Interactions of Lecithin and Pig Apolipoproteins of High Density Lipoproteins at the Surface Monolayer of Reconstituted Very Small Particles

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Cosonication of egg yolk lecithin and triolein with apolipoproteins isolated from pig high density lipoprotein (apoHDL) gave us reconstituted high density lipoprotein particles (r-HDLs) of 9 nm in average diameter. They were smaller than microemulsion particles (MEs) composed of the lipids (35 nm). The protein/ egg yolk lecithin ratio in the fractionated r-HDLs was higher in the smaller particles. Binding of a hydrophobic probe, 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), to MEs, r-HDLs and apoHDL were evaluated on the basis of Halfman and Nishida's method. The reconstitution of apoHDL into MEs led to a 68% reduction in the binding of TNS and a small increase in the α-helix content as compared with free apoHDL. The binding experiments also showed the condensation of lecithin molecules at the r-HDL surface. The amphipathic helixes of apoHDL are located in the surface monolayer of egg yolk lecithin surrounding the triolein core. The intercalation of the hydrophobic residues of apoHDL between egg yolk lecithin molecules brings about a pronounced curvature of the surface and a decrease in the particle diameter.

Keywords high density lipoprotein; apolipoprotein; reconstituted high density lipoprotein; hydrophobic binding probe; amphipathic helix; egg yolk lecithin monolayer

High density lipoproteins (HDLs) are very small particles composed of phospholipids, cholesterol, cholesterylesters, triglycerides and apolipoproteins. Reconstituted particles of the phospholipids and the apolipoproteins have been characterized as phospholipid bilayer discs surrounded by the apolipoproteins.[1-5] The spherical particles reconstituted from the phospholipids, apoproteins and neutral lipids have been regarded as a droplet of neutral lipids covered with a surface monolayer of phospholipids and apolipoproteins.[6,7] However, the total features of the spherical particles, which resemble plasma HDLs,[7,8] are still veiled. Shen *et al.* have proposed a general model for spherical lipoproteins in plasma (chylomicrons, very low density lipoproteins (VLDLs), low density lipoproteins (LDLs) and HDLs) and have correlated the size with the composition.[9] The unfolded apoproteins fill the gaps of the expanded phospholipid monolayer, and free cholesterol is located under the proteins.[9] No solid evidence or criticism of the model has been reported.

Recently, Yang *et al.* showed the complete amino acid sequence of a major protein of VLDLs and LDLs, apoB-100, suggesting the participation of the protein in the core formation of VLDLs and LDLs[10]: The apoproteins of chylomicrons and HDLs, participating in the surface layer formation, have little structural resemblance to the major proteins of VLDLs and LDLs. On the other hand, Derksen and Small showed sharp decreases in the binding capacity of apolipoproteins (A-1 and E-3) to the microemulsion particles (MEs) constituted of lecithin and triglyceride by the addition of cholesterol into the MEs.[7] This finding is in conflict with the cholesterol–protein arrangement in the model proposed by Shen *et al.*[9,11] We have found a condensation of lecithin monolayer in a triolein–saline interface.[12] The area per lecithin molecule of 40 Å^2 at the monolayer is much smaller than the value in the model, 65 Å^2.

In the present work, we have investigated the interactions between pig apo lipoprotein HDL (apoHDL) and lipids in reconstituted spherical particles (r-HDLs) by cosonication of egg yolk lecithin (PC), triolein (TO) and the protein in saline. ApoA-1 is the only major peptide in pig apoHDL, while apoA-1, A-2 and C-3 are main components of human apoHDL.[13]

