High yield overexpression and characterization of human recombinant proapolipoprotein A-I

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Abstract Human apolipoprotein A-I (apoA-I) is the major protein component of high density lipoproteins (HDL) where it defines the particle structure and stability and functions as the main activator of the enzyme lecithin:cholesterol acyltransferase (LCAT). ApoA-I is expressed in the liver as a preproprotein that is targeted to the endoplasmic reticulum for secretion; in plasma, an unknown protease removes the six amino acid long propeptide. In this study, the cDNA coding the human proapoA-I was cloned into an *Escherichia coli* vector; the overexpressed protein was purified to 99% homogeneity and was extensively characterized together with mature apoA-I purified from plasma. SDS-PAGE, mass spectrometry, and Edman sequence analysis showed that the initial Met residue needed for translation in *E. coli* is posttranslationally removed from the N-terminal sequence of the proapoA-I. The structural and functional analyses were carried out on the lipid-free and the lipid-bound proteins. ProapoA-I self associated, interacted with dimyristoyl phosphatidylcholine vesicles, and formed secondary structures very similar to the lipid-free apoA-I. Reconstituted HDL particles made with two initial molar ratios of palmitoyloleoyl phosphatidylcholine/cholesterol/apolipoprotein/Na-cholate had identical particle sizes and distributions when apoA-I or proapoA-I were used. Particles having diameters of 79 Å and 98 Å, containing two apoA-I or proapoA-I molecules per particle, were isolated and characterized. The particles contained the same amounts of α-helical structure, had very similar fluorescence properties, and activated LCAT equally well. We conclude that proapoA-I expressed and purified from *E. coli* is functionally and structurally indistinguishable from mature apoA-I purified from plasma when analyzed in vitro. Therefore, this recombinant proapoA-I and mutants derived from it will be important sources of protein for analyzing apoA-I structure and function, as well as for studies of proapoA-I processing.—**McGuire, K. A., W. S. Davidson, and A. Jonas.** High yield overexpression and characterization of human recombinant proapolipoprotein A-I. *J. Lipid Res.* 1996. **37:** 1519–1528.

Supplementary key words apolipoprotein A-I • reconstituted HDL • phospholipid • lecithin:cholesterol acyltransferase • circular dichroism • fluorescence

Human apolipoprotein (apo) A-I) associates with lipids to form plasma high density lipoproteins (HDL). ApoA-I possesses several important biological functions when associated with lipid. It functions to stabilize the structure of HDL; it is the main activator of the enzyme lecithin:cholesterol acyltransferase (LCAT) which catalyzes the formation of cholesterol ester on the HDL; it participates in the reverse cholesterol transport process; and it may be a ligand for HDL binding to cellular receptors (1–4). Clinical studies have shown that reduced levels of apoA-I and HDL correlate significantly with increased risk of cardiovascular disease. Recent results with transgenic mice that overexpress human apoA-I confirm that the HDL cholesterol levels correlate with the apoA-I levels, suggesting that increasing the apoA-I level in humans may raise the HDL cholesterol levels and improve their lipoprotein profile and resistance to atherosclerosis (5). ApoA-I is synthesized in the liver and small intestine as a 267 amino acid preproapoA-I (6). The proapoA-I protein is secreted into the plasma after an intracellular peptidase cleaves the 18 amino acid signal sequence from preproapoA-I (6). The propeptide containing the amino acids Arg-His-Phe-Trp-Gln-Gln is removed by an unknown protease releasing the 243 amino acid apoA-I protein (6, 7). The majority of the proapoA-I (75–100%) is transformed into mature apoA-I over a period of 10 to 24 h (7–9). The enzyme responsible for the proteolytic conversion of proapoA-I is not affected by the serine protease inhibitor phenylmethylsulfonyl fluoride but is completely inhibited by EDTA (9). In vitro analysis using labeled proapoA-I isolated from rats confirmed the total inhibition of the conversion by EDTA and EGTA and

Abbreviations: apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; rHDL, reconstituted HDL particles; LCAT, lecithin:cholesterol acyltransferase; DMPC, dimyristoyl phosphatidylcholine; BS_3, bis-sulfosuccinimidyl suberate; PVDF, polyvinylidenedifluoride; LB, Luria-Bertani medium; PAGGE, polyacrylamide gradient gel electrophoresis; GdnHCl, guanidine hydrochloride; FC, free or unesterified cholesterol; WMF, wavelength of maximum fluorescence; app. V_{max}, app. K_m, app. V_m/app. K_m, apparent kinetic constants; K_{SV}, Stern-Volmer constant.

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suggested that an unstable plasma factor may be involved in the proteolytic cleavage of proapoA-I (8, 9).

