Structural Properties of High Density Lipoprotein Subclasses Homogeneous in Protein Composition and Size*

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We isolated native high density lipoprotein (HDL) subclasses homogeneous in size and in their protein content with the objective of investigating the differences and similarities in their apolipoprotein AI (apoA-I) structures. Defined particles were isolated from ultracentrifugally prepared HDL by immunoaffinity and gel-filtration chromatography. The isolated 88-Å LpAI, 106-Å LpAII, 96-Å LpAII particles (LpAI, particles contain only apoA-I; LpAII/AII, particles contain apoA-I and apoA-II), together with a 93-Å reconstituted HDL, were analyzed for purity, composition, and content of apolipoprotein molecules per particle, and were examined by far and near circular dichroism and intrinsic fluorescence spectroscopic methods, as well as by reaction kinetics with lecithin:cholesterol acyltransferase.

The spectroscopic analyses indicated that the secondary structures and three-dimensional arrangements of apoA-I in all these particles are remarkably similar: their tryptophan residues are located in similar nonpolar environments and become exposed to increasing concentrations of guanidine hydrochloride in comparable denaturation steps; the 60-66% α-helical content with increasing concentrations of guanidine hydrochloride in the 1ecithin:cholesterol acyltransferase reaction.

Metabolic studies of apoA-I in all these particles are remarkably similar: their tryptophan residues are located in similar nonpolar environments and become exposed to increasing concentrations of guanidine hydrochloride in comparable denaturation steps; the 60-66% α-helical structures and content of apolipoprotein molecules per particle, and were examined by far and near circular dichroism and intrinsic fluorescence spectroscopic methods, as well as by reaction kinetics with lecithin:cholesterol acyltransferase.

The reaction kinetics with lecithin:cholesterol acyltransferase are similar and slow for the isolated HDL particles, reflecting product inhibition, and/or an apoA-I conformation that is unfavorable for the activation of the lecithin:cholesterol acyltransferase reaction.

High density lipoproteins (HDL)* from human plasma contain a heterogeneous population of particles containing different types and amounts of apolipoproteins and lipids. They are mostly spherical particles ranging in diameter from 70–120 Å. Two main subclasses of HDL can be isolated by immunoaffinity chromatography; one of the subclasses contains both apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II) (LpAII/AII), whereas the other subclass contains apoA-I, but no A-II (LpAI) (1–6). Both of these subclasses of HDL include small amounts of other proteins and are homogeneous in size. Several laboratories have investigated the metabolic behavior of these two heterogeneous HDL subclasses (7–11). In vitro studies of the binding of LpAI and LpAII/AII to various cells, and their ability to promote cholesterol efflux from cells enriched in cholesterol, have yielded conflicting results. For example, Barbas et al. (8) found that LpAI and LpAII/AII particles bound equally well to preadipocytes loaded with cholesterol, but that only the LpAI particles promoted cholesterol efflux from the cells. In contrast, Johnson et al. (10), using different cells and experimental conditions, were unable to find a significant difference in the behavior of the two HDL subclasses as cholesterol acceptors. Metabolic studies, in vivo with Rader et al. (11) have shown that the catabolic rate of apoA-I on both types of particles is markedly different. Thus, because of the possible functional differences between the LpAI and LpAII/AII particles it is important to delineate their structural differences, particularly differences in the structure of apoA-I; however, to obtain unambiguous structural information, homogeneous particles are required.

During our investigations of reconstituted HDL (rHDL), we have found that apoA-I can exist in a few well defined conformational states in distinct rHDL particles (12–14). In discoidal rHDL containing apoA-I and palmitoyloleoylphosphatidylcholine (POPC), each apoA-I is arranged into 6 to 8 antiparallel α-helical segments joined by β turns or sheets (12, 15). The nonpolar side of the helices covers the edge of the lipid disc. The discrete diameters of these discoidal rHDL particles are defined by the number of apoA-I molecules per particle (two or more) (12, 13) and by the number of helices in each apoA-I that are in contact with the lipid. The structural differences in the apoA-I molecules were measured by fluorescence and circular dichroism spectroscopic methods, and the distinct structures of apoA-I were correlated with up to 15-fold differences in the reactivity of the rHDL with lecithin:cholesterol acyltransferase (LCAT) (12–14). We have also prepared a spherical rHDL of defined composition and size by reacting the discoidal rHDL with LCAT in the presence of low density lipoprotein (14). The 93-Å rHDL product of this reaction, which contains large amounts of cholesterol ester and appears round by electron microscopy, has three apoA-I molecules in a structure comparable to that in the discoidal rHDL precursors.

