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Lipid–Protein Interactions in High Density Lipoproteins

(recombination of apolipoproteins with phospholipids/circular dichroism and gel chromatography of recombined lipid–protein particles)

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ABSTRACT **Delipidated high density lipoprotein (apoHDL), isolated apolipoproteins apoA-I and apoA-II, *S*-carboxymethylated apoA-II, apoC-III, the NH_2- and COOH-terminal CNBr peptides of apoA-II, and the COOH-terminal CNBr peptide of apoA-I were recombined *in vitro* with [*N*-C^3H_3-*choline*]phosphatidylcholine (PC) and [*N*-$^{14}CH_3$-*choline*]sphingomyelin (SPM). The lipid–protein complexes were analyzed by ultracentrifugal flotation, agarose gel chromatography and circular dichroism. ApoHDL, apoA-II, and *S*-carboxymethylated apoA-II readily recombined with PC or SPM to form particles that were similar in size to native HDL. The COOH- but not the NH_2-terminal CNBr peptide of apoA-II recombined with lipid. ApoA-I and the COOH-terminal CNBr peptide of apoA-I, however, recombined with PC or SPM to only a limited extent, suggesting that protein–protein interactions between apoA-I and apoA-II are important in the integration of apoA-I into recombined lipoprotein particles. Analysis of the recombined lipid–protein complexes by circular dichroism indicated that there was an increase in helical structure concomitant with lipid–protein binding. The reconstituted particles had many of the physical and chemical properties of the native lipoprotein.**

It has been shown (1–4) that the protein moiety of human serum high density lipoprotein (HDL), prepared in its lipid-free form (apoHDL) by treatment with organic solvents (5, 6) retains its capacity to bind lipids, both *in vivo* (7) and *in vitro* (3, 8). About 90% of the protein moiety of apoHDL is composed of two major apoproteins, designated as apoA-I and apoA-II, or, by their COOH-terminal amino acids, as apoGln-I and apoGln-II (9–16); the remaining 10% is mainly composed of three minor apoproteins (apoC-I, apoC-II, and apoC-III), also associated with the very low density lipoprotein family (9). The complete amino-acid sequences of apoA-II, apoC-I, and apoC-III have been reported (17–19). Lipid-free apolipoproteins, when sonicated with or added to (sonicated) HDL lipids, reform lipidated complexes resembling the native molecules (2, 4, 20–23).

The mode of interaction between phospholipids and apolipoproteins remains, however, unknown, and the integration of individual lipid and protein molecules into the structural organization of the HDL macromolecules is not fully understood. To elucidate how the various components are held together in an organized structure and to what extent protein–protein, protein–lipid, and lipid–lipid interactions contribute to the overall structure of HDL, we have examined the interactions of different HDL apolipoproteins, in native and chemically modified form, with phospholipids and neutral lipids. We have studied lipid–protein interactions in HDL utilizing ^{13}C and ^{31}P nuclear magnetic resonance spectroscopy (24–26). Here we report on phospholipid–protein interactions in HDL as assessed by ultracentrifugal flotation, gel chromatography, and circular dichroism (CD).

Abbreviations: HDL, high density lipoproteins; apoHDL, delipidated high density lipoproteins; CD, circular dichroism; PC, phosphatidylcholine; SPM, sphingomyelin.