Inspection of the amino acid sequence of proapoA-I and apoA-I reveals that a large part of the protein evolved by duplication of a primordial sequence encoding a 22 amino acid sequence (22-mer); furthermore, the primordial sequence seems to have evolved by a single duplication of an 11-mer sequence (10). Computer analysis of the 22-mer sequences predicted that each 22-mer can form an amphipathic α-helix. The hydrophobic faces of the α-helices are believed to interact with lipids and the hydrophilic faces with the plasma (10–15). The predicted eight amphipathic α-helices are identical for apoA-I and proapoA-I as they are located between amino acids 45 and 240 in the mature apoA-I sequence. The extra six amino acids in proapoA-I and the next several N-terminal amino acids in the mature sequence are predicted to have a random configuration.

Although most of newly secreted apoA-I and 3% of the plasma apoA-I is proapoA-I, the biological function of proapoA-I has still to be determined. ProapoA-I has been linked to Tangier disease, a disease with abnormally low levels of apoA-I, apoA-II, and HDL (< 5% of normal), and having comparable levels of apoA-I and proapoA-I (9). It was suggested by Gordon et al. (6) that impaired conversion of proapoA-I to apoA-I might be the cause of the disease (6). However, Bojanovski, Gregg, and Brewer (9) isolated proapoA-I and used plasma from Tangier patients and control groups to show that the converting enzyme was normal in Tangier patients. Normal proapoA-I and Tangier proapoA-I were converted to apoA-I equally well by plasma isolated from Tangier patients and controls, indicating that the proteolytic enzyme was not defective (9). The pre-proapoA-I cDNA was later isolated from a Tangier liver and found to contain a single base substitution (G → T) resulting in an amino acid change Glu → Asp in position 120 (16). Currently, it is thought that the differences in the levels of proapoA-I versus apoA-I are a consequence of rapid catabolism of apoA-I in Tangier disease due to an unknown defect in the apoA-I metabolism (16).

Our objective in this study was to characterize in detail the structural and functional differences between apoA-I and proapoA-I in lipid-free and in lipid-bound form. To accomplish this, we developed an efficient overexpression and purification scheme for proapoA-I. A long term goal of this project is to use the proapoA-I overexpression and purification scheme for the production and characterization of proapoA-I point mutations including the Glu_{120} → Asp mutation.

It should be noted that several groups have tried to overexpress mature apoA-I in *E. coli*, but with limited success. The reasons given have been a poor stability of the mRNA and the apoA-I protein in *E. coli* (17, 18). In contrast, proapoA-I has been successfully expressed in *E. coli*, but in some cases retained the initial Met residue. This Met residue had been specifically engineered to allow efficient translation initiation in *E. coli* (19–21). Using one of the most efficient *E. coli* promoters, we obtained high yields of the proapoA-I with the Met residue post-translationally removed. The subsequent characterization showed no significant differences between proapoA-I and apoA-I making this overexpression and purification scheme a powerful tool for future mutagenesis studies.

MATERIALS AND METHODS

Human apoA-I and LCAT were purified from plasma purchased from the Champaign County Blood Bank, Regional Health Resource Center, by the procedures routinely used in our laboratory (13, 14). The purity of the proteins was estimated by Coomassie Blue and silver-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to be more than 95%.

Sodium cholate, crystalline cholesterol (FC), palmitoyloleoyl phosphatidylcholine (POPC), and dimyristoyl phosphatidylcholine (DMPC) were purchased from Sigma Chemical Co. Polyvinylidene difluoride (PVDF) membranes (0.45 μM) were obtained from Millipore. Polyclonal antibodies against apoA-I were purchased from Calbiochem. Radiolabeled [4-^{14}C]cholesterol was obtained from DuPont-New England Nuclear. Sequenase™ Version 2.0 kit was purchased from United States Biochemical Corporation (USB). Radiolabeled [^{35}S-α]ATP for DNA sequencing was purchased from Amersham. Bis-sulfosuccinimidyl suberate (BS_3) was obtained from Pierce Chemical Co. The vector pTrc99A was obtained from Pharmacia Pharmaceutical Co. The cDNA encoding the proapoA-I gene was a gift from Dr. J. I. Gordon, Washington University (22).

SDS-PAGE and Western blotting

The isolation of proapoA-I was followed through the purification steps on 10 or 12% SDS polyacrylamide gels as described by Laemmli (23). Western blotting was performed according to the Protoblot manual by Promega. The SDS gel was blotted onto PVDF membranes using 20 mM Tris, 150 mM glycine, and 20% methanol buffer. The membranes were rinsed with TBST buffer (10 mM Tris, 150 mM NaCl, and 0.05% Tween-20, pH 8.0, and incubated with a 1/1000 dilution of anti-apoA-I antibodies. The antigen–antibody complex was detected by incubating with anti-rabbit IgG antibody that was conjugated to alkaline phosphatase (Sigma Chemical Co.).

