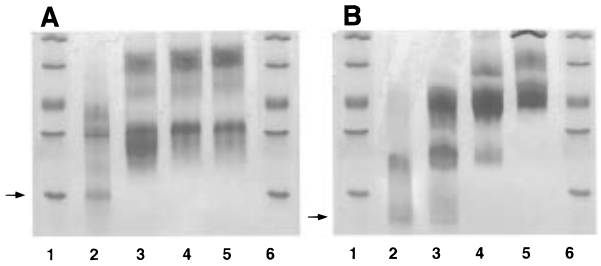

FIGURE 2: Nondenaturing gradient gel electrophoresis of rHDL particles prepared with A-I_M/A-I_M (panel A) and apoA-I (panel B). *Lanes 1* and *6* correspond to protein standards: thyroglobulin (17.0 nm), ferritin (12.2 nm), catalase (9.2 nm), lactate dehydrogenase (8.2 nm), and albumin (7.1 nm). *Lanes 2*−5 correspond to rHDL prepared with POPC:apolipoprotein weight ratios of 1.09:1, 2.17:1, 3.26:1, and 4.34:1, respectively. These values correspond to POPC:apoA-I molar ratios of 40:1, 80:1, 120:1, and 160:1, respectively. The arrows indicate the migration of lipid-free A-I_M/A-I_M (panel A) and apoA-I (panel B).

by Pownall et al. (*18*): $k_{1/2} = 1/t_{1/2}$, where $t_{1/2}$ is the time required for the decrease of half the relative turbidity. The calculated $k_{1/2}$ values were 0.42 ± 0.11 min^{-1} for A-I_M/A-I_M and 0.41 ±0.09 min^{-1} for apoA-I. Therefore, the introduction of an interchain disulfide bridge in apoA-I affects neither the kinetics of interaction with lipids nor the extent of lipid binding.

Preparation of rHDL Containing A-I_M/A-I_M. The sizes and distributions of rHDL particles containing A-I_M/A-I_M and apoA-I were examined by GGE (Figure 2). At a POPC:apolipoprotein weight ratio of 1.09:1 (corresponding to a POPC:apoA-I molar ratio of 40:1), a significant amount of lipid-free apolipoprotein was present in the rHDL preparations containing both A-I_M/A-I_M and apoA-I (Figure 2). Discrete rHDL particles with a diameter of 8.2 and 7.8 nm were also present in preparations containing A-I_M/A-I_M and apoA-I, respectively. At higher POPC:apolipoprotein ratios, the incorporation of A-I_M/A-I_M and apoA-I into stable complexes with POPC appears to be complete, since no protein was found in a position corresponding to the lipid-free apolipoproteins. The distribution of rHDL generated with A-I_M/A-I_M changed with increasing POPC:apolipoprotein ratio from 2.17:1 (w/w) to 4.34:1 (w/w), but the same rHDL species were present in all the samples (Figure 2, panel A). All preparations exhibited rHDL species with diameters of 8.2 and 12.5 nm. A very minor component, with a diameter of 10.8 nm and containing the A-I_M monomer in addition to A-I_M/A-I_M (not shown), was detectable in some samples. In contrast, the average size of rHDL generated with apoA-I increased with increasing POPC:apolipoprotein ratio (Figure 2, panel B) (*25*). An rHDL species with a diameter of 9.6 nm was predominant in all the preparations; at low POPC:apoA-I ratios, a smaller rHDL species with a diameter of 7.8 nm was also present, whereas larger species, with a diameter of 12.7 and 17.6 nm, were generated at high POPC:apoA-I ratios.

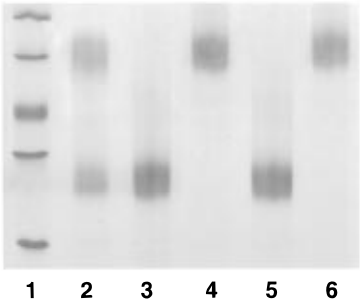

FIGURE 3: Isolation of pure rHDL particles containing A-I_M/A-I_M. POPC−A-I_M/A-I_M mixtures at a 2.17:1 weight ratio were incubated with LDL (rHDL to LDL protein 1:1) for 18 h at 37 °C and applied to three serial Superose 6B columns (1.6 × 100 cm). POPC−A-I_M/A-I_M mixtures at a 3.26:1 weight ratio were directly applied to the Superose columns. *Lane 1*, protein standards: thyroglobulin (17.0 nm), ferritin (12.2 nm), catalase (9.2 nm), lactate dehydrogenase (8.2 nm), and albumin (7.1 nm). *Lane 2*: POPC−A-I_M/A-I_M mixtures (2.17:1, w/w) incubated with LDL. *Lane 3*: A-I_M/A-I_M rHDL particles (7.8 nm) purified by gel filtration from incubated POPC−A-I_M/A-I_M mixtures. *Lane 4*: A-I_M/A-I_M rHDL particles (12.5 nm) purified by gel filtration from nonincubated POPC−A-I_M/A-I_M mixtures (3.26:1, w/w). *Lanes 5*−6: purified apoA-I rHDL particles (7.8 nm and 12.7 nm, respectively).