MATERIALS AND METHODS

Isolation of HDL and HDL Apolipoproteins. The HDL used in these studies was obtained from the plasma of two male volunteers (G.A., K.Z.). The lipoproteins were isolated by preparative ultracentrifugal flotation between KBr densities 1.063 and 1.210 g/ml, and delipidated with chloroform–methanol, 2:1 (6). The procedures used in the isolation and characterization of the individual proteins of apoHDL are described elsewhere (6). ApoA-I was isolated by chromatography of apoHDL in 6 M urea on Sephadex G-200 (Pharmacia, superfine) (13). ApoA-II was isolated by chromatography of apoHDL in 6 M urea on DEAE-cellulose (Whatman, DE-52) (6, 12). ApoC-III was isolated as described (27, 28). Cleavage with CNBr (Eastman, ratio of reagent to peptide, 500:1) was performed in 70% formic acid at 25° for 48 hr (29). The resulting peptide fragments of apoA-II were purified to homogeneity by chromatography on Sephadex G-75 (0.2 M Tris·HCl, pH 8.6, 6 M urea); peptides of apoA-I, by chromatography on Biogel P-10 (25% acetic acid). ApoA-I, apoA-II, COOH- and NH_2-terminal CNBr peptides of apoA-II, and the COOH-terminal peptide of apoA-I were shown to be homogeneous by analytical polyacrylamide gel electrophoresis (30), Edman NH_2-terminal analysis (31), and amino-acid analyses. The latter were performed on a Beckman-Spinco automatic amino-acid analyzer, model 120B or 121, adapted for high sensitivity (32) and a rapid elution schedule (33). Hydrolyses were performed at 110° for 24 hr in constant-boiling HCl (5.7 N) containing 2-mercaptoethanol (1/2000, v/v) (34). ApoA-II was reduced with 2-mercaptoethanol and alkylated with iodoacetic acid (29). ApoA-II was succinylated with a 50-fold molar excess of succinic anhydride in 0.1 M $NaHCO_3$, pH 8.8, for 1 hr at room temperature (35). The incubation mixture was desalted by fractionation over Sephadex G-10 in 0.05 M NH_4HCO_3. ApoA-II was enzymatically digested with trypsin [L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone-treated, Worthington, peptide:enzyme, 30:1] at 37° in 0.1 M trimethylamine the acetate buffer, pH 8.2 (29). Tryptic peptides were isolated by DEAE-cellulose chromatography (29).

Preparation of Lipids. Egg yolk phosphatidylcholine (PC) and sphingomyelin (SPM) were isolated and characterized

(24). An ^{3}H or ^{14}C label was chemically introduced into the choline moieties of PC and SPM (36). [*N-C$^{3}H_3$-choline*]PC (specific activity, 3.5×10^4 cpm/μmol) and [*N-$^{14}CH_3$-choline*] SPM (specific activity, 2.8×10^6 cpm/μmol) were purified by silica gel chromatography (37), and subsequently used for recombination experiments with individual apolipoproteins. The preparation of 1,2-[*dioleoyl-1-*^{14}C]-*sn*-phosphatidylcholine and the procedures used to label cholesterol esters in naturally occurring HDL are described elsewhere (24).

Reassembly Experiments. Sonicated dispersions of PC and SPM were added to individual apoproteins solubilized in 0.1 M NH_4HCO_3 buffer, pH 8.8, by the procedures outlined (24). The lipid–protein samples were adjusted to density 1.063 g/ml with solid KBr and spun for 20 hr in a 65 rotor at 60,000 rpm. The infranatant fractions (3 ml) were adjusted to a density of 1.25 g/ml and spun for 40 hr at 60,000 rpm. Lipid–protein complexes, isolated by ultracentrifugal flotation or agarose gel chromatography (see below) were analyzed for phospholipid by determination of radioactivity, and for protein by the method of Lowry *et al.* (38).

Gel Chromatography. Lipid–protein complexes isolated by ultracentrifugation at density 1.063 g/ml or 1.25 g/ml were chromatographed on a column of Biogel A, 1.5 M (2×90 cm), equilibrated in 10 mM NH_4HCO_3 buffer, pH 8.8; 0.5 ml of the 1.063 g/ml infranatant (3 ml) or 1.25 g/ml supernatant (3 ml) fraction was applied to the column, and the eluate (10 ml/hr) was monitored by A_{280}. Aliquots of each tube were analyzed for phospholipid by determination of radioactivity. Fractions containing protein or protein–lipid were pooled, lyophilized and analyzed by analytical polyacrylamide gel electrophoresis.