Construction of pTKAproapoA-I

The cDNA received from Dr. J. I. Gordon (Washington University) had been modified so that it contained the unique restriction sites *Nco*I and *Hin*dIII localized immediately up- and down-stream from the coding sequence of the human proapoA-I gene (22). The *Nco*I site provided the initial Met residue and changed the first amino acid in the propeptide from Arg to Ala. The proapoA-I cDNA was subcloned into the pTrc99A overexpression vector from Pharmacia placing the gene under control of the IPTG inducible tac promoter and the ribosome binding site eleven nucleotides from the initial Met residue. The distance of eleven nucleotides from the ribosome binding site to the initial Met residue provides an optimal distance for protein expression in *E. coli* (24). The subcloning was verified by restriction analysis and by DNA sequencing using the method of Sanger et al. (25). The plasmid was analyzed in *E. coli* SURE™ for its ability to overexpress the encoded protein. Separation of cell proteins by SDS-PAGE and Western blot analysis revealed optimal induction by IPTG at concentrations from 2 to 5 mM.

Overexpression and purification of proapoA-I

The overexpression plasmid was transformed into *E. coli* SURE™ and grown at 37°C in Luria-Bertani (LB) media supplemented with 100 μg/ml ampicillin (26). Protein synthesis was induced at a cell density corresponding to A_{600} of 0.7 by 2 mM IPTG and grown for 5 h. The cells were harvested from a 6.3 L culture and stored at -80°C.

The purification was carried out on ice or in the cold room (4°C). The cells were resuspended in buffer A containing 20 mM Tris/HCl, pH 8.1, 100 mM NaCl, 1 mM EDTA, and 1 mM PMSF and lysed by intermittent sonication over 30 min. Cell debris was removed by centrifugation at 18,000 *g* for 30 min. A 40% $(NH_4)_2SO_4$ precipitation was performed on the supernatant by adding solid $(NH_4)_2SO_4$ over a period of 30 min followed by a 30- to 60-min precipitation period. The proapoA-I was pelleted by centrifugation. The supernatant was discarded and the pellet resuspended in buffer A (1/5 of the previous volume) and applied to a phenyl Sepharose column (Pharmacia, CL-4B). Weakly bound proteins were removed by washing the column with 10 × buffer A. Contaminating proteins were removed with 60% ethylene-glycol (v/v) and buffer A followed by a wash with 60% propyleneglycol (v/v), and 40% buffer A (both washes were 6 × the column volume). ProapoA-I was eluted with 80% propylene-glycol (v/v), 20 mM Tris/HCl, and 1 mM EDTA, pH 8.1. The fractions containing the highest amounts of proapoA-I, estimated by SDS-PAGE and A_{280}, were pooled and dialyzed against 10 mM NH_4-bicarbonate. The dialyzed protein was lyophilized and stored under N_2 at -20°C.

Electrospray ionization mass spectrometry and Edman sequencing

A VG Quattro (quadrupole-hexapole-quadrupole, QHQ) mass spectrometry system (Fisons Instruments, VG Analytical; Manchester, UK) was used to determine the molecular weight of the purified protein at the Mass Spectrometry Laboratory, University of Illinois School of Chemical Sciences. Prior to analysis, the samples were dialyzed intensively against doubly distilled water. Automated Edman degradation analysis of proapoA-I was performed on an Applied Biosystems Model 477A protein sequencer coupled to a model 120A online PTH analyzer in the Genetic Engineering Facility, University of Illinois Biotechnology Center.

Interaction of apolipoproteins with DMPC

Dry DMPC was dispersed in the standard Tris buffer (10 mM Tris/HCl, 150 mM NaCl, 0.005% EDTA, 1 mM NaN_3, pH 8.0) to form multilamellar liposomes. Experiments were initiated by adding an aliquot of the liposomes to the apolipoproteins in the standard Tris buffer at a 2.5:1 mass ratio of DMPC to protein. The reaction volume was 0.8 ml. The light scattering of the samples at 325 nm was monitored for 120 min at 24.5°C in a Beckman DU-64 spectrophotometer acquiring data points every 2 min. The time required for the reaction to proceed halfway to baseline $t_{1/2}$ was calculated as described by McLean et al. (27).

Preparation and characterization of reconstituted HDL (rHDL) particles

Discoidal particles were reconstituted using the sodium cholate method developed in our laboratory (11–14). Initial mass ratios of POPC to apolipoprotein are shown in Table 1. Pure particles were isolated on a calibrated Superdex 200 HR (10 × 30 mm) gel filtration column (Pharmacia) eluted at 0.5 ml/min with standard Tris buffer. The hydrodynamic diameters of rHDL particles were estimated by native 8–25% polyacrylamide gradient gel electrophoresis (PAGGE) (Pharmacia). The particle migration distances into the gel were determined to within 0.1 mm by computer image digital analysis as previously described (28). Purified rHDL particles were analyzed using the Markwell et al. modification of the Lowry protein assay (29) while phospholipids were determined as inorganic phosphorus by the method of Sokoloff and Rothblat (30). The number of apoA-I molecules per particle as well as self-association properties of the lipid-free proteins were determined by cross-linking the apolipoproteins with BS_3

(31). The rHDL charge characteristics were measured and calculated as described by Sparks and Phillips (32).