Experimental

Materials Pig HDLs of a density range of 1.063—1.21 g/ml were isolated from plasma using standard ultracentrifugal techniques.[13,14] Gel filtration in saline (0.15 M NaCl, 0.01 M Tris–HCl, pH 7.4) was performed to purify the HDLs on Sepharose CL-6B (Pharmacia). ApoHDL was purified by delipidation of the HDLs with ethanol–diethyl ether, 3 : 2 (v : v) at −20 °C.[15] The purity of the resultant residue was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands on the polyacrylamide gel were stained with coomassie blue in 10% acetic acid. Protein was determined by the Lowry procedure.[16] PC was kindly presented by Asahi Kasei Co. The purity (over 99.5%) was determined by thin layer chromatography. TO, obtained from Taiyo Chemical Co., was purified by silica gel (Wakogel C-200, Wako Pure Chemicals) column chromatography to remove fatty acids, diglycerides and monoglycerides by using chloroform/methanol (99/1) as an eluent. The purity of TO thus obtained was over 99%. 2-*p*-Toluidinylnaphthalene-6-sulfonate (sodium salt) (TNS) was supplied by Aldrich Chemical Co., Inc. Water was doubly distilled with a quartz still.

Preparation of MEs and r-HDLs MEs: PC and TO at various mole ratios were dissolved in chloroform. After evaporation of the solvent the mixtures were dried *in vacuo* for 15 h. The saline of 10 ml was added to the dried lipid mixture at 4 °C. The total concentration of PC and TO was kept at 0.1 mM. The suspension was briefly vortexed and then sonicated for 15 min under a stream of nitrogen gas at 4 °C. The probe-type sonicator used was a UD-200 from Tomy Seiko Co., Ltd. The lipid dispersed solution (microemulsion) was centrifuged for 10 min to remove the titanium dust. r-HDL: apoHDL in saline was added to a dried mixture of PC and TO. The dispersion was briefiy vortexed and sonicated under the same conditions as that of MEs except the sonication period was 5 min. The particles thus prepared were fractionated on a Sepharose CL-6B column. The protein and PC contents were determined by the methods of Lowry *et al.*[16] and Bartlett,[17] respectively.

Electrron Microscopy MEs or r-HDLs were diluted as much as 50- fold in 1% potassium phosphotungstate and were applied to collodion-coated, carbon-stabilized copper grids and examined using a Hitachi H-500 electron microscope. 200 particles per sample were measured and average particle diameters were determined.

Binding Measurements Binding of TNS to MEs, apoHDL, r-HDLs, or pig HDLs in the saline were determined by fluorometric assay using a Jasco FP-550A spectrofluorometer at 25 °C.[18] The fluorescence intensity at 420 nm with excitation at 366 nm was monitored as a function of the TNS concentration ($<10^{-5}$ M). The binding isotherms or TNS were evaluated on the basis of Halfman and Nishida's method.[19]

Circular Dichroism (CD) Spectra CD spectra of apoHDL and r-HDLs in the saline were recorded on a Jasco J-600 instrument at 25 °C. The saline was used as a blank. The instrument outputs were calibrated

by using non-hygroscopic ammonium *d*-camphor-10-sulfate. The contents of helix, beta, turn and unordered structures were estimated on the basis of a constrained least-square calculation[20] with a set of reference spectra by Chang *et al.*[21] A mean helical length of 20 residues was assumed.[22]

Results and Discussion

Reconstitution of r-HDL After delipidation of pig HDLs, the purified apoproteins gave one major band at 26—27 kirodaltons (kDa) on the polyacrylamide gel (Fig. 1a). Pig HDLs have one major apoprotein, which has been identified as apoA-1 (total amino acid residues 230 and molecular weight 26000) and is similar to human apoA-1 (total amino acid residues 241 and molecular weight 27900).[13] Figure 2 shows electron microscopic pictures of the unfractionated MEs (PC/TO mole ratio = 3/2) and the unfractionated r-HDLs (PC/TO/apoHDL mole ratio = 3/2/0.1). The latter composition is close to that of HDLs. The average weight-diameters were 34.0 and 9.0 nm, respectively. Israelachivili *et al.* have investigated the correlation between the structure of lipid assembly and the molecular structure of the lipid.[23] Huang and Mason have discussed the size of the assembly of lecithin.[24] These studies have shown the minimun size of the PC bilayer vesicle to be 25 nm. Therefore, the apoHDL plays important roles in the formation of the smaller particles of PC (9 nm).

