Structural and functional properties of reconstituted high density lipoprotein discs prepared with six apolipoprotein A-I variants

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Abstract Six apolipoprotein A-I (apoA-I) variants containing the following amino acid changes: $Pro_3 \rightarrow Arg$, $Pro_4 \rightarrow Arg$, $Lys_{107} \rightarrow 0$ (Lys deletion) $Lys_{107} \rightarrow Met$, $Pro_{165} \rightarrow Arg$, and $Glu_{198} \rightarrow Lys$, and the corresponding normal allele products, were isolated by preparative isoelectric focusing from heterozygous individuals. The apoA-I samples were reconstituted with palmitoyloleoyl phosphatidylcholine (POPC) or dipalmitoyl phosphatidylcholine (DPPC), and small amounts of cholesterol, into discoidal high density lipoprotein (HDL) complexes in order to examine their lipid binding and structural properties as well as their ability to activate lecithin:cholesterol acyltransferase (LCAT). Starting with initial molar ratios around 100:5:1 for phosphatidylcholine–cholesterol–apolipoprotein, all the normal and variant apoA-Is were completely incorporated into reconstituted HDL (rHDL). The rHDL particle sizes and their distributions were examined by nondenaturing gradient gel electrophoresis, before and after incubation with LDL, to assess the folding of apoA-I in the complexes. Intrinsic Trp fluorescence properties of the rHDL were measured, as a function of temperature and guanidine hydrochloride concentration, to detect conformational differences in the apoA-I variants. In addition, the LCAT reaction kinetics were measured with all the rHDL, and the apparent kinetic constants were compared. In terms of the structure of the rHDL particles, all the normal variant apoA-Is had similar sizes (94, 96 Å) and size distributions, and indistinguishable fluorescence properties, with the exception of the $Lys_{107} \rightarrow 0$ mutant. This variant formed slightly larger particles that were resistant to rearrangements in the presence of LDL, and had an altered apoA-I conformation in the vicinity of the Trp residues. The kinetic experiments with LCAT indicated that the apoA-I variants, $Lys_{107} \rightarrow 0$ and $Pro_{165} \rightarrow Arg$, in rHDL particles had statistically different (30 to 90%) kinetic constants from the corresponding normal allele products; however, the variability in the kinetic constants among the normal apoA-I products was even greater (40 to 430%). Therefore, we conclude that the effects of these six mutations in apoA-I on the activation of LCAT are minor, and that the structural effects on rHDL, and possibly native HDL, are insignificant with the exception of the $Lys_{107} \rightarrow 0$ mutation. —**Jonas, A., A. von Eckardstein, K. E. Kézdy, A. Steinmetz, and G. Assmann.** Structural and functional properties of reconstituted high density lipoprotein discs prepared with six apolipoprotein A-I variants. *J. Lipid Res.* 1991. **32:** 97–106.

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Apolipoprotein A-I (apoA-I), the major protein component of high density lipoproteins (HDL), determines the structure and the stability of HDL in plasma. Furthermore, apoA-I is the best activator of the lecithin cholesterol acyltransferase (LCAT) reaction in vitro (1), and possibly is a mediator of the interactions of HDL with cell surface receptors (2). The amino acid sequence of human apoA-I, initially determined by chemical analysis, appears to contain amphipathic α-helical regions that are responsbile for the lipid binding and self-association properties of this apolipoprotein (3). More recent studies of the structure of the apoA-I gene revealed that the 3rd and 4th exons give rise to the mature protein (4), and that the 4th exon encodes a sequence with characteristic 22 amino acid repeats also found in other water-soluble apolipoproteins (5). These repeats may give rise to eight amphipathic α-helical segments in apoA-I, starting at residue 44

Abbreviations: apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; rHDL, reconstituted HDL; IEF, isoelectric focusing; POPC, palmitoyloleoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; GGE, gradient gel electrophoresis; LDL, low density lipoprotein; PC, phosphatidylcholine; Gnd HCl, guanidine hydrochloride; app. V_{max}, app. K_m, app. V_{max}/app. K_m, apparent kinetic constants; r, correlation coefficient; rHDL-POPC, rHDL prepared with POPC; rHDL-DPPC, rHDL prepared with DPPC; HPLC, high performance liquid chromatography.

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and ending a few residues from the C-terminus of the protein. Many of the 22 amino acid repeats start with Pro residues which are thought to participate in bends between adjacent α-helical segments, changing their relative orientation and optimizing interactions with lipids (6). Lysine residues appear frequently at interfaces between the polar and nonpolar sides of the amphipathic helices; they may contribute to the interactions with lipids through the hydrocarbon portions of their side chains (7). Acidic residues are usually found near the middle of the polar face of the helices, but two Glu residues, at positions 78 and 111 on the hydrophobic sides of two helices, have been postulated to play a role in LCAT activation (8).

To date, 22 naturally occurring structural variants of apoA-I have been discovered. Most have been identified by electrophoretic screening in apparently normal individuals and two were detected in individuals suffering from familial hypoalphalipoproteinemia or from familial amyloidotic polyneuropathy (9, 10). For the most part, the variants appear to be benign; nevertheless, some may have functional deficiencies that are compensated for by other apolipoproteins. For example, it has been shown that esterification of cholesterol by LCAT proceeds in cases of apoA-I deficiency (11).

The objective of this study was to examine the relationships between the structure and function of six apoA-I variants ($Pro_3 \rightarrow Arg$, $Pro_4 \rightarrow Arg$, $Lys_{107} \rightarrow 0$, $Lys_{107} \rightarrow Met$, $Pro_{165} \rightarrow Arg$, $Glu_{198} \rightarrow Lys$) and the corresponding normal apoA-I from the same heterozygous individuals. We set out to compare their lipid binding properties, their effects on the structure of reconstituted HDL (rHDL), and the reactivity of the rHDL with LCAT.