Circular dichroism and isothermal denaturation studies

The average α-helix contents of proapoA-I and apoA-I in the lipid-free and lipid-bound forms were determined by circular dichroism (CD) spectroscopy using a Jasco J-720 spectropolarimeter located in the Laboratory for Fluorescence Dynamics at the University of Illinois. Spectra were measured at 25°C in a 0.1 cm path length quartz cuvette as described previously (13). The percent α-helix was determined from the molar ellipticities at 222 nm (33). The effect of guanidine HCl (GdnHCl) on the α-helix content of the rHDL particles was used to obtain the free energy of unfolding of apoA-I as proposed by Aune and Tanford and modified by Sparks, Phillips, and Lund-Katz (34).

Fluorescence spectroscopy

For all fluorescence studies in this report, the concentration of protein was 0.1 mg/ml in standard Tris buffer. The wavelengths of maximum fluorescence of the tryptophan residues in apoA-I and proapoA-I were determined from uncorrected spectra using a Perkin-Elmer MPF-66 fluorescence spectrophotometer with 4 nm excitation and emission slit widths. The samples were excited at 295 nm (to avoid tyrosine fluorescence), and the emission was scanned from 305 to 375 nm at room temperature. Fluorescence quenching experiments were carried out on an ISS PC1 photon counting spectrofluorimeter at the Laboratory for Fluorescence Dynamics at 25°C using increasing concentrations of KI (0–0.5 M). The 5 M KI stock solution contained 1 mM $Na_2S_2O_4$ in order to prevent the formation of I_3^-. Aliquots of the stock were added to a constant volume of protein sample, and the resulting fluorescence intensities were corrected for the minor dilution of the protein. Spectra corrected for the contribution of buffer were then used to determine the quenching parameters using the Stern-Volmer equation as modified by Lehrer (35):

$$Fo/\Delta F = 1/f_a + 1/f_a K_{sv}[KI]$$

where Fo is the integrated fluorescence intensity in the absence of quencher, ΔF is the difference between Fo and the observed intensity at each concentration of KI, f_a is the fraction of Trp residues that is accessible to the quencher and the K_{sv} is the Stern-Volmer constant.

Reaction with lecithin:cholesterol acyltransferase

The LCAT assay and isolation of LCAT have been described in detail previously (11). Purified rHDL particles that had been reconstituted with [^{14}C]cholesterol (typically 2.5×10^4 cpm/μg cold FC) were incubated at 37°C with a constant amount of LCAT (0.025 μg/ml) over a concentration range of 2.0×10^{-7} to 2.0×10^{-6} M of apolipoprotein. Also present in the reaction mixture were 4% (v/v) delipidated BSA and 4 mM β-mercaptoethanol in a total volume of 0.5 ml of standard Tris buffer. The observed initial velocities at each concentration were analyzed on Lineweaver-Burk plots and the apparent V_m and apparent K_m values were obtained by linear regression.

RESULTS

Overexpression and purification of proapoA-I from *E. coli*

The overexpression and purification of proapoA-I resulted in 39 mg of pure protein from a 6.3 L culture. **Figure 1** shows the SDS-PAGE analysis of the purification steps for proapoA-I. The cell suspension contained 4.5 g of total protein and approximately 0.32 g of proapoA-I (100% yield). The centrifugation step gave 0.23 g of proapoA-I or a 72% yield. After the AS precipitation step (Fig. 1, lane 4), 0.48 g of total protein was recovered and 80 mg of proapoA-I (25% yield). The phenyl Sepharose elution gave 99% pure proapoA-I (Fig. 1, lanes 5 and 6) in a yield of 12%.

Electrospray ionization mass spectrometry of the purified proapoA-I gave a molecular mass of 28,878 Da, one mass unit lower than the calculated molecular weight. Edman degradation analysis provided the first ten amino acids of proapoA-I (A-H-F-W-Q-Q-D-E-P-P)

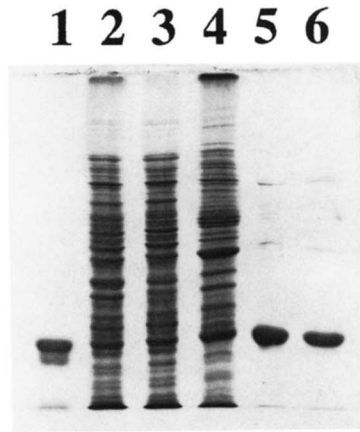

Fig. 1. Purification of proapoA-I analyzed by SDS-PAGE. Lane 1, apoA-I purified from plasma; lane 2, crude cell extract; lane 3, supernatant after the cell debris has been removed; lane 4, resuspended pellet from 40% ammonium sulfate precipitation; lanes 5 and 6, proapoA-I eluted from the phenyl Sepharose column; lane 6, purified proapoA-I after solubilization in 3 M GdnHCl.