A major objective of the present investigation was to compare the structure of A-I_M/A-I_M and apoA-I when bound to rHDL particles of comparable size. Two different approaches were applied to prepare small and large rHDL containing A-I_M/A-I_M or apoA-I. Essentially pure 7.8 nm rHDL particles were isolated following the incubation of the original apolipoprotein−POPC samples, prepared with a 2.17:1 (w/w) POPC:apolipoprotein ratio, with LDL (*9*). As previously shown by Jonas and co-workers (*9*), the incubation of apoA-I rHDL with LDL leads to the rearrangement of the original rHDL into limiting particles with defined apolipoprotein structures (*11*). Incubation of A-I_M/A-I_M rHDL caused minor changes in the size distribution of the original rHDL: the 12.5 nm rHDL did not change, whereas the 8.2 nm rHDL disappeared with the generation of a particle with a diameter of 7.8 nm. Therefore, the incubated mixture displayed two rHDL species with diameters of 7.8 and 12.5 nm (Figure 3). The 7.8 nm rHDL displayed a lower phospholipid content than the 8.2 nm rHDL isolated from nonincubated mixtures (POPC/A-I_M/A-I_M = 1.04 vs 1.33), while the large particles had identical compositions (POPC/A-I_M/A-I_M = 2.05 and 2.08 for rHDL isolated from nonincubated and incubated mixtures). Pure 7.8 nm A-I_M/A-I_M and apoA-I rHDL were isolated from incubated mixtures by gel filtration chromatography (Figure 3). rHDL particles with a diameter of 12.5−12.7 nm were instead purified by gel filtration from nonincubated POPC−apolipoprotein mix-

Table 1: Compositions and Sizes of rHDL

particle	POPC:apolipoprotein[a]		molecules/particle		diameter (nm)		
	mass ratio (w/w)	mole ratio (mol/mol)	apolipoprotein[b]	POPC	GGE[c]	column[d]	EM[e]
7.8 nm A-I_M/A-I_M rHDL	1.04(±0.05):1	76(±4):1	1	76	7.8	8.2	8.7 ± 1.8
12.5 nm A-I_M/A-I_M rHDL	2.05(±0.11):1	151(±8):1	2	302	12.5	12.7	15.1 ± 1.8
7.8 nm apoA-I rHDL	1.21(±0.11):1	45(±4):1	2	90	7.8	8.0	ND[f]
12.7 nm apoA-I rHDL	4.02(±0.03):1	148(±1):1	3	444	12.7	13.0	ND

[a] Determined on three independent reconstitution experiments (±SD). [b] Determined by SDS−PAGE of delipidated particles after cross-linking with DMS. [c] Determined by nondenaturing polyacrylamide gradient gel electrophoresis (±0.1 nm, SD). [d] Calculated from the elution volume from a calibrated Superose 6 gel filtration column (±0.7 nm, SD). [e] Average major diameter of 100 particles determined from negative staining electron microscopy (±SD). [f] ND: not determined.

tures prepared with a 3.26:1 POPC:apolipoprotein weight ratio (Figure 3).

Characterization of A-I_M/A-I_M rHDL. The chemical composition and size of the isolated rHDL containing A-I_M/A-I_M or apoA-I are shown in Table 1. Incubation of rHDL with LDL caused an exchange of phospholipids and a net transfer of cholesterol from LDL to rHDL (*9*). Since the content of cholesterol in the isolated 7.8 nm rHDL was below the detection limit of the enzymatic assay in both A-I_M/A-I_M and apoA-I rHDL, and more than 90% of the phospholipids was POPC (*9*), these compositional changes can be neglected, and the experimental and calculated compositions of A-I_M/A-I_M or apoA-I rHDL can be compared. As indicated by the low standard deviations, the composition of rHDL is highly reproducible. rHDL containing apoA-I displayed lipid and protein stoichiometries similar to those previously reported (*9, 26*). It is noteworthy that although the starting preparations had identical POPC:apolipoprotein contents, rHDL with different lipid contents were generated with A-I_M/A-I_M compared with apoA-I (Table 1). The 7.8 and 12.5 nm A-I_M/A-I_M rHDL contained 15% and 30% less POPC molecules than the corresponding apoA-I rHDL.