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The abbreviations used are: HDL, high density lipoproteins; apoA-I, apolipoprotein AI; apoA-II, apolipoprotein AI; LpAII/AII, subclass of HDL containing both apoA-I and apoA-II; LpAI/AII, subclass of HDL containing both apoA-I and apoA-II, also designated HDL (AI with AII); LpAI, subclass of HDL containing apoA-I, but no apoA-II; LpAII, also designated HDL (AI without AII); rHDL, reconstituted HDL; POPC, palmitoyloleoylphosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; GdnHCl, guanidine hydrochloride; BSb, bis(sulfosuccinimidyl)suberate; GGE, non-denaturing gradient polyacrylamide gel electrophoresis; Trp, tryptophan residues.
Since we have firmly established, by using the rHDL models, that apoA-I can exist in well defined conformational states which can determine the functional properties of the rHDL, we set out in this work to isolate native LpAI and LpAI/AlI particles of uniform size, by immunofluorescence chromatography and gel filtration, with the objective of characterizing their structures and establishing whether apoA-I exists in different conformations in different native particles.

 MATERIALS AND METHODS

Apolipoprotein A-I, LDL, and LCAT were prepared by routine methods (16-18) from human plasma donated by the Champaign County Blood Bank-Regional Health Resource Center. The purity of apoA-I and LCAT was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and the gels were stained with either Coomassie Blue or silver stain using the Phast System (Pharmacia LKB Biotechnology). Sodium cholate, crystalline cholesterol, POPC, and bovine serum albumin (BSA) were purchased from Sigma; [4-14C]cholesterol was purchased from Du Pont-New England Nuclear. Sodium cholate (DgnHCl) was the electrophoresis-grade product from Fisher; bis(sulfosuccinimidyl)suberate (BSs) was purchased from Pierce.

 Preparation of Anti-AI and Anti-AlI Immunosorbents—Monoclonal antibodies against apoA-I and apoA-II were prepared by intraperitoneal immunization of BALB/c mice with intact HDL, and characterized as previously described (19). A mixture (100 mg) of three different monoclonal antibodies (A05, A17, and A30) which recognize all forms of plasma apoA-I was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia LKB) as instructed by the manufacturer except that a ratio of 3.5 mg of antibodies/g of gel was used. Three different monoclonal antibodies (G03, G05, and G11), which recognize all forms of plasma apoA-II, was coupled to CNBr-Sepharose (3.5 mg/g gel). The immunosorbents were packed into borosilicate glass columns of 5-cm internal diameter.

Preparation of Native HDL and rHDL—LpAI and LpAI/AlI were isolated using a modification of the Cheung and Albers method (3). Human HDL (d = 1.063-1.21 g/ml) obtained by ultracentrifugal flotation was used in a two-step immunofluorescence chromatography procedure at 4° C. Typically, 2.5 mg of HDL protein were injected on the anti-apoA-II column connected in series with the anti-apoA-I column at a slow flow rate (0.2 ml/min) during a 15-h period. Then the individual columns were extensively washed (2 ml/min) and each retained fraction, LpAI and LpAI/AlI, was eluted with 20 ml of 3 M NaSCN. The eluted fractions were immediately desalted on Sephadex G-25 columns at a flow rate of 2 ml/min in order to minimize the time of contact with NaSCN. In all cases, more than 75% of the protein applied to the columns was retained by the immunospecific columns, and essentially all of the retained HDL protein was eluted with the NaSCN and recovered after the desalting step. The LpAI and LpAI/AlI were concentrated using Centricon 10 concentrators (Amicon) to 1 mg/ml and finally applied to a Superose 6 gel filtration column for further fractionation by size using a Pharmacia FPLC system. The losses of HDL protein on the Superose 6 column were insignificant, but each of the fractions with uniform sizes used in the subsequent experiments represented only 10-20% of the HDL protein applied to this column. The samples were stored in a standard 10 mM phosphate buffer, pH 7.4, and 150 mM NaCl, 1 mM EDTA, and 1 mM Na2S2O3. This buffer was used for all the experiments performed in this study unless otherwise specified.

In a separate experiment, we tested the effects of NaSCN on HDL particle size distribution and reactivity with LCAT. High density lipoprotein (2.5 mg of protein) was incubated with 3 mg NaSCN for 5 h at 4 °C. After removal of the NaSCN by dialysis, the particle size distribution for exposed and unexposed HDL was shown to be essentially identical by gradient gel electrophoresis (method described below). Also the reaction kinetics with LCAT (described below) were found to be the same for HDL which had been exposed or not exposed to NaSCN. Therefore, we concluded that the effects of this salt on the structural and functional properties of HDL are insignificant as judged by these sensitive tests.

The 93-A spherical reconstituted HDL (rHDL) was synthesized using the sodium cholate dialysis method (20, 21). An rHDL mixture containing 5 mg apoA-I/g of phosphate buffer was prepared by a modification of the method of Lowry et al. (23). rHDL was incubated with LDL (1:2 weight ratio of rHDL to LDL protein) in the presence of 12 mg/ml BSA, 4 mM β-mercaptoethanol, and 20 μg of LCAT at 37 °C for 48 h. Ultrafiltration was performed first at a density of 1.070 g/ml for two 24-h periods in order to remove the LDL, and then at a density of 1.21 g/ml for 24 h to remove rHDL. Residual LDL and small amounts of a 78-A particle were removed by gel filtration on a Sephrose 6 column as was previously described (14).