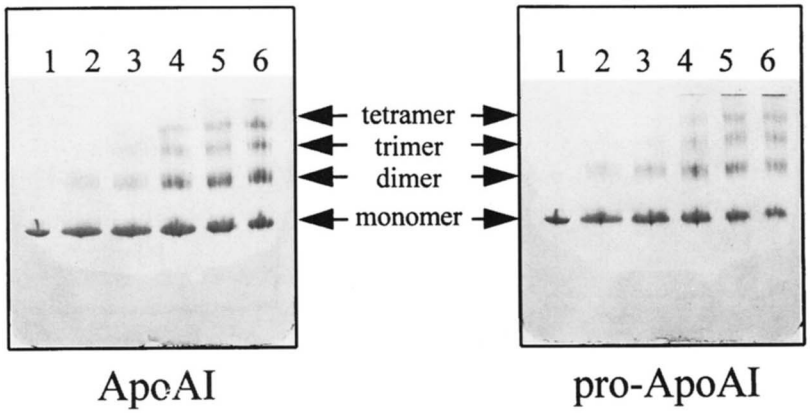

Fig. 2. SDS-PAGE analysis of the self-association properties of apoA-I and recombinant proapoA-I studied by BS_3 cross-linking. Samples were loaded onto the gels at the top and the molecules migrated towards the bottom. The concentrations of protein present in 50 mM phosphate buffer, pH 7.4, were: lane 1, 0.1 mg/ml protein, not cross-linked; lane 2, 0.1 mg/ml; lane 3, 0.2 mg/ml; lane 4, 0.8 mg/ml; lane 5, 1.4 mg/ml; and lane 6, 2.0 mg/ml for each type of protein. In lanes 2–6, the proteins have been cross-linked.

and confirmed that the initial Met residue had been post-translationally removed in agreement with the molecular weight determined by mass spectrometry.

Self-association of apoA-I and proapoA-I

The ability of lipid-free apoA-I and proapoA-I to self associate in phosphate buffer was determined by BS_3 cross-linking. The SDS-PAGE gels in **Fig. 2** show the oligomerization patterns of the apolipoproteins present in the solution at concentrations of 0.1 to 2.0 mg/ml. Both proteins self-associate in a concentration-dependent manner with substantial formation of trimers and tetramers occurring at concentrations over 0.7 mg/ml. It is clear from visual inspection of Fig. 2 as well as densitometric analysis of these gels (data not shown) that the presence of the propeptide in proapoA-I does not change its tendency for self-association relative to native apoA-I. This suggests that the regions of apoA-I that are required for self-association are likely to be in similar conformations in both proteins, and that the extreme N-terminal sequences are not critical for self-association in vitro or for protein–protein interactions in complexes with lipid.

Interaction of apoA-I and proapoA-I with DMPC

To determine the effect of the presence of the propeptide on the ability of proapoA-I to bind to and reorganize phospholipid, the abilities of native apoA-I and proapoA-I to clear multilamellar DMPC liposomes were determined. As shown in **Fig. 3,** DMPC liposomes incubated in the absence of protein had a slow reduction in the light scattering ($t_{1/2}$ = 600–700 min); this was likely due to the slow settling of the large liposomes. It is clear from Fig. 3 that both apoA-I and proapoA-I were effective in clearing the liposomes when combined in a 2.5:1 weight ratio (DMPC to apolipoprotein). The $t_{1/2}$ values (± 1 SD) for apoA-I and proapoA-I were 28 ± 7 and 21 ± 8 min, respectively. These results indicate that the regions of the apoA-I that are important for interaction with lipids are similarly effective in both proteins and are not affected by the presence of the propeptide. If anything, it seems that clearance of DMPC is slightly enhanced by the presence of the propeptide.

Physical properties of reconstituted HDL particles

To determine the relative abilities of the mature and the proform of apoA-I to form rHDL particles, reconstitutions were carried out with POPC at initial POPC:FC:protein ratios of 100:5:1 and 40:3:1 (mol:mol) that have been shown with native plasma apoA-I to result primarily in 96 and 78 Å particles containing two mole-