METHODS

Preparation of the normal and variant apoA-I isoforms

We have previously reported nine different structural defects in 16 apoA-I variants that were detected by electrophoretic screening (9, 12). Heterozygote individuals for the amino acid changes in apoA-I: $Pro_3 \rightarrow Arg$ (sample 1B), $Pro_4 \rightarrow Arg$ (sample 2B), $Lys_{107} \rightarrow 0$ (sample 3B), $Lys_{107} \rightarrow Met$ (sample 4B), $Pro_{165} \rightarrow Arg$ (sample 5B), and $Glu_{198} \rightarrow Lys$ (sample 6B) (9), donated plasma by plasmapheresis. Normal (series A) and variant (series B) apoA-I allele products from each donor were prepared in parallel by a procedure that has been reported previously (12, 13). Briefly, HDL were isolated from plasma by sequential ultracentrifugation and were delipidated with ethanol–ether 3:1 (v/v). The precipitated apolipoproteins were solubilized in 5 mM sodium phosphate buffer, containing 1% decylsulfate (w/w), 100 mM dithiothreitol, and 6 M urea (pH 8.0). The apoA-I isoproteins were separated by isoelectric focusing (IEF) on preparative gels with immobilized pH-gradients (Pharmacia-LKB, Bromma, Sweden), ranging from pH 4.5 to pH 5.5 for anodic variants, and from pH 5 to pH 6 for cathodic variants. The gels were rehydrated with 6 M urea and 15% glycerol (w/v). After overnight IEF at 2,000 V, 15 mA and 5 W, and an additional 4 h at 3,000 V, the normal and variant apoA-I isoforms were seen in the unstained gels as opalescent lines. Gel strips containing the apoA-I isoproteins were cut out of the gel without prior staining. After electroelution for 24 h in a 0.05 M Tris-HCl buffer (pH 9.0) the proteins were dialyzed against 0.01 M NH_4HCO_3 (pH 7.8) for 24 h. Subsequent lyophilization and solubilization in 0.01 M NH_4HCO_3 (pH 7.8) was following by purification of the protein samples by reversed phase HPLC. Contamination of the apoA-I preparations with other proteins was excluded by performing analytical IEF on 20-μl aliquots of each one of the samples. Because of their identical isoelectric points, contamination of normal apoA-I isoproteins with the deamidated isoforms of cathodic variants, as well as contamination of anodic variant apoA-I isoforms with the corresponding deamidated isoprotein of normal apoA-I could not be prevented. However, deamidated isoproteins in HDL probably represent less than 5% of the nondeamidated isoprotein as determined by scanning densitometry of normal apoA-I separated by analytical IEF.

Preparation and characterization of rHDL particles

The rHDL complexes were prepared by the sodium cholate dialysis method described by Jonas (14), with the modification that the apoA-I stock solutions (containing ~1 mg of apoA-I) were prepared directly in 0.20 ml of a sodium cholate solution (30 mg/ml) in 10 mM Tris-HCl buffer, pH 8.0, 0.15 M NaCl, 1mM NaN_3, 0.01% EDTA. The same buffer was used throughout the subsequent experiments. The rHDL particles prepared with palmitoyloleoyl phosphatidylcholine (POPC) contained 80:4:1 molar ratios of POPC–cholesterol–apoA-I, corresponding to weight ratios (in mg) of 1.09:0.028:0.5 in a final volume of buffer of 0.250 ml. Each sample also included 4000 cpm of [4-^{14}C]cholesterol (New England Nuclear) per nmol of this lipid.

The rHDL samples containing dipalmitoyl phosphatidylcholine (DPPC) were prepared from reaction mixtures that had molar ratios of 100:5:1 DPPC–cholesterol–apoA-I, corresponding to weight ratios (in mg) of 1.31:0.035:0.5, in a volume of buffer equal to 0.250 ml. The same relative amounts of radiolabeled cholesterol were included in these samples as in those containing POPC. After exhaustive dialysis against buffer, intended to remove the Na cholate (30 mg/ml) that had been added to each reaction mixture to give a molar ratio of 1:1 relative to the PC, the clear rHDL samples were diluted to apoA-I concentrations in the range from 0.260 to 0.370 mg/ml. The rHDL samples were stored at 4°C in the standard buffer, and were analyzed within 8 weeks of their preparation.

From previous experience we determined that most rHDL samples can be stored for about 2 months without major changes in their properties.

The sizes and size distributions of the rHDL particles were determined by nondenaturing gradient gel electrophoresis (GGE) using PAA 4/30 gels from Pharmacia LKB Biotechnology Inc., run in a Tris-boric acid, pH 8.4 buffer, at 150 V for 19 h. The gels were stained with Coomassie Blue stain and were scanned with an LKB Ultro Scan XL laser densitometer. Protein standards: bovine serum albumin, lactate dehydrogenase, catalase, horse ferritin, and thyroglobulin, supplied in the Pharmacia Fine Chemicals calibration kit, were included in each gel.

A second series of GGE experiments was performed on the rHDL samples after they had been incubated with human low density lipoprotein (LDL) for 24 h at 37°C. This treatment has been shown previously by us to deplete rHDL particles of some of their phospholipid, and in the case of the POPC-containing samples, to alter their structure, forming limiting particles of sizes around 78 and 108 Å in diameter (15, 16). Since these particle transformations are connected with major structural rearrangements of the apoA-I, it was of interest to establish whether or not the mutant apoA-I samples can undergo similar transformations. In these experiments rHDL samples (0.020 mg of apoA-I) were incubated with purified, heat-inactivated LDL (0.040 mg of protein). After 24 h at 37°C, the samples were applied directly to the gels for nondenaturing GGE, under the same conditions as the rHDL samples alone. The LDL was completely separated from the rHDL particles during the electrophoresis run, and remained on the top of the gels.