The r-HDLs were delipidated and the purified protein was again subjected to electrophoresis. It showed one major band similar to the original protein (Fig. 1b). Weak bands at the higher molecular weight regions are of the dimeric and trimeric apoHDL, which may be formed in the cosonication process. No free fatty acids resulting from hydrolysis of the lipids during sonication were detected by thin-layer chromatography. Klein's oxidation index[25] of the lipids extracted from the r-HDL showed no lipid oxidation. Therefore, the 5 min-cosonication for reconstitution under nitrogen atmosphere did not lead to any degradations of the lipid components.

The cosonication products of lipids and apoHDL mixture (PC/TO/apoHDL mole ratio = 1/1/0.01) were fractionated and examined by electron microscopy. Table I shows the variation in the average diameter of the fractionated particles by the PC/apoHDL mole ratio. The particle size decreases with an increase in the mole fraction of apoHDL.

Binding of TNS to Reconstituted Particles Halfman and Nishida's method of determination of ligand binding to macromolecule is based on an assumption that the equilibrium of ligand between bound and free states is established in an entire solution.[19] The total concentration of ligand, C_t, is a sum of concentrations of the free and the bound ligands, C_f and C_b, respectively.

$$C_t = C_f + C_b = C_f + \nu C_M \quad (1)$$

Here, ν and C_M are the number of bound ligand molecules per macromolecule and the concentration of the macromolecule, respectively. The latter can be represented either in molar concentration of PC or in that of apoHDL. The fluorescence intensity of the TNS solution (F) arises from the bound TNS molecule to MEs or r-HDL and the intensity due to the free TNS molecule is negligible[18] *i.e.* $F \propto \nu C_M$. At a constant value of F/C_M (constant ν value), Eq. 1 gives a linear relation between C_t and C_M because C_f is a function of ν. The slope and the intercept to the C_t-axis (ordinate) show the ν and the C_f values, respectively.[19,26] The macromolecular concentration, C_M, is represented in two ways: moles of PC or apoHDL in 1 dm^3 of solution. Accordingly, the binding isotherms of TNS are shown in

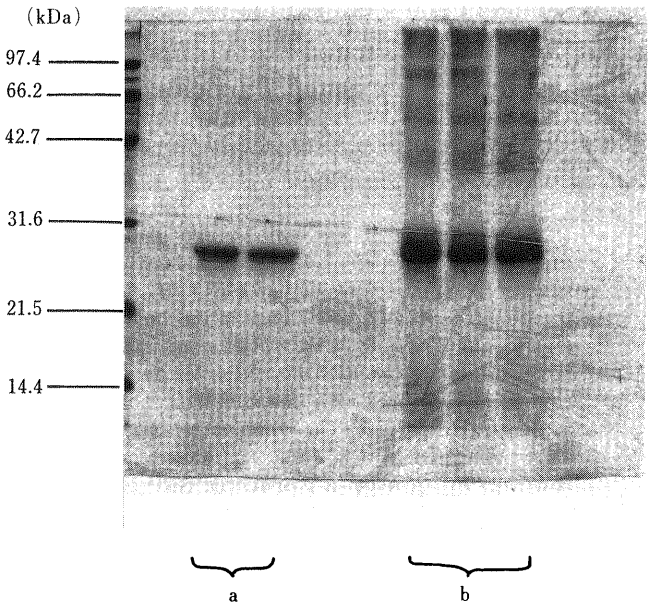

Fig. 1. SDS Gel Electrophoresis of Isolated apoHDL from Pig Plasma HDL (a), and the Protein Separated from r-HDL by Delipidation (b)
r-HDL: PC/TO/apoHDL = 3/2/0.1 in mole ratio.