Electrophoretic Analyses of the rHDL Particle and the Native HDL Subfractions—The Stokes diameters of the rHDL and native HDL subfractions isolated after immunofluorescence chromatography were determined by nondenaturing gradient polyacrylamide gel electrophoresis (GGE) on Pharmacia precast PAA 4/30 gels as described (22). The following proteins were used as standards for the Stokes diameters of the HDL subclasses: BSA, 71 Å; lactate dehydrogenase, 82 Å; horse ferritin, 122 Å; and thyroglobulin, 170 Å. The apolipoprotein composition of the native HDL subfractions was determined by SDS-PAGE and silver staining on 20% gel slabs.

Lipoprotein Analysis—The protein concentration was determined using the method of Lowry et al. (23) and the absorbance at 280 nm with an extinction coefficient of 1.13 ml/mg-cm for the rHDL and LpAI particles and an estimated extinction coefficient of 0.98 ml/mg-cm for the native HDL subclasses. The lipid content was estimated by assuming that the LpAI/AlI particle contains an equimolar quantity of apoA-I and apoA-II and using molar extinction coefficients of 51,700 M-1 cm-1 and 12,000 M-1 cm-1 (24) and molecular weights of 28,000 and 17,500 for apoA-I and apoA-II, respectively. The phospholipid content was determined by an enzyme-linked immunossorbent assay (25). The Chen et al. method (26) was used to determine the phospholipid content, extending the standard curve down to 0.16 μg of inorganic phosphate. Total cholesterol was determined using the enzymatic assay of Heider and Boyett (27).

The number of apoA-I and apoA-II molecules per particle for the native HDL subfractions was determined by cross-linking with 10 mM BSs, for 3.5 h in a modified version of the Staros technique (28). The cross-linking reaction mixture was quenched using 250 mM ethanolamine and subsequently run on SDS-PAGE (10-30%). Free apoA-I and apoA-II, as well as a 1:1 weight ratio of apoA-I and apoA-II, were also used for cross-linking with BSs and SDS-PAGE in order to serve as standards. In addition, mixtures of apoA-I to apoA-II in different molar ratios were run on the SDS-PAGE gel along with the 98-A LpAI/AlI particle. The intensity of the stained bands was quantitatively assessed using a Pharmacia LKB Ultrascan XL laser densitometer. The observed intensities indicated that the apoA-I and apoA-II were present in a 1:1 molar ratio on the 98-A LpAI/AlI. The 93-A spherical rHDL has been previously shown to contain 3 apoA-I molecules per particle (14).

Fluorescence Spectroscopy—Uncorrected tryptophan (Trp) fluorescence levels with both a 933 spherical rHDL and apoA-I in the absence of apoA-II in the perkin-Elmer MPF-36 fluorescence spectrophotometer at 25 °C with an SLM model 400 fluorescence polarization instrument using a 280-nm exciting light, 4-nm slit widths, and Corning glass 0-54 emission filters. Denaturation with solid GdnHCl was monitored by following the change in the wavelength of maximum fluorescence of the Trp emission with both a 933 spherical rHDL and apoA-I in the perkin-Elmer MPF-36 fluorescence spectrophotometer at 25 °C. An excitation wavelength of 280 nm and 4-nm slit widths were used. The samples were adjusted to an absorbance value of approximately 0.05 at 280 nm with standard salt buffer which was shown to contribute little to the spectral region of interest, 350-355 nm. The same samples were also used to measure the intrinsic fluorescence polarization values at 25 °C with an SLM model 400 fluorescence polarization instrument using a 280-nm exciting light, 4-nm slit widths, and Corning glass 0-54 emission filters. Denaturation with solid GdnHCl was monitored by following the change in the wavelength of maximum fluorescence of the Trp emission with both a 933 spherical rHDL and apoA-I in the perkin-Elmer MPF-36 fluorescence spectrophotometer at 25 °C. An excitation wavelength of 280 nm and 4-nm slit widths were used. The samples were adjusted to an absorbance value of approximately 0.05 at 280 nm with standard salt buffer which was shown to contribute little to the spectral region of interest, 350-355 nm. The same samples were also used to measure the intrinsic fluorescence polarization values at 25 °C with an SLM model 400 fluorescence polarization instrument using a 280-nm exciting light, 4-nm slit widths, and Corning glass 0-54 emission filters. Denaturation with solid GdnHCl was monitored by following the change in the wavelength of maximum fluorescence of the Trp emission with both a 933 spherical rHDL and apoA-I in the perkin-Elmer MPF-36 fluorescence spectrophotometer at 25 °C. An excitation wavelength of 280 nm and 4-nm slit widths were used. The samples were adjusted to an absorbance value of approximately 0.05 at 280 nm with standard salt buffer which was shown to contribute little to the spectral region of interest, 350-355 nm. The same samples were also used to measure...
wavelength \( \lambda \) is the optical path in cm, \( c \) is the concentration in g/ml, and MRW is the mean residue weight. We used a value of 115 g/residue for the rHDL and LpAI subfractions and a value of 114.6 g/residue for the single purified subfraction of LpAI/AII (assuming that apoA-I and apoA-II are equimolar in this particle). The percentage of \( \alpha \)-helix was calculated from the empirical expression of Chen et al. (29). Denaturation with GdnHCl was also monitored using the change in observed ellipticity at 222 nm. The conditions used were the same as those in the fluorescence denaturation experiment described above except that the absorbance at 280 nm of all samples was adjusted to approximately 0.1. This experiment was repeated on two separate preparations of particles with very similar results.