Spectroscopic analysis of the rHDL complexes

The intrinsic fluorescence of the Trp residues of apoA-I in rHDL complexes was measured with a Perkin-Elmer MPF-66 Fluorescence Spectrophotometer using 280 nm exciting light, and 5 nm excitation and emission slit widths. Spectra were recorded from 290 to 450 nm. The wavelengths of maximum fluorescence were obtained from uncorrected spectra at regulated temperatures between 10 and 40°C. For the denaturation experiments fluorescence spectra were recorded immediately after adjusting the rHDL solutions to a 3 M guanidine hydrochloride (Gdn HCl) concentration (initial, 0 h) and after 24 h at 25°C.

Fluorescence polarization measurements were performed with the SLM, Model 400 fluorescence polarization instrument using a 280 nm excitation wavelength, 4-nm slit widths, and 0-54 Corning glass filters in the path of the emitted light. The temperature was regulated, and measurements were taken at 10°C intervals between 10 and 40°C.

Reaction of the rHDL complexes with LCAT

The purification of LCAT and the assay of the enzymatic activity has been described in our previous work (17). The preparation of LCAT used in these experiments had a specific activity of 34,000 nmol cholesteryl ester formed/h per mg and a concentration of 0.05 mg/ml. Its activity remained essentially constant for 6 months, including the period of these experiments. In the kinetic experiments, three concentrations of each sample in the range from 2.6×10^{-7} to 2.7×10^{-6} M apoA-I were used with a constant enzyme concentration of 0.025 μg/ml, known to give a linear production of cholesteryl ester over 15 min of reaction, at 37°C. The observed initial velocities as a function of apoA-I concentration were analyzed by the double reciprocal Lineweaver-Burk plot using normal Michaelis-Menten kinetics. Linear regression analysis was used to obtain the apparent (app.) kinetic constants: app. V_{max}, app. K_m, and app. V_{max}/app. K_m. All the kinetic experiments were repeated, and the reported apparent kinetic constants are the average of two measurements. In the linear regression analysis all the correlation coefficients (r) ranged from 0.980 to 1.000, except for the 3B samples which had r values from 0.896 to 0.994.

The comparisons of the particle sizes and the mean values of the apparent kinetic constants for the different samples were performed by using the Student's t statistical distribution test and a level of significance, $\alpha = 0.01$ (18). For the comparison of the kinetic parameters among different individuals and within pairs, we determined the standard error of measurement for each one of the kinetic parameters using the ratio of the duplicated data. The mean of these ratios was near 1.0 for the 12 samples, and gave standard deviations ranging from 0.08 to 0.430. The standard deviations were as follows, for the rHDL-POPC samples: 0.18 for app. V_{max}, 0.08 for app. V_{max}/app. K_m, 0.28 for app. K_m; and for the rHDL-DPPC samples: 0.33 for app. V_{max}, 0.19 for app. V_{max}/app. K_m, 0.43 for app. K_m. Comparisons between the desired kinetic parameters were then based on the difference between their ratio and 1.0 using the Student's t-test statistics for a significance level, $\alpha = 0.01$ (18).

RESULTS

Particle sizes and size distributions

The GGE patterns (**Fig. 1**). for the rHDL particles prepared either with POPC or the disaturated DPPC give no indication of any free apoA-I; therefore, the incorporation of the variant or "normal" apoA-I into rHDL particles appears to be complete. Regardless of the type of mutation present in these apoA-I samples, the lipid binding ability of the apolipoprotein is retained. In all cases the