Circular Dichroism. All samples analyzed by CD were exhaustively dialyzed against 0.05 M sodium phosphate buffer, pH 8.0. Spectra were recorded using a Cary 60 spectropolarimeter equipped with a model 6001 CD accessory and calibrated with *d*-10-camphosulfonic acid. The spectra reported are the mean of two to four repetitive analyses of each preparation. The signal-to-noise ratio was always greater than 10:1. Base line runs containing 0.05 M sodium phosphate buffer, pH 8.0, were repeated twice for each preparation. The mean residue ellipticity in units of deg cm^2 dmol^{-1} was calculated from $[\theta] = (\text{MRW})\ (\theta)°/10\ lc$ where $(\theta)_\lambda°$ is the observed ellipticity in degrees at wavelength λ; l, the optical path in cm; c, the concentration in g/ml; and MRW, the mean residue weight calculated from amino-acid analyses (ApoHDL, 115; apoA-I, 119; apoA-II, 112; succinylated apoA-II, 113; CNBr peptides of apoA-II:NH_2-terminal 110, COOH-terminal, 112; COOH-terminal CNBr peptide of apoA-I, 112).

The degree of α helical structure in the individual samples was estimated at 222 nm assuming a value of 29,000 for a completely helical protein and little or no residues in β-structure (39, 40).

RESULTS

Reassembly Experiments. The results of the recombination of PC and SPM with individual apolipoproteins and peptide fragments are summarized in Table 1. The lipid-to-protein weight and molar ratios are presented for the 1.25 g/ml of supernatant fraction (3 ml); no lipid was detectable in the 1.25 infranatant fraction (3 ml). The protein not incorporated into the lipid–protein complex in the 1.25 supernatant fraction was recovered in the 3-ml infranatant fraction. When sonicated dispersions of labeled lipid were ultracentrifuged (density 1.063) in the absence of protein all lipid radioactivity was present in the 3-ml supernatant fraction. Recoveries of protein and lipid for individual ultracentrifugation steps were greater than 90%.

TABLE 1. *Recombination of apolipoproteins and peptide fragments with phospholipids*

	Recombination mixture		1.25 g/ml of supernatant*		Lipid to protein ratio†	
	Lipid (mg)	Protein (mg)	Lipid (mg)	Protein (mg)	Weight	Molar
ApoHDL + [^{14}C]SPM‡						
Exp. I	41.0	10.0	9.1	3.7	2.5	
Exp. II	16.8	7.5	6.2	3.0	2.1	
ApoHDL + [^{3}H]PC						
Exp. I	60.0	10.0	6.6	3.5	1.9	
Exp. II	22.0	7.5	5.8	2.9	2.0	
ApoA-II + [^{3}H]PC						
Exp. I	22.0	3.0	2.6	2.1	1.2	25.7
Exp. II	15.0	5.0	6.7	4.5	1.5	30.9
Exp. III	5.5	5.1	5.3	4.7	1.1	23.2
Exp. IV	13.8	4.9	6.6	4.5	1.5	30.0
ApoA-II + [^{14}C]SPM						
Exp. I	54.0	25.0	40.8	21.0	1.9	40.2
Exp. II	12.8	5.0	8.3	4.6	1.8	37.3
Cys(Cm)apoA-II + [^{14}C]SPM	65.0	9.0	31.2	8.6	3.6	37.5
Succinylated apoA-II + [^{3}H]PC	15.0	5.0	0.7	1.1	0.7	13.5
COOH-terminal apoA-II + [^{3}H]-PC	5.5	3.7	3.4	3.3	1.0	7.0
NH_2-terminal apoA-II + [^{3}H]PC	5.5	4.1	—	0.2	—	—
ApoA-I + [^{3}H]PC						
Exp. I	22.0	3.0	3.0	0.4	7.5	238
Exp. II	15.0	5.0	1.3	0.6	2.1	67.0
Exp. III	27.5	5.2	1.9	0.5	3.8	132.0
ApoA-I + [^{14}C]SPM						
Exp. I	6.0	5.0	4.1	0.5	8.2	291.0
Exp. II	54.0	9.0	6.0	0.6	10.0	327.0
COOH-terminal apoA-I + [^{3}H]PC	15.0	4.0	0.5	1.0	0.5	6.4
COOH-terminal apoA-I + [^{14}C]SPM	6.0	4.0	1.0	1.1	0.9	11.5
ApoC-III + [^{3}H]PC	22.0	3.0	4.0	0.9	4.4	43.0
ApoC-III + [^{14}C]SPM	16.0	3.0	8.1	1.1	7.4	71.0

* 1.25 g/ml of supernatant = supernatant fraction (3 ml) from the ultracentrifugation of recombined lipid–protein complexes at density 1.25 g/ml.