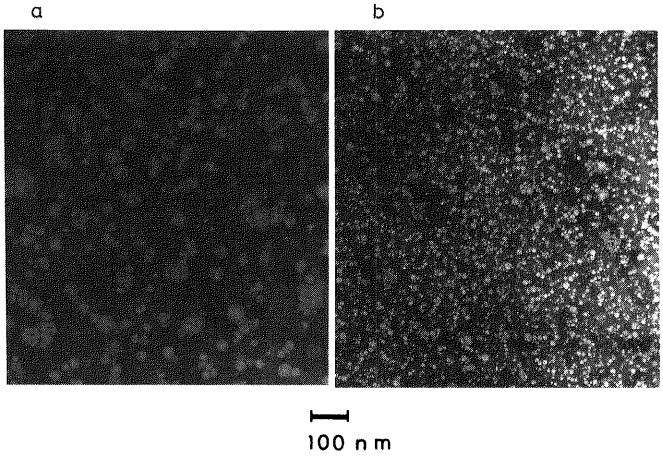

Fig. 2. Electron Microscopic Pictures of Microemulsion (MEs, a) and r-HDL(b)
Unfractionated MEs: PC/TO = 3/2, unfractionated r-HDL: PC/TO/apoHDL = 3/2/0.1 (in mole ratio).

TABLE I. Correlation between Diameter and Composition of r-HDL[a]

Composition PC/apoHDL (n_L/n_P) (mole ratio)	Diameter of r-HDL[a] (nm)
100/0	34
134/1	23
100/1	21
68/1	15
34/1	9

a) A mixture of PC/TO/apoHDL (1/1/0.01 in mole ratio) was cosonicated and the resultant particles were fractionated on a Sepharose CL-6B column.

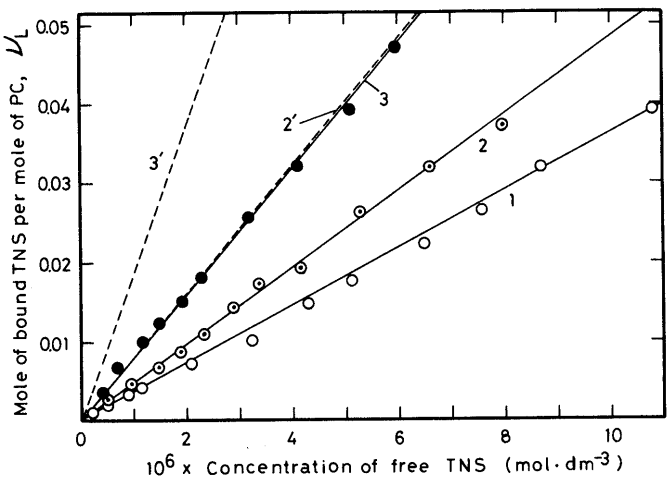

Fig. 3. TNS Binding Isotherms

The mole of bound TNS per mole of PC (ν_L) is represented as a function of the concentration of free TNS in saline (C_f). Saline: 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4. Macromolecules: 1 MEs (PC/TO=3/2), 2 fractionated r-HDL (PC/TO/apoHDL=3/2/0.055, $n_P/n_L=1/55$, diameter: 13 nm), 3 fractionated r-HDL (PC/TO/apoHDL=3/2/0.15, $n_P/n_L=1/20$, diameter: 9 nm). The broken lines show the isotherms calculated at $n_P/n_L=1/55$ (2') and 1/20 (3'), by assuming that the additivity of bound moles of TNS to PC and apoHDL of r-HDL is valid.