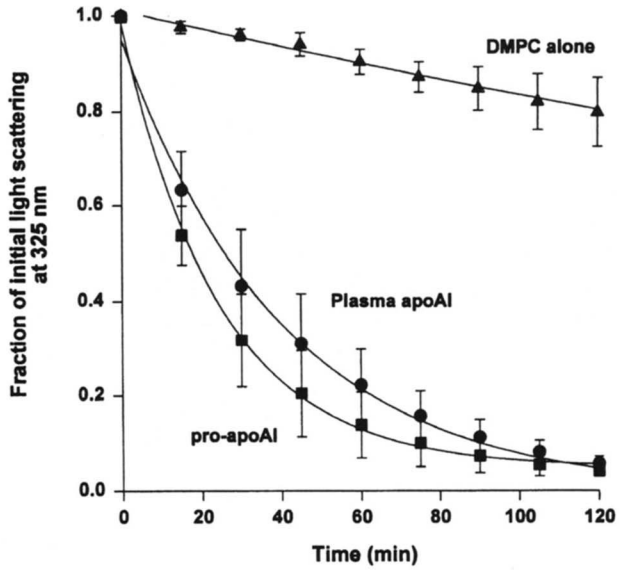

Fig. 3. Interaction of apoA-I and recombinant proapoA-I with DMPC liposomes. Multilamellar DMPC liposomes were added to the proteins in Tris buffer at pH 8.0 at a 2.5:1 mass ratio of phospholipid to protein (the reaction volume was 0.8 ml and the concentration of DMPC was 0.5 mg/ml). Absorbance at 325 nm was measured as a function of time at 24.5°C. The samples shown are: (▲), DMPC alone, (●) native plasma apoA-I, and (■) recombinant proapoA-I. The curves were derived from nonlinear regression (see Methods) through the data which is an average from two independent experiments.

TABLE 1. Composition and size of discoidal rHDL particles made with human plasma apoA-I and recombinant proapoA-I

Protein Component of rHDL Particle	Molar Composition[a] (POPC:FC:ApoA-I) Initial	Final	# Protein/ Particle[b]	Diameter (Angstroms)[c]
Plasma apoA-I	100:5:1	84 ± 17:4:1	2	98 ± 2.2
Plasma apoA-I	40:3:1	57 ± 10:2:1	2	79 ± 0.2
ProapoA-I	100:5:1	94 ± 17:3:1	2	100 ± 2.7
ProapoA-I	40:3:1	60 ± 17:4:1	2	79 ± 0.2

[a]Determined by Markwell/Lowry protein assay and analysis of phosphorous on three independent reconstitution experiments (± 1 SD). Final values were obtained after purification by gel-filtration chromatography on a Superdex 200 column.
[b]Determined from SDS-polyacrylamide gel electrophoresis of delipidated apoA-I or proapoA-I after cross-linking with BS_3.
[c]Determined from nondenaturing gradient gel electrophoresis using reference globular proteins.

cules of protein per complex (36). **Table 1** shows the final compositions of these particles after purification by gel filtration chromatography and their hydrodynamic diameters calculated from non-denaturing PAGGE. At both initial ratios, mature plasma apoA-I and proapoA-I formed rHDL particles within the expected size ranges. Furthermore, the compositions of the isolated homogeneous particles made with either protein were not significantly different. These data show that the ability of apoA-I to bind to and organize phospholipids into discoidal particles of defined sizes is not perturbed by the presence of the prosegment in proapoA-I.

Secondary structure and conformational stability of lipid-free apolipoproteins and rHDL particles

Circular dichroism (CD) was used to estimate the average α-helical content of the lipid-free forms of both apolipoproteins as well as the two isolated rHDL size classes (**Table 2**). The calculated α-helix content showed no significant differences between apoA-I and proapoA-I in all forms studied. The relative stability of the two proteins in the various states was determined by isothermal denaturation studies. In these studies the degree of α-helicity of apolipoproteins was monitored in the presence of increasing concentrations of GdnHCl. ApoA-I and proapoA-I exhibited similar free energies of denaturation. Taken together, the similarities in α-helix content and the thermodynamic stability strongly suggest that both proteins adopt very similar conformations in each of the three states and indicate that the pro-segment in proapoA-I does not have a significant effect on the overall conformation of the proteins.

Fluorescence spectroscopy of the lipid-free and rHDL particles

To obtain further information on the conformation of apoA-I and proapoA-I, the fluorescence properties of the Trp residues were studied (**Table 3**). The average wavelength of maximum fluorescence (WMF) is a sensitive indicator of the relative hydrophobicity of the environment of the four Trp residues in the N-terminal half of apoA-I (position 8, 50, 72, 108). ProapoA-I contains a fifth Trp residue at position -3 in the propeptide. Therefore, the fluorescence properties observed for this protein are due to the average contribution from five Trp residues rather than four as in apoA-I. Table 3 shows that native apoA-I exhibited a WMF of about 333–334 nm in the lipid-free form and was blue-shifted by a nm or so when present in rHDL of both sizes. The WMF values of proapoA-I in each case were slightly higher (2 to 3 nm) than the corresponding apoA-I values. The increased WMF in proapoA-I suggests that the Trp residue in the propeptide is in a slightly more polar environment than the average environment of the remaining four Trp residues. Despite the differences in WMF, both proteins exhibit similar changes in WMF in response to lipid binding, indicating that the Trp residues undergo similar changes in environment in both proteins. Fluorescence quenching experiments with KI were used to determine the exposure of the Trp residues to the solvent (Table 3). The K_{sv} term reflects the relative

TABLE 2. Alpha helix content and GdnHCl denaturation of human plasma apoA-I and recombinant proapoA-I in the lipid-free state and in two defined discoidal rHDL[a]