For the CD measurements in the near-ultraviolet region (250–320 nm) a 10-mm path length quartz cell was used, and the optical density of all samples was adjusted to approximately 0.2 at 280 nm. The near-ultraviolet CD spectra are the average of eight scans. Base-line runs were made prior to each sample run and the base-line was subtracted to obtain the final spectrum. The spectra were reproducible for at least two separate preparations of particles.

**LCAT Reaction Kinetics**—The rHDL and native HDL particles were incubated with aliquots of \(^{14}C\)cholesterol dispersed in 2% BSA containing approximately \( 4 \times 10^6 \text{ cpm} \) for 3 h at 37 °C, in order to label the substrate particles (30). The reaction mixtures for the kinetic analysis contained substrate concentrations ranging from \( 8 \times 10^{-8} \) to \( 2 \times 10^{-6} \) M apoA-I or apoA-I plus apoA-II, 2 mg of defatted BSA, 4 mM \( \beta \)-mercaptoethanol, and 0.4 or 0.5 \( \mu \)g of pure LCAT. The mixtures were incubated at 37 °C for 2 h. Lineweaver-Burk plots were constructed from data for four particle concentrations and the corresponding initial velocity results. The inverse slope of the Lineweaver-Burk plot gives the \( V_{\text{max(app)}}/K_{\text{m(app)}} \), an indicator of the overall reactivity of the particles. The \( V_{\text{max(app)}}/K_{\text{m(app)}} \) was adjusted for any differences in enzyme concentration.

**RESULTS**

This work represents one of the first attempts to isolate and characterize distinct size classes within the two main fractions of HDL, LpAI, and LpAI/AII. Fig. 1 shows a photograph of a 4–30% polyacrylamide gradient gel containing the LpAI and LpAI/AII fractions isolated by gel filtration. Panel A shows that LpAI has been successfully separated into two discrete sizes of 106 and 88 Å as determined by gradient gel electrophoresis. These particles are free of contaminating apoA-I which appears as a double band at the bottom of the gel. Panel B illustrates the fractionation of the LpAI/AII. There appear to be three main size classes in this fraction including 96-, 87-, and 80-Å particles. Lane F represents the 96-Å size class which was purified and used in subsequent studies. The smaller particles shown in lane E were not effectively separated. Similar size classes of HDL have been observed in other laboratories starting with either plasma or ultracentrifugally isolated HDL in the immunoaffinity chromatography separation procedure (3, 6, 31–33). Cheung and Albers (3) reported two mean Stokes diameters for LpAI of 10.8 and 8.5 nm and three mean Stokes diameters for LpAI/AII including 9.6, 8.9, and 8.0 nm which agree very well with our results.

The photograph in Fig. 2 represents a silver-stained SDS-PAGE of the isolated HDL subclasses. Lanes D and E represent the LpAI/AII and LpAI fractions, respectively, obtained by immunoaffinity chromatography. Clearly the LpAI is completely depleted of apoA-II. After further fractionation by gel filtration of the LpAI, the isolated 88-Å LpAI subfraction (lane B) and the 106-Å LpAI subfraction (lane C) contain only apoA-I. By overloading an SDS-PAGE gel, we estimated that greater than 95% of the protein in these subfractions was apoA-I; apoE and apoC bands are not detectable. After gel filtration, apoA-I and apoA-II together represent greater than 90% of the 96-Å LpAI/AII subfraction (data not shown). The identity of the other protein bands in lanes E, D, and F, which were largely eliminated by the gel filtration step, were not investigated, but should include the C apolipoproteins, and apoE.

Table I lists the sizes of the native HDL subfractions and the reconstituted rHDL sphere as determined by GGE. The 93-Å spherical rHDL has been previously synthesized and characterized in our laboratory (14). Table I also contains the composition of the native and synthetic particles, as well as the number of molecules of apoA-I and apoA-II determined by cross-linking with BSs.