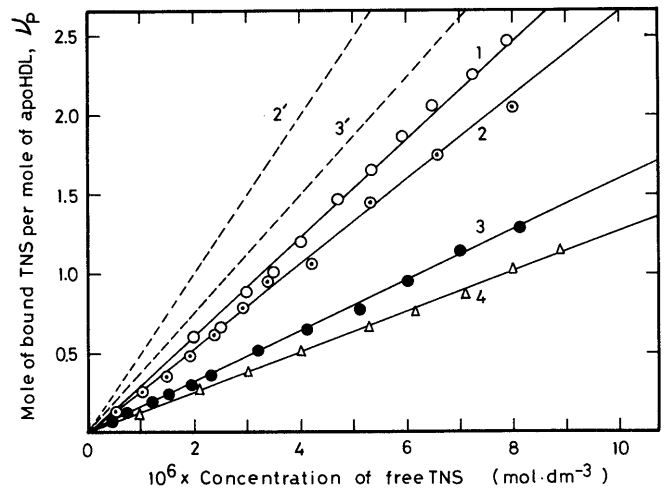

Fig. 4. TNS Binding Isotherms

The mole of bound TNS per mole of apoHDL (ν_P) is represented as a function of the concentration of free TNS in solution (C_f). Macromolecules: 1 apoHDL, 2 fractionated r-HDL ($n_P/n_L=1/55$), 3 fractionated r-HDL ($n_P/n_L=1/20$), 4 pig HDLs ($n_P/n_L=1/23$). The broken lines show the isotherms calculated, $n_P/n_L=1/55$ (2') and 1/20 (3') by assuming that the additivity of bound moles of TNS to PC and apoHDL of r-HDL is valid.

two ways. Mole of bound TNS per one mole of PC (in MEs or r-HDLs), ν_L, and mole of bound TNS per one mole of apoHDL (in solution or r-HDL), ν_P, are shown in Figs. 3 and 4, respectively, as a founction of C_f. The binding isotherms of TNS to MEs and the free apoHDLs observed were linear:

$$\nu_L = K_L C_f \quad (2)$$

$$\nu_P = K_P C_f \quad (3)$$

Here, ν_L and ν_P are the numbers of bound TNS per PC and per apoHDL molecules, respectively. K_L and K_P are binding constants and are equal to the magnitude of TNS binding × number of binding site. The experimental values of K_L and K_P are 3.6×10^3 and 3.0×10^5 dm^3/mol, respectively. The variation in PC/TO ratio (3/2—3/3) of MEs had little

Table II. Contents[a] of α-Helix, Beta, Turn and Unordered Structures of ApoHDL in Saline and r-HDLs[b]

	α-Helix	Beta	Turn	Unordered
ApoHDL in saline	0.41	0.00	0.22	0.37
ApoHDL in r-HDLs	0.49	0.00	0.20	0.31
ApoHDL (treated with 2 M urea and dialyzed against saline)	0.27	0.10	0.28	0.35

a) Contents of secondary structures were estimated by using a constrained least-square calculation[20] with a set of reference spectra by Chang *et al.*[21] *b*) Composition: PC/TO/apoHDL=3/2/0.1 in mole ratio.

effect on the K_L value. Assuming that the additivity of TNS bindings to PC and apoHDL of r-HDLs is valid, we obtain the mole of bound TNS to r-HDL, TNS_b, as:

$$TNS_b = (n_L K_L + n_P K_P) C_f \quad (4)$$

Here, n_L and n_p are moles of PC and apoHDL in the solution respectively, and

$$\nu_L = (TNS_b/n_L) = [K_L + K_P(n_P/n_L)] C_f \quad (5)$$

or

$$\nu_P = (TNS_b/n_P) = [K_L(n_L/n_P) + K_P] C_f \quad (6)$$

The broken lines in Figs. 3 and 4 show the isotherms at $(n_P/n_L)=1/20$ and 1/55, calculated by Eqs. 5 and 6 respectively. The experimental values of the bound amount were remarkably smaller than the calculated ones, thus indicating a reduction in the binding capacity of PC and/or apoHDL by the recombination. Introducing the experimental values of ν_L/C_f at $(n_P/n_L)=1/20$ and 1/55 to Eqs. 5, we obtained K_L of 3.2×10^3 and K_P of 9.7×10^4 dm^3/mol. The K_L value of r-HDLs was 10% smaller than that of MEs, and the K_P value of r-HDL was one third of that of the free apoHDL. The large reduction in the TNS binding to apoHDL in r-HDL is due to either a large conformational change (folding) or the concealment of hydrophobic regions of the protein in the reconstituted HDL particles. The binding isotherm was estimated also for pig HDLs (lecithin/apoHDL=23/1 in mole ratio) in Fig. 4. The lower binding capacity, as compared with r-HDLs (the line 3 in Fig. 4), indicates some differences in the surface structure between r-HDLs and pig HDLs.

Table II shows the contents of helix, beta, turn and unordered structures of apoHDL in solution and r-HDL (PC/TO/apoHDL=3/2/0.1 in mole ratio and $n_P/n_L=1/30$). The 8% increase in α helix content and the 6% decrease in unordered structure content by the reconstitution are comparable to the changes of human apoA-1 by similar recombination.[5] This result indicates that a rather small change occurs in the secondary structure of the protein by the reconstitution.

Israelachivili *et al.*[23] and Ruckenstein and Nagarajan[27] have shown that the volume ratio of hydrophobic and hydrophilic residues in a lipid molecule plays important roles in the shape-determination of the lipid assembly. In the formation of r-HDL (diameter < 10 nm), the binding of apoHDL to the lipid assembly involves the transfer of hydrophobic residues of the amphipathic helixes (6—7 helixes[22]) of the protein to the hydrophobic region of the PC monolayer: the intercalation of the helixes between the

PC molecules at the surface. The polar residues (70% of residues in the helixes) distributed on the opposite sides of the helixes, contribute to the expansion of the hydrophilic area of monolayer surrounding a core of the neutral lipid (TO), and thereby to the formation of very small particles of a pronounced curvature. Thus, the higher content of the protein resulted in the formation of the smaller particles. The concealment of hydrophobic residues of apoHDL causes the large reduction in number of the binding site for TNS. The small change in the secondary structures of apoHDL in the course of reconstitution is interpreted by the unfolding of the folded amphipathic helixes in solution and their binding to the lipid by hydrophobic interactions. The unfolding of the protein molecule at the PC monolayer has been evaluated on the basis of our interfacial tension measurements (unpublished data). Yokoyama *et al.* reconstituted very small particles having a diameter less than 10 nm with peptides designed for optimizing amphipathic helix and phospholipids.[28] We cosonicated an amphipathic β-structure forming peptide, gramicidin S, with PC and TO in the saline. The planar peptide, which had a hydrophobic surface and a hydrophilic surface at the opposite sides of the molecule, had little effect on the preparation of very small particles of a pronounced curvature.

r-HDLs were reconstituted with apolipoprotein and lipids without cholesterol. In the spherical lipoprotein model of Shen *et al.*,[9] cholesterol was considered as playing an important role in the constitution of the lipoproteins. The expanded PC monolayer of the outer surface of small unilamellar liposomes (SUV) are unstable and are easily attacked by apoproteins of HDL to give micelle-like particles (HDL particles).[29,30] PC molecules in liposomes, low or very low density lipoproteins, are transferred to HDL particles through the physicochemical potential gradient.[31-33] The potential of PC is lowest in the HDLs due to the strong interaction between the lipid and apoHDL molecules. The 10% reduction in the TNS binding to PC shows the condensation of the PC molecules during the recombination with apoHDL. We found correlations between the stability of MEs and the condensation of the phospholipid monolayer at the TO–saline interface.[12] The recombination of PC and apoHDL will bring about the close packing of the lipid and protein molecules in the curved surface of r-HDL and stabilize the particles.

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