	Alpha Helix Content[b] (%)	$D_{½}$[c] (M GdnHCl)	Δn[d] (mol GdnHCl/mol ApoA-I)	ΔG_D^0 (kcal/mol ApoA-I)[e]
ApoA-I lipid-free	56 ± 5	1.1 ± 0.2	12.9 ± 0.5	2.5 ± 0.1
ApoA-I rHDL-98	77 ± 8	3.0 ± 0.4	6.4 ± 1.6	2.6 ± 0.9
ApoA-I rHDL-79	64 ± 5	2.7 ± 0.1	5.2 ± 1.0	1.8 ± 0.2
ProapoA-I lipid-free	57 ± 8	1.2 ± 0.2	10.4 ± 1.0	2.7 ± 0.9
ProapoA-I rHDL-100	73 ± 5	3.1 ± 0.5	8.5 ± 1.4	3.1 ± 0.5
ProapoA-I rHDL-79	64 ± 5	2.5 ± 0.3	5.1 ± 0.5	1.7 ± 0.1

[a]Values in this table are derived from three independent rHDL reconstitution experiments.
[b]Determined from molar ellipticities at 222 nm (± 1 SD).
[c]GdnHCl concentration for 50% denaturation of the protein (± 1 SD).
[d]GdnHCl bound to each molecule of protein during the denaturation (± 1 SD).
[e]Standard free energy change of denaturation (± 1 SD).

TABLE 3. Tryptophan fluorescence parameters and charge characteristics of human plasma apoA-I and recombinant proapoA-I in the lipid-free form and in two discoidal rHDL particles[a]

	Wavelength of Maximum Fluorescence (nm)	K_{sv}[b] (M^{-1})	fa[c]	Surface Potential (mV)	Valence (e)[d]
ApoA-I lipid-free	333 ± 0.7	6.9 ± 1.0	0.65 ± 0.04	-8.2 ± 0.2	-3.2 ± 0.1
ApoA-I rHDL-98	332 ± 0.8	3.7 ± 1.9	0.59 ± 0.07	-7.8 ± 0.5	-7.5 ± 0.5
ApoA-I rHDL-79	333 ± 0.9	1.5 ± 0.2	1.03 ± 0.01	-9.1 ± 0.3	-6.1 ± 0.2
ProapoA-I lipid-free	336 ± 1.0	5.4 ± 0.9	0.65 ± 0.06	-8.3 ± 0.1	-3.2 ± 0.1
ProapoA-I rHDL-100	334 ± 0.1	3.7 ± 0.9	0.55 ± 0.06	-7.6 ± 0.4	-7.6 ± 0.3
ProapoA-I rHDL-79	334 ± 0.8	1.03 ± 0.4	1.02 ± 0.03	-9.3 ± 0.4	-6.6 ± 0.3

[a]Values in this table are derived from three independent rHDL reconstitution experiments.
[b]Stern-Vomer constant indicating relative exposure of quenchable fluorescence to the quenching agent (KI) (± 1 SD).
[c]Fraction of tryptophan fluorescence that is quenchable by KI.
[d]Surface potential is the charge potential as determined by 0.5% agarose gel electrophoresis at pH 8.6 and 25°C (± 1 SD). The electrophoresis buffer contained 5,5-diethylbarbituric acid (10 mM), ionic strength 0.05. The valence is the excess negative charge per particle in electronic units (± 1 SD).

accessibility of Trp residues whereas the f_a term indicates the fraction of residues that are accessible to solvent. As expected, the lipid-free proteins had the highest K_{sv} values. Both apoA-I and proapoA-I had similar K_{sv} values in all forms studied. The fractional accessibility of the Trp residues in these proteins was also similar in all three states. This is strong evidence that the three-dimensional conformations in the N-terminal halves of these proteins are similar despite the presence of the prosegment in proapoA-I.

Charge properties of lipid-free apolipoproteins and rHDL

The charge characteristics of the lipid-free forms of apoA-I and proapoA-I along with the two rHDL particles made with each apolipoprotein are summarized in Table 3. Both proteins and the rHDL exhibited surface potential values that were in the pre-β migration range (32). The valence, or net excess negative charge per particle, was identical for lipid-free apoA-I and proapoA-I. As the propeptide is essentially uncharged under the conditions of the experiment, it can be concluded that the ionization states of the charged residues in both proteins are similar. Similar values were also observed between these two proteins in the rHDL particles. These results agree with the data from the fluorescence studies and confirm that the conformations of both proteins are similar in both the lipid-free and lipid-bound states.

Reaction with lecithin:cholesterol acyltransferase (LCAT)

To date, the best characterized function of apoA-I is the activation of LCAT. To determine the effect of the prosegment in proapoA-I on this function of apoA-I, 96 Å rHDL particles were formed containing [^{14}C]cholesterol and POPC and were reacted with LCAT. The reaction conditions were chosen so that the initial reaction velocities could be measured and analyzed by standard Michaelis-Menten kinetics. The kinetic parameters derived for the particles made with apoA-I and proapoA-I were all very similar (see **Table 4**). These data complement the structural data presented above and further demonstrate that the propeptide has little effect on the structure of the region(s) of apoA-I that are involved in LCAT activation. It is also clear that the prosegment does not interfere with LCAT activation in other ways such as sterically interfering with LCAT interactions with the rHDL surface.