The cross-linking results indicate that the LpAI 88-Å particle contains 3 apoA-I molecules while the 106-Å particle contains 4 apoA-I. The cross-linking of the 96-Å LpAI/AII gives a cross-linked protein with an equivalent migration to 2 apoA-I plus 2 apoA-II molecules or 3 apoA-I plus 1 apoA-II molecules on SDS-PAGE. In order to determine which of these ratios was correct, the intensity of protein staining for the 96-Å LpAI/AII particle versus that of standard molar

![Fig. 1. Nondenaturing gradient gel electrophoresis of the native HDL subfractions. Panel A shows the LpAI fractions, panel B shows the LpAI/AII fractions. Lanes A and G correspond to the LpAI and LpAI/AII fractions, respectively, before gel filtration; lanes B, C, E, and F represent the native HDL subfractions after gel filtration: 106-Å LpAI, 88-Å LpAI, 87- and 80-Å LpAI/AII; 96-Å LpAI/AII, respectively. Lanes D and H contain the standard proteins, from top to bottom: thyroglobulin, ferritin, catalase, lactate dehydrogenase, and albumin.](image1)

![Fig. 2. SDS-polyacrylamide gel electrophoresis of the apolipoproteins of native HDL, the LpAI and LpAI/AII fractions, and the 88- and 106-Å LpAI subfractions. Lanes A and G contain the apoA-II and apoA-I standards, respectively; lanes B and C represent the isolated 88- and 106-Å LpAI subfractions, respectively; lane F represents native HDL; lanes D and E correspond to the LpAI/AII and LpAI immunoaffinity column fractions, respectively.](image2)
331 to 334 nm, the Trp residues must reside in a fairly nonpolar environment especially compared to free apoA-I. The intrinsic fluorescence of the Trp residues and their fluorescence lifetimes. As previously observed, the free apoA-I has the highest polarization value. The 93-Å rHDL and the native HDL subfractions seem to be similar in the dynamic behavior of their tryptophan residues.

The α-helical content is 55% for the free apoA-I and 65% for the 93-Å rHDL. These values are within the expected error of the results published previously by our laboratory (12, 14). The LpAI subfractions are quite similar in their α-helical content while the 96-Å LpAI/AII particle has much lower α-helicity. This lower α-helicity is due to the presence of apoA-II, since it is well established that the percentage of α-helix is lower in apoA-II than apoA-I (37, 38). Stoffel and Preissner (38) have shown that apoA-II in aqueous solution has 27% α-helical structure, and upon recombination with phospholipids, increases in helix content to 40%.

### Homogeneous Native HDL

**Table I**

| Size and composition of the isolated rHDL and HDL subclasses | Apo-A-I; apoA-II/HDL/M | Apo-A-I; apoA-II/HDL/M |
|------------------------------------------------------------|------------------------|------------------------|
| rHDL or HDL diameter | Composition PL/TC/Prot | apo-A-I; apoA-II/HDL/M |
|----------------------|------------------------|------------------------|
| 93-Å rHDL            | 42/15/1*                | 3.0                    |
| 88-Å LpAI            | 20/15/1                 | 3.0                    |
| 106-Å LpAI           | 23/19/1                 | 4.0                    |
| 96-Å LpAI/AII        | 25/23/1                 | 2.2                    |

* The diameters were obtained by GGE (±2 Å).
* Phospholipid (PL), total cholesterol (TC), and protein (Prot) were determined in duplicate for a representative preparation.

### Apolipoprotein Conformation Based on Spectroscopic Results

Determined by cross-linking the apolipoproteins with bis(sulfosuccinimidyl) suberate (28) and analysis by SDS-PAGE. The previously determined composition for a similar 93-Å rHDL particle was 44/24/1 (14).

* An estimated extinction coefficient of 0.96 ml/mg-cm and the combined molecular weights of apoA-I and apoA-II were used in calculating the composition ratios. We assume two protein units (apoA-I plus apoA-II) per particle.

### Denaturation Studies with Guanidine Hydrochloride

Fig. 3 shows the results of GdnHCl denaturation of free apoA-I, the 93-Å rHDL, and the native HDL subfractions monitored by the change in the wavelength of maximum fluorescence. Free apoA-I denatures quite readily in 2 M GdnHCl as expected (12, 14, 39, 40). The results in Table II indicate that 50% denaturation has occurred at a 1.06 M GdnHCl concentration. The 88-Å LpAI subfraction is more readily denatured than the 106-Å LpAI and 96-Å LpAI/AII subfractions. It is possible that the apoA-II on the LpAI/AII subfraction stabilizes the structure of apoA-I. The 98-Å rHDL is the most stable particle requiring 4.8 M GdnHCl in order to attain 50% denaturation (Table II). From the plateaus in the denaturation curves one may speculate that the different regions of apoA-I containing the 4 Trp residues are denaturing independently from each other.

The results of the denaturation with the same concentrations of GdnHCl, monitored by circular dichroism, are given in Fig. 4. It appears that the loss of secondary structure followed by the change in the ellipticity does not occur at the same rate as the change in the wavelength of maximum fluorescence. Fifty percent of the secondary structure is lost at GdnHCl concentrations less than 2 M for the native HDL subfractions. Again, the 93-Å rHDL appears more resistant to denaturation by GdnHCl, since it requires greater concentrations of GdnHCl to reach the 50% denaturation point.