Electron microscopy confirmed that A-I_M/A-I_M rHDL are homogeneous and discoidal in appearance with an average width of 4.1 nm. As expected, the electron micrographs showed the characteristic stacks of discoidal particles upon staining with phosphotungstate (Figure 4). The electron microscopic diameter of rHDL was larger than the hydrodynamic diameter determined by GGE, as reported by others (*11, 27*). Application of the correction introduced by Nichols et al. (*27*) to our EM results gives diameters that are in good agreement with those determined by GGE and gel filtration. Therefore, the particle diameters calculated from GGE were used in further calculations.

Chemical cross-linking of A-I_M/A-I_M in rHDL and analysis of the products by SDS−PAGE indicated that the two rHDL with diameters of 7.8 and 12.5 nm contained 1 and 2 A-I_M/A-I_M molecules/particle, respectively (Figure 5). As expected, 7.8 and 12.7 nm apoA-I rHDL contained 2 and 3 apoA-I molecules/particle, thus belonging to the previously described Lp2A-I and Lp3A-I subclasses (*26*).

Spectral Properties of A-I_M/A-I_M and ApoA-I rHDL. The results for CD, fluorescence, and UV spectroscopy are given in Table 2. Reference values for the lipid-free apolipoproteins are also reported. As expected, apoA-I rHDL had higher α-helix contents than the lipid-free apoA-I, with the 12.7 nm rHDL displaying the highest α-helix content. Therefore, increases in the particle size and POPC content of apoA-I rHDL appear to be directly related to increases in the α-helix content in apoA-I (Table 2). In contrast, A-I_M/A-I_M rHDL had an α-helix content similar to that of the lipid-free apolipoprotein, thus confirming that minor changes occur

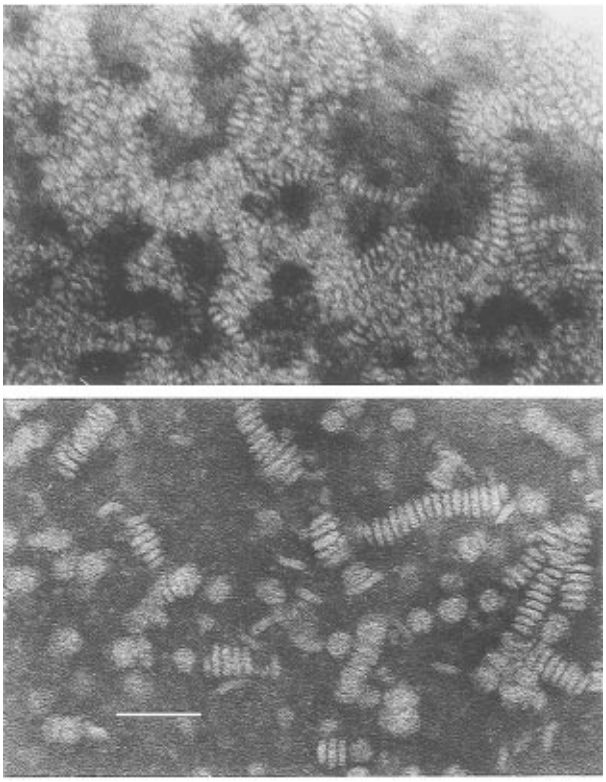

FIGURE 4: Electron micrographs of purified 7.8 nm (*top*) and 12.5 nm (*bottom*) A-I_M/A-I_M rHDL. The bar marker represents 50.0 nm and is the same for both micrographs.

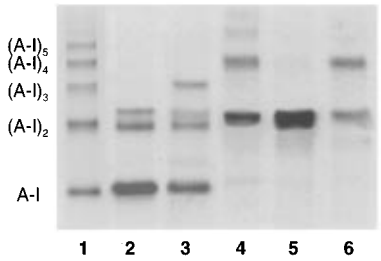

FIGURE 5: Cross-linking of A-I_M/A-I_M, apoA-I, and their rHDL as analyzed by SDS−PAGE. *Lane 1*: lipid-free apoA-I. *Lane 2*: 7.8 nm apoA-I rHDL. *Lane 3*: 12.7 nm apoA-I rHDL. *Lane 4*: lipid-free A-I_M/A-I_M. *Lane 5*: 7.8 nm A-I_M/A-I_M rHDL. *Lane 6*: 12.5 nm A-I_M/A-I_M rHDL.

in A-I_M/A-I_M secondary structure upon interaction with phospholipids, either POPC or DMPC (*15*). Moreover, small and large A-I_M/A-I_M rHDL displayed similar α-helix contents, which is indicative of a reduced ability of A-I_M/A-I_M to rearrange in response to changes in particle size.