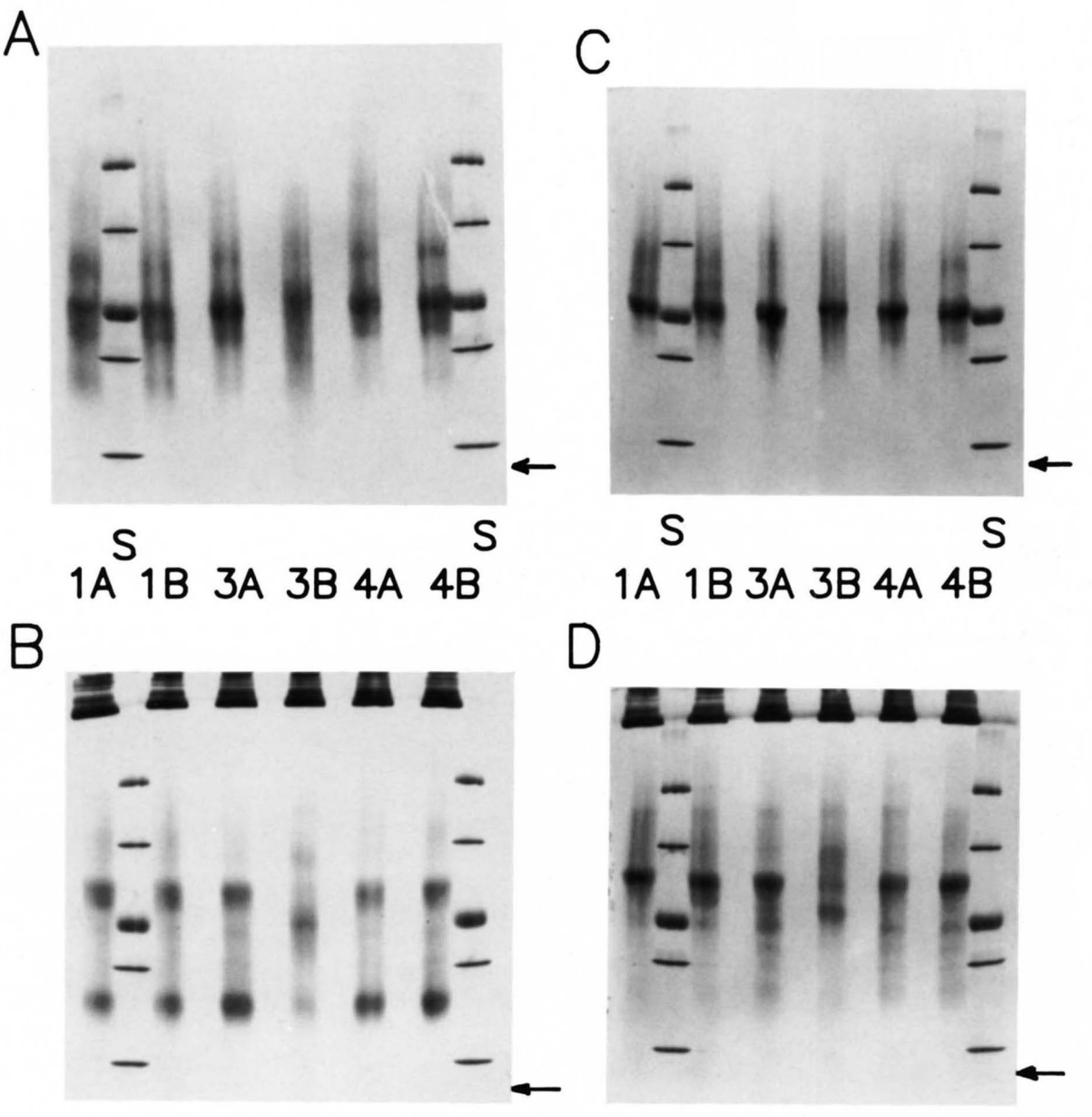

Fig. 1. Nondenaturing gradient gel electrophoresis (GGE) patterns stained for protein for selected rHDL complexes: A, rHDL complexes with POPC; B, the same rHDL-POPC complexes after incubation with LDL for 24 h at 37°C; C, rHDL complexes prepared with DPPC; D, the same rHDL-DPPC complexes incubated with LDL for 24 h at 37°C. The standard proteins (S) are, from top to bottom: thyroglobulin, horse ferritin, catalase, lactate dehydrogenase, and bovine serum albumin. The arrow indicates the migration position of free apoA-I.

particles formed have diameters in the range from 77 to 150 Å, corresponding to the size classes and subclasses of discoidal rHDL that have been previously studied in our laboratory (16, 19).

The densitometer scans (**Fig. 2A**) show, superimposed, the traces for the A/B pairs of complexes (rHDL) prepared with POPC. In all cases the major peak corresponds to particles with diameters in the range from 93.2 to 96.1 Å. The mean size of the "normal" particles is 94.0 Å with a standard deviation of 0.52 Å (n = 6). At the significance level of $\alpha = 0.01$, the only particles that are distinct from this mean are the 3B, and 6A species. The 3B particles stand out as visibly larger compared to the rest.

The particles prepared with DPPC have the densitometric patterns shown in **Fig. 3A**. The main peak corresponds to rHDL with diameters from 94.5 to 97.6 Å. The mean size of the "normal" particles in 95.4 Å with a standard deviation of 0.65 Å (n = 6). At the significance level

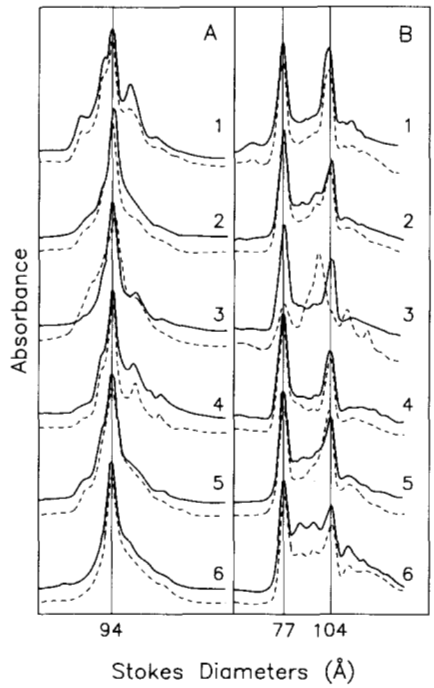

Fig. 2. Densitometer scans of the GGE patterns for rHDL-POPC complexes: In panel A are shown the rHDL-POPC complexes as originally prepared from reaction mixtures containing POPC-cholesterol-apoA-I 80:4:1 (mol/mol); In panel B are the same complexes after incubation with LDL at 37°C for 24 h. The numbers 1 through 6 represent the individual donors of apoA-I; the full traces (—) the rHDL prepared with "normal" apoA-I; and the dashed traces (---) the rHDL prepared with the corresponding variant apoA-I. The Stokes diameters were determined relative to the standard proteins listed in the legend to Fig. 1.

of α = 0.01, only the rHDL particles prepared from samples 3B, 5A, and 5B are distinct from the mean. When the differences between the rHDL sizes of each A/B pair are considered, only the 3A/3B pair has a significant difference in complex sizes with both lipids; the 6A and 6B pair has a small difference in the size of the rHDL particles containing POPC. All the other rHDL pairs have indistinguishable sizes for the main peak, and similar particle size distributions.

After incubation of the rHDL-POPC complexes with LDL, the expected particle rearrangements are observed for the majority of the samples; the main peak decreases in intensity and is replaced by particles with mean diameters of 77.5 and 104.4 Å with a 0.5 Å standard deviation in each case. From our previous work (16) we known that this transformation is accompanied by major structural rearrangements in apoA-I that are consistent with the removal of one or two α-helical segments of apoA-I from contact with the acyl chains of the POPC and their rearrangement into a different compact structure. Although the extent of this transformation varies for the different samples, as seen in Fig. 2B, it is clear that it is very similar within each A/B pair, with the exception of the 3A/3B pair. In this case the transformation of the 3A sample is normal, while the rearrangement of the 3B variant ($Lys_{107}\rightarrow 0$) is inhibited: there are only modest amounts of 78 Å particles, and 94 Å particles remain the major species, 104 Å particles are hardly detectable, and some 116 Å particles appear.