### Near-ultraviolet Circular Dichroism

The near-ultraviolet (250-320 nm) CD spectra (Fig. 5) were normalized to an optical density of 0.1 (at 280 nm) for the three native HDL subclasses, the total HDL, the 93-Å rHDL, and the free apoA-I. Two peaks at 284 and 291 nm are evident for the different native HDL and rHDL subfractions. The maximum at 284 nm for the 88-Å LpAI is weaker. One minimum at 296 nm is observed for all of these particles, and a broad negative band with several extrema between 260 and 280 nm is observed for the particles that contain only apoA-I. A strong minimum at 272 nm characterizes the spectrum of the 96-Å LpAI/AII particle. The spectra of the 96-Å LpAI/AII and the total HDL (1.065 g/ml < d < 1.31 g/ml) are very similar except that the strong minimum is blue-shifted at 269 nm for the total HDL. The spectrum of the free apoA-I is quite different. The peaks observed for the HDL particles at 291 and 284 nm are replaced by a minimum at 293 nm and a shoulder at 287 nm, and the minimum at 296 nm is no longer present. Also a positive plateau of low ellipticity replaces the negative band centered between 252 and 272 nm. As the extracted lipids of HDL have no optical activity between 250 and 320 nm, the observed differences in the spectra can be attributed to differences in protein conformation (37).
The samples were adjusted to an absorbance value of 0.05 at 280 nm. The values shown are similar for the different particles and are very low compared to discoidal rHDL substrates of fluorescence spectroscopy. The fluorescence of the apoA-I Trp residues is shown as a function of subfractions. Independent of maximum fluorescence is f2 nm.

Reactivity with LCAT—Table III gives the results for the reactivity of LCAT with the 93-Å rHDL and the native HDL subfractions. Independent $V_{\text{max(app)}}$ and $K_{\text{m(app)}}$ values were not obtained since the particle concentrations were too low relative to the $K_{\text{m(app)}}$. The specificity constants, $V_{\text{max(app)}}/K_{\text{m(app)}}$, were determined from the inverse slopes of Lineweaver-Burk plots. The values shown are similar for the different particles and are very low compared to discoidal rHDL substrates of LCAT (12, 14). The 106-Å LpAI particles appear to be the least reactive and the 96-Å LpAI/AII the most reactive particles in these preparations.

DISCUSSION

Prior to structural analysis of the three isolated subclasses of HDL, we needed to establish their homogeneity. They were shown (Fig. 1) to be quite homogeneous in size by nondenaturing GGE. Further proof of the homogeneity of the purified particles was the detection of a single band upon cross-linking of the proteins followed by analysis on SDS-PAGE. By SDS-PAGE we have also established that the LpAI fraction was completely depleted in apoA-II (Fig. 2, lane B) and that apoA-I represented greater than 95% of the proteins contained in the two size subclasses of LpAI (88 and 106 Å). By the same technique apoA-I and apoA-II have been shown to represent greater than 90% of the proteins in 96-Å LpAI/AII particles.

The sizes given in Table I are similar to those reported by other laboratories (3, 6, 31–33). However, comparisons between our compositional data and those of other laboratories are not easily accomplished since we have isolated pure size classes within the LpAI and LpAI/AII fractions where most other research groups have not. Only Nichols et al. (3) have reported the composition of a purified 10.6-nm LpAI size subclass. Our 106-Å LpAI subfraction contains a similar amount of phospholipid, but less total cholesterol and more protein. These differences in composition may be due to differences in the homogeneity of the isolated particles and to the inherent ability of a fixed protein framework to contain more or less lipid, as shown for discoidal rHDL particles (14).

The number of apoA-I molecules per LpAI 106-Å particle agrees with the results of Nichols et al. (41) showing that 4 apoA-I molecules per particle are present on adult HDL or LpAI. We found 2 apoA-I and 2 apoA-II molecules per particle (i.e., a 1:1 molar ratio) within the 96-Å LpAI/AII subfraction. Other investigators have determined this molar ratio to range from 0.8:1 to 3:1 for ultracentrifugal fractions of HDL (31).
The present 93-Å rHDL particle preparation contains a similar amount of phospholipid compared to our previous ones, but a substantially lower amount of cholesterol (14). Compared with the native HDL subclasses, the rHDL contains considerably more phospholipid and less total cholesterol. In the past, we considered the 93-Å rHDL to be a good model for mature HDL in the plasma compartment; however, due to this difference in lipid composition, the 93-Å rHDL probably represents an intermediate in the formation of a mature spherical rHDL. The synthesis of the 93-Å rHDL involves adding exogenous heat-inactivated LDL as a source of free cholesterol, LCAT, and BSA to an incubation mixture at 37 °C. It is possible that the absence of lipid transfer proteins and other sources of cholesterol prevent the full maturation of the rHDL.