TABLE 4. Reaction of rHDL particles made with plasma apoA-I and recombinant proapoA-I with lecithin:cholesterol acyltransferase

Apolipoprotein Component of rHDL[a]	Apparent V_{max}[b] (nmol CE/h)	Apparent K_m (μM)	App. V_{max}/App. K_m (nmol CE/h · M × 10^{-6})
ApoA-I rHDL-98	4.3 ± 0.3	2.3 ± 0.2	1.9 ± 0.1
ProapoA-I rHDL-100	3.7 ± 0.7	1.9 ± 0.5	2.0 ± 0.1

[a]Values in this table are derived from two observations in each of two separate experiments.
[b]The apparent kinetic parameters (± 1 SD) were determined by a least squares linear regression analysis using a Lineweaver Burk plot of the reciprocals of the reaction velocity vs. the substrate concentration (see Methods).

DISCUSSION

In this work, we describe a new, high yield expression system for the production of a variant of human proapoA-I in *E. coli.* The overexpressed protein was purified from the cytoplasm of cells. Earlier attempts to produce apoA-I in *E. coli* from the cDNA of the mature protein had limited success attributed to the reduced stability of the mRNA and the rapid degradation of the apoA-I protein during expression (17, 18). Apparently, the presence of a prosegment or a leader sequence targeting proteins to the periplasmatic space of *E. coli* stabilizes the expression products (21, 22). In fact, there is a recent report by Calabresi et al. (37) on the industrial-scale overexpression of the apoA-I_{Milano} mutant in *E. coli.* According to the report, this recombinant protein was excreted into the cell medium in the mature protein form.

Our results on the lipid-free proteins clearly show that the structure and stability of proapoA-I are essentially identical to those for mature apoA-I. If intracellular stabilization of the proapoA-I is indeed due to the presence of the prosegment, then interaction with other intracellular proteins, rather than intrinsic protein stabilization by the prosegment, must be involved. It is likely that in hepatocytes and intestinal cells, the propeptide of apoA-I directs intramolecular interactions and traffic rather than the folding of the polypeptide chain. Another interesting observation is that the lipid-binding properties and LCAT activation of the proapoA-I and mature apoA-I are indistinguishable in vitro. Therefore, the removal of the prosegment in plasma is not a prerequisite for the full expression of the known functions of apoA-I. It would be interesting to compare the kinetics of proteolytic cleavage for the lipid-free and lipid-bound proapoA-I in order to estimate the relative accessibility of the prosegment to the protease and to gain a better understanding of the events of apoA-I maturation and assembly with lipids in plasma.

The solution properties of proapoA-I and mature apoA-I, including self-association, stability to denaturation, secondary structure, and three-dimensional structure in the N-terminal half of the molecule, as reported by the intrinsic fluorescence parameters, are very similar, indicating that the folding of the proteins is not affected by the prosegment. A minor difference in the spectroscopic properties of the apolipoproteins is due to the presence of the additional Trp residue in position -3 of the prosegment that shifts the average WMF by 2–3 nm to the red. This suggests that the Trp residue in the prosegment is in a considerably more polar environment than the rest of the Trp residues (334 nm), and likely forms part of a more disorganized and accessible region of the protein. In fact, if identical contributions to fluorescence intensity and spectral distributions are assumed for all the Trp residues of mature apoA-I, a fifth Trp residue with a WMF of 347 nm (352 nm for a Trp completely exposed to water) would account for the observed red shift.

The substitution of Ala in this study for an Arg in the native proapoA-I should not have any significant structural effects, as the charged Arg is expected to be even more exposed to solvent than is Ala. However, this amino acid substitution could have an effect on intramolecular interactions.

This study also shows that there are no discernible differences in the lipid-binding properties nor LCAT activation between proapoA-I and apoA-I. The structure and stabilities of both types of rHDL particles (79 Å and 98 Å in diameter) are very similar. From our past work (28), we know that these two subclasses of particles contain two distinct conformation of apoA-I that activate LCAT very differently (15- to 20-fold) and are interconverted by removal of phospholipid. From this work, it is clear that the extreme N-terminus of apoA-I is not involved in this conformational adaptation.

As suggested by the essentially identical structure of proapoA-I and apoA-I in the lipid-bound forms, their ability to activate LCAT was also the same. This confirms the observation of other investigators that binding of monoclonal antibodies specific for N-terminal sequences of apoA-I do not affect LCAT activation (38, 39).

From the practical standpoint, our extensive characterization of the product of this expression system by comparison with mature apoA-I has demonstrated the usefulness of this system for the production of significant amounts of proapoA-I mutants for the study of the structure and function of apoA-I.

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