Binding of both A-I_M/A-I_M and apoA-I to POPC was associated with a shift of tryptophan fluorescence maxima to lower wavelengths from 333−334 nm to 329−330 nm (Table 2), suggesting that lipid binding of both apolipopro-

Table 2: Spectral Properties of A-I_M/A-I_M and ApoA-I in rHDL and Free in Solution

particle	α-helix[a] (%)	λ_{max}[b] (nm)	Tyr exposure[c] (α)
7.8 nm A-I_M/A-I_M rHDL	61	330	0.57
12.5 nm A-I_M/A-I_M rHDL	62	329	0.62
A-I_M/A-I_M	66	333	0.60
7.8 nm apoA-I rHDL	61	330	0.45
12.7 nm apoA-I rHDL	71	329	0.41
apoA-I	43	334	0.88

[a] Determined from the 190−250 nm CD spectrum. Values are representative of three different preparations of rHDL (±3%, SD). [b] From uncorrected fluorescence spectra, excited at 280 nm (±1 nm, SD). [c] Determined by second-derivative ultraviolet spectroscopy (±0.02, SD).

teins results in a similar translocation of tryptophan residues to a more hydrophobic environment. As shown by others (*25, 28*), rHDL particles with different diameters displayed similar fluorescence maxima. Lipid-free A-I_M/A-I_M consistently had a slightly shorter fluorescence maximum than apoA-I, suggesting that the tryptophan residues in A-I_M/A-I_M are located in a more hydrophobic environment than those in apoA-I. This would be consistent with previous data indicating a more folded tertiary structure in A-I_M/A-I_M than in apoA-I (*15*). However, the difference in wavelength maxima is very close to the experimental error, making it difficult to draw definite conclusions from the fluorescence measurements. It is noteworthy that two of the four tryptophan residues of apoA-I are located in the N-terminal portion of the molecule, which is predicted to have a low content of amphipathic structure and associates weakly with phospholipids (*29*). Therefore, one can postulate that differences in the tertiary structure between A-I_M/A-I_M and apoA-I involve mostly the central and C-terminal portions of the molecule, where the interchain disulfide bridge is located.

Tyrosine exposure in A-I_M/A-I_M and apoA-I rHDL was determined by second- derivative ultraviolet spectroscopy. ApoA-I binding to POPC resulted in a significant decrease of fractional tyrosine exposure (Table 2), as previously described (*15*), confirming a translocation of tyrosine residues to a more hydrophobic environment, i.e., consistent with the fluorescence data. In contrast, the fractional tyrosine exposure in A-I_M/A-I_M was poorly affected following interaction with POPC, as already shown for the binding to DMPC (*15*). Particle size did not affect tyrosine exposure in both A-I_M/A-I_M and apoA-I; very similar values, corresponding to the exposure of 3 out of the 7 apoA-I tyrosines and of 8 out of the 14 A-I_M/A-I_M tyrosines, were measured for small and large rHDL.

DISCUSSION

In this study, we used the recombinant A-I_M/A-I_M dimer to investigate the effects of the introduction of an interchain disulfide bridge on the lipid-binding properties of apoA-I. This work demonstrates that the dimerization of apoA-I (1) does not affect the rate of association of apoA-I with phospholipids and (2) restricts rHDL particle size heterogeneity.

Binding of ApoA-I to Phospholipids. The interaction of amphipathic apolipoproteins with liposomes is characterized by two major kinetic events: the initial recognition and binding of the polypeptide to the lipid bilayer (*30, 31*), followed by the modification of the overall packing of the phospholipids due to the penetration of the amphipathic helices into the "holes" of the lipid bilayer (*30*). Several factors are known to influence the kinetic behavior of lipid−apolipoprotein interactions: the association rate is faster near the transition temperature of the phospholipid (*32*), and increases with decreasing molecular weight of the apolipoprotein (*18*) and with increasing number of hydrophobic residues exposed on the apolipoprotein surface (*18*). Therefore, A-I_M/A-I_M would associate with DMPC at a slower rate than apoA-I, because of the higher molecular weight and lower exposure of hydrophobic residues (*15*). Indeed, self-associated apoA-I, which resembles the disulfide-linked A-I_M/A-I_M in secondary and tertiary structure (*15*), associates with DMPC at a slower rate than monomeric apoA-I (*33*), while the A-I_M monomer, which displays a lower content of secondary structure and an increased exposure of hydrophobic residues than apoA-I, associates at a faster rate with DMPC liposomes (*16*).