The transformation of the DPPC particles, in the presence of LDL has not been characterized or modeled at the molecular level to the same extent as the rHDL-POPC species. However, it is interesting to note that the formation of the 78 Å particles is minimal, and the main change is into a mean particle size of 107.4 Å with a standard deviation of 0.5 Å (Fig. 3B). Comparisons within pairs show, once again, that the transformation of the 3B particles ($Lys_{197}\rightarrow 0$) is impaired relative to their "normal" (3A) counterparts. **Table 1** summarizes the particle sizes and the appropriate statistics.

Fluorescence studies

The wavelengths of maximum fluorescence of Trp residues at each of four temperatures (from 10 to 40°C) are shown in **Fig. 4**; they are essentially the same for the A series of rHDL and the corresponding B series of samples, with the exception of the 3B rHDL ($Lys_{107}\rightarrow 0$) complex. For this variant there are 2 nm shifts to longer wavelengths at all the temperatures measured, indicating

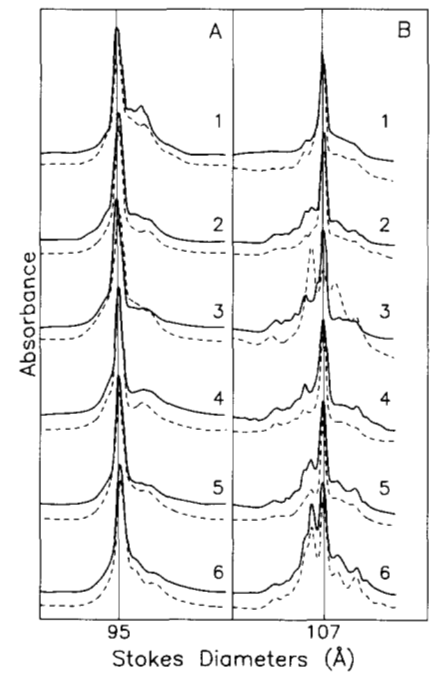

Fig. 3. Densitometer scans of the GGE patterns for rHDL-DPPC complexes. In panel A are shown the rHDL-DPPC complexes as originally prepared from reaction mixtures containing DPPC-cholesterol-apoA-I 100:5:1 (mol/mol). In panel B are the same complexes, after incubation with LDL at 37°C for 24 h. The numbers 1 through 6 represent the individual donors of apoA-I; the full traces (—) the rHDL prepared with "normal" apoA-I; and the dashed traces (---) the rHDL prepared with the corresponding variant apoA-I.

TABLE 1. Summary of rHDL particle sizes obtained from GGE experiments

Samples	Sizes of the Major Particles[a]			Standard Deviation[a]
	Å			Å
rHDL-POPC	94.0		(110)	0.52
3B[b]	96.1			
rHDL-POPC after exposure to LDL	77.5	104.4		0.50
3B[b]	(78) 93.8		116	
rHDL-DPPC	95.4		(115)	0.65
3B[b]	97.6			
rHDL-DPPC after exposure to LDL		107.4		0.51
3B[b]	98	107	118	

[a]The mean sizes and standard deviations are given for the particle populations of the rHDL-POPC and rHDL-DPPC complexes prepared with the "normal" apoA-I. The sizes of minor rHDL components are given in parentheses.
[b]Particles prepared with the 3B ($Lys_{107}\rightarrow 0$) variant of apoA-I.

that the average environment of the Trp residues is slightly more polar than in all the other rHDL-POPC complexes. Similar results are obtained with the series of particles (A and B) prepared with DPPC. Again, the 3B sample shows somewhat different properties, with wavelengths shifted to the red by about 2–3 nm.

Measurements of the wavelength of maximum fluorescence of Trp in 3 M Gnd HCl solutions of the rHDL complexes immediately after the addition of the Gnd HCl, and 24 h later, provide an indication of the extent and rate of denaturation of the normal and variant apoA-Is in the rHDL complexes. The particles containing POPC denature more rapidly and more extensively than those containing DPPC; and only the 3B sample, incorporated into particles with DPPC, appears to denature more readily than the other rHDL particles. At times of 0 and 24 h of exposure to 3 M Gnd HCl, the maximum fluorescence wavelengths for both series of particles prepared with DPPC were 335 and 338, respectively, compared to 338 and 340 for the 3B sample. The intrinsic fluorescence polarization measurements at several temperatures (10 to 40°C) gave comparable results for the A and B series of particles for both lipids, including the 3B sample. Clearly, the order and mobility of the Trp residues is not measurably affected in the variants compared to the normal apoA-Is in the rHDL particles.

Thus, only the 3B samples show minor conformational differences in the apoA-I that affect the average polarity of the Trp residue environment, and increase slightly the susceptibility of the apoA-I to denaturation by Gnd HCl.

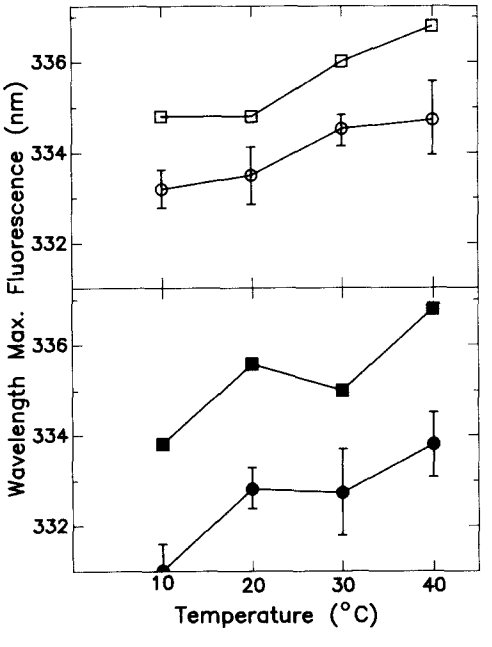

Fig. 4. Wavelengths of maximum fluorescence as a funciton of temperature for: (top) rHDL-POPC complexes and (bottom) rHDL-DPPC complexes. The means (○, ●) and standard deviation bars are shown for the complexes prepared with the six normal apoA-Is. The rHDL complexes prepared with the 3B ($Lys_{107}\rightarrow 0$) variant apoA-I (□, ■) are significantly different from the mean. The values for the other variant products did not differ significantly from the mean of the normal products and are not shown.