The fluorescence spectroscopy results indicate no significant differences in the environment and the mobility of the tryptophan residues of the rHDL and native HDL subfractions including the 96-Å LpA1/AII particle. Using Trp fluorescence quenching experiments with iodine, Talussot and Ponsin (42) also did not observe any differences between rHDL containing apoA-I and rHDL containing apoA-I and apoA-II. The circular dichroism spectra in the far-ultraviolet region showed ellipticity minima at 222 and 208 nm and maxima in the 190–195-nm region indicating that α-helix is the major secondary structure of the 93-Å rHDL and the native HDL subfractions. The content of α-helical secondary structure did not differ markedly (61–65%) for the HDL particles except for the 96-Å LpA1/AII particle which had an α-helix content of 52%. The lower percentage of α-helix in the 96-Å LpA1/AII particle is the result of the equimolar amounts of apoA-II and apoA-I in the particle. As the α-helicity of apoA-II complexed with phospholipid has been established at about 40% (38), we can estimate from the total α-helicity of the 96-Å LpA1/AII that the α-helix content of apoA-I at about 60%. Thus, the presence of apoA-II seems not to alter the secondary structure of apoA-I in the native 96-Å LpA1/AII.

In order to examine the structural stability of the apolipoproteins in the native HDL subfractions, the denaturation by GdnHCl was assessed by the change in the wavelength of maximum fluorescence and the ellipticity at 222 nm. The wavelength of maximum fluorescence monitors the NH2-terminal region of the apoA-I (up to residue 108) which contains the 4 Trp residues. Since apoA-II does not contain any Trp
residues, only the structural changes of the apoA-I molecules are monitored in the 96-Å LpAI/AII particle in the fluorescence experiment. The ellipticity change at 222 nm follows the loss in secondary structure upon denaturation. The secondary structure represented mainly by α-helical segments is thought to start at residue 44 and to extend to the carboxyl terminus of the apoA-I. The apoA-I structure as previously described (12-14) and as shown in Figs. 3 and 4 is clearly stabilized by lipids while free apoA-I completely denatures by 2 M GdnHCl. Both Figs. 3 and 4 show a general pattern in the stability of the 93-Å rHDL and the native HDL subclasses; the 88-Å LpAI particles are the least stable followed by the 106-Å LpAI particles. The 96-Å LpAI/AII particles are more resistant to GdnHCl than the LpAI subfractions but less so than the 93-Å rHDL. Possibly the 93-Å rHDL are the most stable particles because of their higher content of surface lipids, and the absence of polyunsaturated lipids which may decrease the hydrophobic interactions within the particles. The apoA-II, even though it does not seem to alter the conformation of the apoA-I on the 96-Å LpAI/AII particle, may stabilize the apoA-I conformation and the particles in general. Nichols et al. (43) have reported that ultracentrifugation of HDL in GdnHCl causes the dissociation of apoA-I alone between 2 and 3 M GdnHCl and the dissociation of apoA-I together with apoA-II between 5 and 6 M GdnHCl. Perhaps these separate pools of apoA-I represented LpAI and LpAI/AII and their relative stability due to apoA-II. In fact, Cheung and Wolf (32) investigating the stability of LpAI and LpAI/AII to ultracentrifugation found that the LpAI fraction lost more protein than the LpAI/AII fraction and changed the relative proportions of the different size subclasses. The LpAI/AII remained essentially unchanged. These reports and our results with the 96-Å LpAI/AII particles support the hypothesis that the LpAI/AII particles are more stable than the LpAI subclasses.

The curves in Figs. 3 and 4 suggest a multiphasic denaturation. Clearly, the secondary structure is lost before the apoA-I is completely denatured according to the fluorescence wavelength experiments. The results show a similar pattern of denaturation for apoA-I in all of the particles suggesting once again a similar protein structure. The first step in the denaturation curves shown in Fig. 3 may represent the exposure of 1 Trp residue in a rather unstable region of the protein. We propose that this region involves Trp-108 which is located in one of the α-helical segments of apoA-I. Recent work in our laboratory on the denaturation behavior of the Lys-107 deletion mutant of apoA-I in discoidal rHDL complexes indicated that this section of apoA-I is structurally flexible, and is readily denatured (44, 45). The next denaturation step in Fig. 3 from about 2.5 to 4.5 M GdnHCl, is still accompanied by changes in secondary structure and represents a wavelength change that could account for the exposure of the 2 Trp residues to solvent; therefore, we speculate that the Trp-50 and Trp-72 residues may be involved, since they are located in putative α-helical segments. Finally, we propose that the Trp-8 residue is exposed only at GdnHCl concentrations above 5 M. At this denaturant concentration all secondary structure is lost, yet one of the Trp residues is still protected, suggesting that the possible candidate is Trp-8 in a region of apoA-I which has no predicted α-helical structure. Furthermore, human apoA-IV, which has a linear sequence very homologous to apoA-I (46) and forms comparable rHDL particles to apoA-I (47), has a single Trp at position 12 which behaves in the presence of GdnHCl very much like the most stable Trp in apoA-I. While the secondary structure of apoA-IV in rHDL complexes is lost with 5.5 M GdnHCl, the single Trp residue in the NH2-terminus of the molecule is not yet exposed to solvent (47). These results strongly suggest a very stable NH2-terminal domain in apoA-IV rHDL complexes. Similarly it is likely that the 93-Å rHDL and the native HDL subclasses contain apoA-I with a stable NH2-terminal domain.