Recent studies with recombinant apoA-I mutants (*34*), proteolytic apoA-I fragments (*28*), or synthetic apoA-I peptides (*35*) revealed that the two C-terminal helices of apoA-I participate in the initial binding of the apolipoprotein to the phospholipid bilayer. This would trigger the formation of an amphipathic protein sheet, possibly via cooperative interhelical salt bridge interactions (*12, 35*), and the generation of stable lipid−apolipoprotein complexes. The lack of effect of apoA-I dimerization on the association rate with DMPC suggests that, despite the presence of the interchain disulfide bridge, the amphipathic helical segments of A-I_M/A-I_M, and in particular the two C-terminal helices, are relatively free to reorientate, associate with the lipid surface, and trigger phospholipid binding. Studies with a C-terminally truncated A-I_M/A-I_M would help in solving this issue.

Structure of A-I_M/A-I_M in rHDL. Studies on apoA-I rHDL have demonstrated that discretely sized particles within each rHDL class have unique apoA-I conformations, that are repeated in the homologous particles of the next class (*9, 26*). Changes in size between rHDL of the same class require the incremental addition of two helices per apoA-I molecule to the disc periphery (*9*). The apoA-I rHDL we used in this study have been characterized before (*9, 26*), and our results are consistent with the current model for discoidal apoA-I rHDL in which each apoA-I molecule rearranges into six or eight antiparallel helices around the disc, depending on particle size (*9*).

Perhaps the most significant new result from the present study is that A-I_M/A-I_M forms rHDL with size distributions distinct from those of apoA-I rHDL (Figure 2); in particular, the introduction of an interchain disulfide bridge in A-I_M/A-I_M remarkably limits the size distribution of rHDL particles. It is generally accepted that the structure of the apolipoprotein determines the overall structure of the rHDL particle, and that the number of potential helical segments accommodated around the discoidal rHDL determines the size of rHDL (*13, 34*). Assuming that the diameter of each helical segment at the periphery of rHDL is 1.5 nm (*9*), we calculated that A-I_M/A-I_M 7.8 and 12.5 nm rHDL, which contain 1 and 2 A-I_M/A-I_M molecules/particle, accommodate 12 and 24 helices around the discs, respectively. Therefore, changes in particle size among A-I_M/A-I_M rHDL only reflect a variation in the particle apolipoprotein content, with a constant number of helices per apolipoprotein molecule in contact with the lipid bilayer. It is noteworthy that in the

carriers' plasma the A-I_M/A-I_M is exclusively present in a single HDL species (L. Calabresi and G. Franceschini, unpublished data), thus confirming that, even *in vivo*, A-I_M/A-I_M has a limited capacity to generate HDL subspecies which are heterogeneous in size.

Physiological Significance. The HDL fraction in human plasma is heterogeneous, with a number of particles differing in size, density, and apolipoprotein composition (*36*). There is mounting evidence that specific HDL subpopulations have differing functions, and that the ability of apoA-I to rearrange in response to changes in particle size and lipid content plays a major role in modulating the function of specific HDL subpopulations (*37*). A minor subpopulation of very small, apoA-I-containing HDL has been identified as the preferred initial acceptor for cell cholesterol in the first step of reverse cholesterol transport (*38*). Native and reconstituted HDL of different size display marked differences in their ability to act as substrates for the LCAT enzyme (*4*, *39*). Possibly because of these differences, distinct HDL subpopulations may differ in their ability to protect against coronary heart disease (*40*).

The present data suggest that the introduction of an interchain disulfide bridge in apoA-I remarkably limits HDL heterogeneity and thus would have a significant impact on some major functions of these lipoproteins. Indeed, A-I_M/A-I_M rHDL particles are 40−70% less reactive than those with apoA-I as substrates for the LCAT enzyme (*41*); native HDL containing A-I_M/A-I_M are more resistant to remodeling by LCAT and CETP (*42*), and are cleared from the circulation at a much slower rate than apoA-I-containing particles (*43*). A-I_M/A-I_M would thus behave as a stable form of apoA-I, which might explain its powerful antiatherogenic activity in animal models (*44*) and, possibly, humans (*45*).

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