Reaction with LCAT

A sample Lineweaver-Burk plot of the kinetic results for two rHDL pairs, prepared with POPC, is shown in **Fig. 5**. The good reproducibility and linearity of the data is evident in this example.

Table 2 and **Table 3** give the results from the linear regression analysis of the kinetic data: correlation coefficients, apparent V_{max}, app. V_{max}/app. K_m, and app. K_m parameters, as well as the appropriate statistical values for the rHDL samples prepared with POPC or DPPC, respectively, for the A and B series of samples. These results show that the mean values of the kinetic constants for all six samples are not significantly different for the variants compared with the "normal" apoA-Is. Furthermore, it is clear that even normal apoA-Is can have 2- to

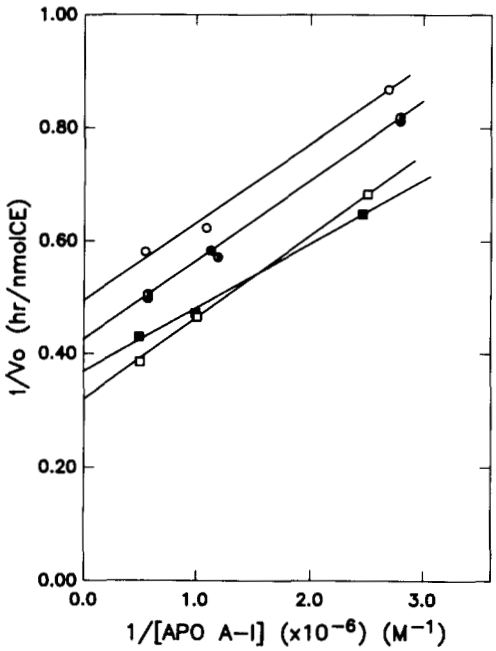

Fig. 5. Lineweaver-Burk plots giving the inverse of the initial velocity ($1/v_o$) as a function of the inverse of the substrate concentration (1/[apoA-I]) for the reaction of LCAT with selected rHDL complexes. The complexes shown were prepared with POPC and the following apoA-I samples: (■) 2A; (□) 2B; (●) 4A; (○) 4B; (◑), 4A, measured on a different day.

5-fold differences in kinetic parameters when compared among themselves.

To detect differences among the kinetic constants for normal apoA-Is and within A/B pairs, we had to determine first the measurement errors, as described in the Methods section. Then we used the values of the kinetic constants in Tables 2 and 3 to calculate the ratios of the constants being compared, and estimated the significance of their difference from 1.0 taking into account the appropriate standard error for the measurement. These estimates indicate that there are significant differences ($P < 0.01$) in two of the kinetic parameters within the number 3 and 5 pairs of samples in complexes prepared with POPC (30 to 72%) and with DPPC (37 to 87%). Pairs 4 and 6 had detectable differences only in the app. V_{max}/app. K_m value for the rHDL-DPPC complexes (34%) and for the rHDL-POPC complexes (18%), respectively.

The physiological significance of these differences, however, is questionable because a similar comparison of the extreme values of the kinetic constants for samples prepared with normal apoA-I gave differences in the range from 42 to 430%. Thus, the variability in the measured kinetic constants between individuals is considerably larger than the variability within normal/variant samples from the same individual. Since the differences in kinetic parameters within most pairs are smaller than those between samples obtained from different individuals, it appears that the preparation of the rHDL is not responsible for this functional variability of the "normal" apoA-I samples. Rather, it is possible that inter-individual differences arise from apoA-I microheterogeneity that is intrinsic, or is due to preparation or storage conditions. In fact, previous unpublished studies in our laboratory (A. Jonas and K. E. Kézdy, 1988) have shown that apoA-I samples obtained from several laboratories in the United States and several of our own preparations give differences in the kinetic parameters similar (3- to 4-fold) to those observed in this study when analyzed in terms of the reactivity of rHDL preparations with LCAT.

The results of this study can be summarized as follows: *1*) All the apoA-I samples were completely incorporated into rHDL complexes with POPC or DPPC; therefore, all the variants retained lipid binding properties. *2*) The sizes and size distributions of the rHDL particles, before or after exposure to LDL, were quite similar for most A/B pairs, and comparable for the six samples, with the clear exception of the 3A/3B pair. The 3B variant ($Lys_{107} \rightarrow 0$)

TABLE 2. Apparent kinetic constants for the rHDL-POPC complexes

rHDL Sample	Correlation Coefficient	app. V_{max}[a]	app. V_{max}/app. K_m[a]	app. K_m[a]
		nmol/h	*nmol/h · M*	*M*
1A	0.997	4.007	6.658×10^6	6.018×10^{-7}
1B	0.996	3.966	6.293×10^6	6.302×10^{-7}
2A	0.998	2.948	8.500×10^6	3.468×10^{-7}
2B	1.000	3.107	7.821×10^6	3.974×10^{-7}
3A	0.997	3.032[b]	7.805×10^{6}[b]	3.885×10^{-7}
3B	0.896	2.172[b]	5.405×10^{6}[b]	4.018×10^{-7}
4A	1.000	2.358	7.148×10^6	3.299×10^{-7}
4B	0.995	2.031	7.736×10^6	2.625×10^{-7}
5A	0.999	3.502[b]	5.974×10^6	5.862×10^{-7}[b]
5B	0.999	2.148[b]	6.296×10^6	3.412×10^{-7}[b]
6A	0.998	2.889	6.423×10^{6}[b]	4.498×10^{-7}
6B	0.996	2.476	5.432×10^{6}[b]	4.558×10^{-7}
n[c]				
A		6	6	6
B		6	6	6
$\bar{X}$[d]				
A		3.123	7.085×10^6	4.505×10^{-7}
B		2.650	6.498×10^6	4.148×10^{-7}
S[e]				
A		0.566	0.936×10^6	1.187×10^{-7}
B		0.752	1.068×10^6	1.242×10^{-7}

[a]From linear regression analysis of duplicates of kinetic data as shown in Fig. 5, for three apoA-I concentrations: app. V_{max} is the inverse of the y-intercept; app. K_m/app. V_{max} is the slope of the line; and app. K_m is calculated from the other two parameters.