† The values for protein have been set at 1 in all cases.

‡ Abbreviations: Cys(Cm)apoA-II = *S*-carboxymethylated apoA-II. COOH and NH_2 terminal = COOH- and NH_2-terminal CNBr peptide.

Complexes with lipid-to-protein ratios (by weight) of between 1.9/1 and 2.4/1 were isolated after recombination of apoHDL with various weight ratios of SPM (4.1/1, 2.2/1) and PC (6/1, 3/1) (Table 1). About 30% of the protein in the incubation mixture was incorporated into lipid–protein particles.

When pure apoA-II was incubated with PC or SPM between 70 and 90% of the protein was isolated in the lipid–protein complexes. The molar ratio of PC to protein in the isolated complexes varied from 23/1 to 30/1; the molar ratios

of SPM to protein were 37/1 and 40/1 (Table 1). Reduction and carboxymethylation of apoA-II did not significantly alter the molar ratio of SPM to protein (38/1) observed in the recombined complex. Succinylation of apoA-II, however, resulted in a decrease in lipid binding, only 22% of the succinylated apoA-II being incorporated into the lipid–protein complex. The molar ratio of PC to protein (13/1) obtained was about half that observed in the native apoA-II–PC complex. Recombination of the isolated COOH-terminal CNBr peptide of apoA-II with PC resulted in a complex containing 98% of the protein used in the incubation mixture. The molar ratio of lipid to protein in the complex was 7/1, which is about one-third that observed in the native apoA-II–PC complex. The NH_2-terminal CNBr peptide and the isolated tryptic peptides of apoA-II did not bind phospholipid.

Recombination of apoA-I with PC or SPM resulted in the formation of lipid–protein complexes characterized by a high molar ratio of lipid to protein (67/1–327/1) (Table 1). The amount of protein recovered in this complex, however, was only 10% of the apoA-I initially used in the incubation mixture. Whether this small amount of protein recombined in a specific manner with phospholipid or represented aggregated or trapped protein contained in a lipid complex could not be determined.

The majority of PC (97%) and SPM (84%) used in the recombination mixture with the COOH-terminal CNBr peptide of apoA-I were isolated, unrecombined with protein, in the 1.063 supernatant fraction. The significance of the lipid-poor particles isolated by ultracentrifugal flotation at density 1.25 g/ml was not established (Table 1).

ApoC-III, which interacts strongly with PC (22), also recombines with SPM. The recombined particle contained 30% of the protein used in the recombination mixture, and had a molar ratio of lipid to protein of 43/1 for PC and 71/1 for SPM (Table 1). The value determined for PC is in agreement with a value reported by Morrisett *et al.* (22).

Gel Chromatography. Native HDL and recombined lipid-protein particles were also analyzed by gel chromatography (Fig. 1). Aliquots of either the 1.063 g/ml infranatant or 1.25 g/ml supernatant were chromatographed. In all gel chromatography column runs, more than 80% of the lipid radioactivity and more than 85% of the protein were recovered. The chromatographic behavior of aliquots of the 1.063 g/ml infranatant and 1.21 g/ml supernatant of several different recombined particles (Fig. 1, Exps. 4, 5, and 12, 23) indicated that no significant destruction of the recombined particles isolated in the 1.063 infranatant fraction occurred during the subsequent ultracentrifugation at density 1.25 g/ml (Fig. 1 and Table 1).

Native HDL isolated by ultracentrifugal flotation separated reproducibly into two fractions, with fraction b containing principally apoA-I (Fig. 1). These subfractions will be analyzed elsewhere (Assmann, G. & Brewer, H. B., manuscript in preparation).