The near-ultraviolet circular dichroism spectra of the HDL particles and free apoA-I are very similar to the spectra measured by Lux et al. (37). The spectra of the different HDL particles display maxima at 284 and 291 nm and a minimum at 296 nm (see Fig. 5). On the basis of their location and characteristic spacing, these maxima have been assigned to 1 or more of the Trp residues of apoA-I and correspond to two different vibrational states of the 1Lb electronic transition of Trp (37, 48-50). From studies with Trp model compounds, the minimum at 296 nm in HDL has been attributed to the 1Lb transition of Trp (50). The absence of 284- and 291-nm maxima (37, 51) in the free and in the lipid-bound apoA-II, which contains no Trp residues, confirms their assignment to the Trp residues. In free apoA-I, these two peaks are reversed in sign and red-shifted to 287 and 293 nm, showing that the Trp residues responsible for the spectra have a different average conformation and are in a more polar environment.

Tryptophans, tyrosines (37, 48-50), and disulfides (52) may all contribute to the circular dichroism signal in the 260-280-nm region. However, since Lux et al. (37) have shown that an increase in pH from 9.5 to 12.6 is accompanied by major changes of the ellipticity between 260 and 280 nm, we suggest that there is an important contribution of the tyrosine residues to the ellipticity in this region. Lux et al. (37) showed that the repopulation of HDL apolipoproteins with phosphatidylcholine alone was able to restore the 284- and 291-nm maxima which were inverted in the free apolipoprotein. The addition of cholesterol esters intensified these bands and was required to reproduce the broad negative band between 260 and 280 nm present in native HDL. It is of interest to note that the intensity of the maxima at 284 nm for the HDL particles (Fig. 5) seem to be proportional to the total phospholipid content (Table 1). The presence, in the total HDL and in the 96-Å LpAI/AII, of apoA-II containing 4 tyrosines per monomer contributes to the strong negative ellipticity observed at 269 and 272 nm, respectively. The addition of one part of the 88-Å LpAI, one part of the 106-Å LpAI, and two parts of the 96-Å LpAI/AII spectra between 250 and 315 nm approximately simulates the HDL spectrum, including the strong minima from 262 to 272 nm. The near-ultraviolet circular dichroism spectra of the 88- and 106-Å LpAI subfractions are very similar and indicate that the tertiary structure of the apoA-I in both these subclasses of LpAI is essentially identical. The section of the spectrum in Fig. 5 from 280 to 320 nm, due to the electronic transitions of the apoA-I Trp residues, is also similar for the 96-Å LpAI/AII as well as the 93-Å rHDL. The shape, sign, characteristic wavelengths, and spacing of the spectrum for the 93-Å rHDL particles is identical to the native LpAI spectra; only the intensity of the 284-nm band for the 93-Å rHDL is somewhat higher probably because the rHDL contains double the amount of phospholipid per apoA-I compared to the 88-Å LpAI particle. Our results suggest very similar conformations of apoA-I in all of these native subfractions and the 93-Å rHDL, including the same number of α-helical segments.

Finally, the LCAT reactivity studies indicate that the 93-Å rHDL and the native HDL subfractions are all poor substrates for LCAT compared to the 96-Å rHDL discs which are at least 30-fold more reactive (14). Overall this low reactivity may be due to product inhibition by the cholesterol esters in these particles and/or to an unfavorable apoA-I structure for
the activation of the LCAT reaction. The 106-Å LpAI is somewhat less reactive than the 88-Å LpAI particle, which agrees with, but does not explain, the differential reactivity of HDL2 versus HDL3 with LCAT (53). At this point we have no explanation for the marginally higher reactivity of the 96-Å LpAI/AII subfractions as compared to both LpAI and HDL, with LCAT.

From the above results the major differences between the 93-Å rHDL and each of the native HDL subclasses is their phospholipid composition. Nevertheless, the 93-Å rHDL may still be a good model for native HDL since the apoA-I structure appears to be quite similar for all of the particles studied. The major difference found is the increased stability of the 96-Å LpAI/AII subfractions as compared to both LpAI and HDL. The major differences between the major differences between the HDL subclasses is their phospholipid composition. Nevertheless, the 93-Å rHDL may still be a good model for native HDL since the apoA-I structure appears to be quite similar for all of the particles studied. The major difference found is the increased stability of the 96-Å LpAI/AII subfractions as compared to both LpAI and HDL. This property may underlie different functions of LpAI and LpAI/AII in the circulation and may be responsible for their different catabolism.

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