[b]The ratios within these pairs were found to be significantly different from 1.0 ($P < 0.01$) using the standard deviations for the measurements given in the Methods section and the Student's *t*-statistical test.

[c]n, Number of samples.

[d]$\bar{X}$, Mean.

[e]S, Standard deviation.

TABLE 3. Apparent kinetic constants for the rHDL-DPPC complexes

rHDL Sample	Correlation Coefficient	app. V_{max}[a]	app. V_{max}/app. K_m[a]	app. K_m[a]
		nmol/h	*nmol/h · M*	*M*
1A	1.000	2.202	3.571×10^5	6.166×10^{-6}
1B	0.993	1.853	4.560×10^5	4.064×10^{-6}
2A	1.000	1.852	5.470×10^5	3.386×10^{-6}
2B	0.999	1.987	5.663×10^5	3.509×10^{-6}
3A	0.998	1.428[b]	6.689×10^{5}[b]	2.135×10^{-6}
3B	0.994	0.765[b]	4.876×10^{5}[b]	1.569×10^{-6}
4A	0.998	0.849	5.924×10^{5}[b]	1.433×10^{-6}
4B	0.998	1.257	4.412×10^{5}[b]	2.849×10^{-6}
5A	1.000	1.021[b]	4.832×10^{5}[b]	2.113×10^{-6}
5B	1.000	0.747[b]	3.278×10^{5}[b]	2.279×10^{-6}
6A	0.995	0.852	3.877×10^5	2.198×10^{-6}
6B	1.000	0.654	4.187×10^5	1.562×10^{-6}
n[c]				
A		6	6	6
B		6	6	6
$\bar{X}$[d]				
A		1.367	5.060×10^5	2.897×10^{-6}
B		1.210	4.496×10^5	2.639×10^{-6}
S[e]				
A		0.564	1.203×10^5	1.699×10^{-6}
B		0.590	0.787×10^5	1.026×10^{-6}

[a]From linear regression analysis of duplicates of kinetic data as shown in Fig. 5, for three apoA-I concentrations: app. V_{max} is the inverse of the y-intercept; app. K_m/app. V_{max} is the slope of the line; and app. K_m is calculated from the other two parameters.

[b]The ratios within these pairs were found to be significantly different from 1.0 ($P < 0.01$), using the standard deviations for the measurements given in the Methods section and the Student's *t*-statistical test.

[c]n, Number of samples.

[d]$\bar{X}$, Mean.

[e]S, Standard deviation.

gave slightly larger rHDL particles, that appeared to be resistant to the structural rearrangements induced by the phospholipid transfers to LDL. *3*) The rHDL-POPC substrates were about 15-fold more reactive with LCAT compared to the rHDL-DPPC substrates, as previously reported (20). *4*) The kinetic constants for rHDL prepared with "normal" apoA-I from six individuals cover a 1.4- to 4.3-fold range of values, indicating a substantial effect of the microheterogeneity of apoA-I on the reaction of the rHDL substrates with LCAT. The source of the microheterogeneity is unknown. *5*) For the rHDL particles prepared with the A/B pairs of apoA-I the variability in the kinetic constants (18 to 87%) was, in general, less than the variability among the rHDL containing "normal" apoA-Is from different individuals. *6*) The ratios of the kinetic constants for A/B pairs of samples were significantly different ($\alpha = 0.01$) from 1.0 for samples 3 and 5 (from 30 to 87% different in at least two kinetic constants). *7*) Similar estimates from the ratios of the kinetic constants for the other A/B pairs indicated no significant differences for samples 1 and 2; and a differences only in the app. V_{max}/app. K_m parameter (18, 34%) within samples 4 and 6.

DISCUSSION

The six apoA-I variants examined in this work correspond to amino acid changes in positions 3 (Pro→Arg), 4 (Pro→Arg), 107 (Lys→0, Lys→Met), 165 (Pro→Arg), and 198 (Glu→Lys) in the sequence of apoA-I. All of the variants contain charge modifications that could lead to destabilization of the protein structure or alteration in its functional properties if critical amino acids were involved. However, the present study has shown that most of these mutations have essentially no structural nor functional consequences: the apoA-I variants are completely incorporated into complexes with lipids, have similar rHDL particle sizes and distributions, are similarly resistant to denaturation in 3 M Gnd HCl, have comparable local protein conformations in the vicinity of Trp residues, and do not show appreciable differences in the reaction kinetics with LCAT. The only clear exception is variant 3, the Lys_{107}→0 deletion, which shows significant structural alterations and measurable differences in its kinetic parameters (28 to 87%) relative to the corresponding normal apoA-I. The Pro_{165}→Arg variant only showed (37 to 72%) changes in kinetic parameters and no changes in rHDL structure.

The insensitivity of the apoA-I structure and function to five of these mutations is probably connected to the high amino acid variability in apoA-I ($0.8/10^6$ residues) which reflects a weak active selection against apoA-I variants. In contrast, other proteins exhibit considerably lower amino acid variability (e.g., albumin, $0.2/10^6$ residues; globins and transferrin $0.5/10^6$ residues) (9). Of the 22 apoA-I structural variants studied to date, only a few are associated with lipid disorders, for example, the apoA-I (Arg_{173}→Cys) (21) and apoA-I (Pro_{165}→Arg) (12) which do not appear to be deleterious. However, there are apoA-I deficiencies that are associated with premature myocardial infarction (11); and with lethal familial amyloidotic polyneuropathy (22) (the apoA-I, Gly_{26}→Arg variant).

Earlier in vitro studies of the apoA-I variants: Lys_{107}→0, Pro_{143}→Arg, and Arg_{173}→Cys, led to the conclusion that these proteins were dysfunctional in their activation of the LCAT reaction (12, 23–25). However, the decrease in cholesterol esterification was at most 50%, and the variability in the LCAT kinetics of normal apoA-Is was not fully appreciated at that time. In vivo these mutations are not expected to affect cholesterol esterification to a significant extent because of the activation of the LCAT reaction by the normal apoA-I present in the heterozygote and by other apolipoproteins.

The likely explanation for the relative insensitivity of

apoA-I to the substitution of charged amino acids is that they do not affect the lipid binding properties of the protein, which depend on the hydrophobic residues in the nonpolar faces of the amphipathic helices. Thus, it is not surprising that the Glu_{198}→Lys and Lys_{107}→Met variants that replace charged groups on the polar face or interface have essentially no effect on the structure and functional properties of apoA-I.

On the other hand, Pro residues are known to have important roles in the structure of proteins by producing bends in helical domains and by changing the direction of the polypeptide backbone. In the sequence of apoA-I there is a group of Pro residues near the amino terminus. The other Pro residues occur in the region corresponding to the 4th exon, and are evenly spaced between most of the 22 amino acid repeated sequences (5). These prolines are thought to join segments of amphipathic α-helical structure and to determine their relative orientations. Recently it has been shown (26) that in discoidal rHDL the helical segments of apoA-I run parallel to the lipid acyl chains and probably to each other, implying that the Pro bends completely reverse the direction of the helices. Furthermore, Chung and his coworkers (6) have shown that synthetic, amphipathic peptides of 18 amino acids joined by a Pro residue are more effective in binding to lipid and in activating LCAT than the 18-mers.

Our present results indicate that the substitutions of Arg for Pro in the 3 and 4 positions of the apoA-I sequence have no structural or functional effects. Evidently the N-terminal region with a low potential for α-helix formation is not critical for the function of apoA-I. Interestingly, the Pro_{165}→Arg substitution in the putative region of lipid binding does not have a significant effect on the rHDL structure as detected by the GGE and fluorescence methods. Previously, we had speculated that the elimination of a β-turn between two adjacent α-helices might be responsible for altered lipid binding and/or LCAT activating properties of apoA-I (Pro_{165}→Arg), thus leading to HDL deficiency in the heterozygous variant carriers (12). However, a change in the rHDL particle sizes or their distribution would be predicted if one or more α-helical segments would no longer bind to lipid. Apparently this is not the case with the Pro_{165}→Arg mutant: the same particle diameters, 94 and 96 Å, are observed with the variant as with the normal rHDL, compatible with eight α-helices per apoA-I in contact with lipid (16). In the presence of LDL, the rHDL-POPC particles rearrange to the 78 Å and 104 Å products which could contain six α-helices per apoA-I in contact with lipid (16). Therefore, we conclude that an amino acid other than Pro (here Arg) can participate in the required turn between adjacent helices that are responsible for the lipid–protein interactions and for stabilizing the rHDL structure. However, we cannot exclude the possibility that apoA-I (Pro_{165}→Arg) could have a different structure in native HDL particles.

Of the six variants examined in this study, the Lys_{107}→0 deletion produces measurable effects on the structure of the rHDL complexes, their rearrangements in the presence of LDL, and the local protein configuration near one or more Trp residues. Since Trp 108 is adjacent to the site of this deletion, it is not surprising to find changes in the spectral properties of this apoA-I variant. The slightly larger diameters of the rHDL prepared with this variant probably indicate that the α-helix, including the Lys_{107}→0 deletion, occupies a little more space (~2 Å) on the periphery of the rHDL discs, perhaps as a result of a slight mismatch with the adjacent helices. In fact, the helical wheel representations of the segment between residues 100 and 117, in the sequence of the normal and variant apoA-Is, show that the variant still has a hydrophobic face, but that it is about 90° rotated relative to the hydrophobic face of the normal segment (23).

The conversion of the 94 Å rHDL-POPC into the 78 and 104 Å particles in the presence of LDL was suggested by us (16) to result from a loss of POPC to the LDL. We proposed that, in the process, the structure of apoA-I rearranges from eight to six α-helices in contact with lipid. Since this rearrangement could be detected by fluorescence changes of the Trp residues, we postulated that the dissolution of the two helices occurred in the N-terminal half of the protein sequence. Evidently the Lys_{107}→0 deletion inhibits this structural rearrangement and more directly implicates the 99–120 region in this process. Whether or not such rearrangements take place in vivo is not known, but in vitro they result in a 15-fold reduction in the reactivity of the rHDL with LCAT, going from 94 Å to the 78 or 104 Å complexes (16).

Regarding the reactivity with LCAT, none of the variant rHDL showed major differences compared to the normal rHDL substrates. Although statistically significant differences could be detected within the two variant and normal pairs including the Lys_{107}→0 and Pro_{165}→Arg mutations (pairs 3A/3B and 5A/5B), the variability among the kinetic constants for the six "normal" rHDL samples was much larger. Thus, any discussion of the differences seen with the Lys_{107}→0 and Pro_{165}→Arg variants is futile in view of the unexplained origin of the variability observed with the "normal" apoA-I. The nature of the heterogeneity of the "normal" apoA-I, whether due to deamidation, carbamylation, oxidation of Met, or covalent modification with lipids or their degradation products, is not known at this time, but we do know that it is a prevalent problem in apoA-I samples prepared throughout the United States and in Germany, and in repeated preparations in the same lab (A. Jonas). Nevertheless, it is clear from our results that the differences in the reactivity of LCAT with these apoA-I variant products are relatively small, and cannot involve amino acid residues that are specifically required in LCAT activation or that affect structure sufficiently to prevent effective activation.

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