Recombination of apoHDL with SPM, and apoA-II with PC or SPM produced lipid–protein complexes which had a particle size resembling that of native HDL (Fig. 1). Reduction and carboxymethylation of the disulfide bridge of apoA-II did not affect the binding of lipid or the particle size (Fig. 1 and Table 1). When a mixture of CNBr peptides of apoA-II was incubated with PC and an aliquot of the 1.063 infranatant fraction analyzed by gel chromatography, three distinct subfractions were obtained (Fig. 1, Exp. 10). Fraction a

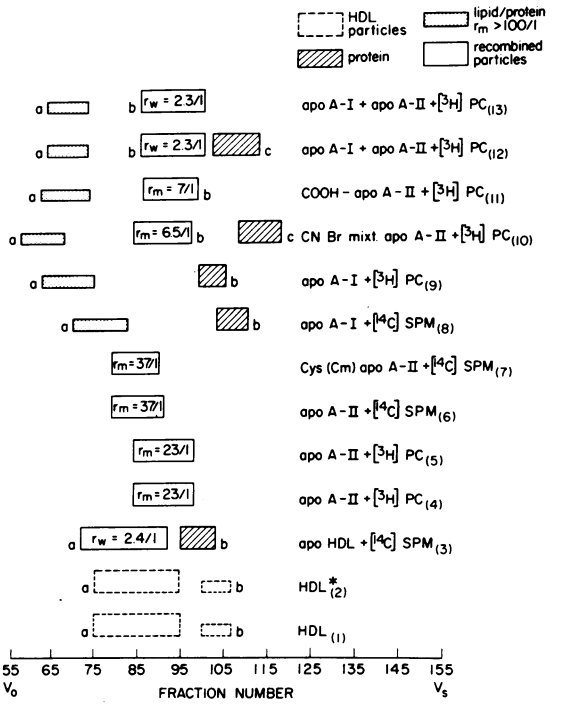

FIG. 1. Gel chromatography of native and recombined HDL particles. Abbreviations: See Table 1; r_m, molar ratio; r_w, weight ratio; HDL V_0 and V_s are the excluded and included volumes, respectively (see *Methods*). Experiments are indicated by subscript numbers in parentheses. Exps. 1, 2, 4, 6, 7, 11, and 13: Native or reassembled lipoproteins isolated as the supernatant fraction (3 ml) after ultracentrifugation at density 1.25 g/ml. Exps. 3, 5, 8, 9, 10, and 12: Reassembled lipoproteins isolated as infranatant fraction (3 ml) after ultracentrifugation at density 1.063 g/ml. $HDL_{(2)}$*: native HDL labeled in its cholesterol ester moiety using 1,2-[*dioleoyl-1-*^{14}*C*]-*sn*-phosphatidylcholine as substrate for lecithin-cholesterol-acyltransferase (24).

contained less than 1% of the protein. Fraction b contained 60% of the protein with a molar ratio of lipid to protein of 6.5/1. Fraction c contained no lipid and 30% of the protein applied to the column. Amino-acid analyses of these fractions indicated that fraction b was the COOH-terminal and fraction c the NH_2-terminal CNBr peptide of apoA-II (29). The particle isolated by gel filtration after the recombination of the isolated COOH-terminal peptide of apoA-II and PC (Fig. 1, Exp. 11) was similar in size and ratio of lipid to protein to that obtained after the recombination of PC with the unfractionated CNBr peptides.

In all recombination experiments with apoA-I lipid–protein complexes with a high molar ratio of lipid to protein and large particle size were observed (Fig. 1, Exps. 8 and 9). The isolated apoA-I–PC or apoA-I–SPM complexes contained only a small fraction (<10%) of the protein initially used, and the significance of these fractions could not be determined. When apoA-I was preincubated with apoA-II (ratio by weight of 3/1) and the mixture then added to PC (Fig. 1, Exps. 12 and 13) a significant amount of apoA-I was incorporated into a complex similar in particle size to the apoA-II–PC complex. Based on analytical polyacrylamide electrophoresis, the ratio of apoA-I to apoA-II in these lipoprotein particles was about 1:1.

Circular Dichroism. The circular dichroic spectra of HDL, apoHDL, apoA-II, the CNBr peptides of apoA-II, the

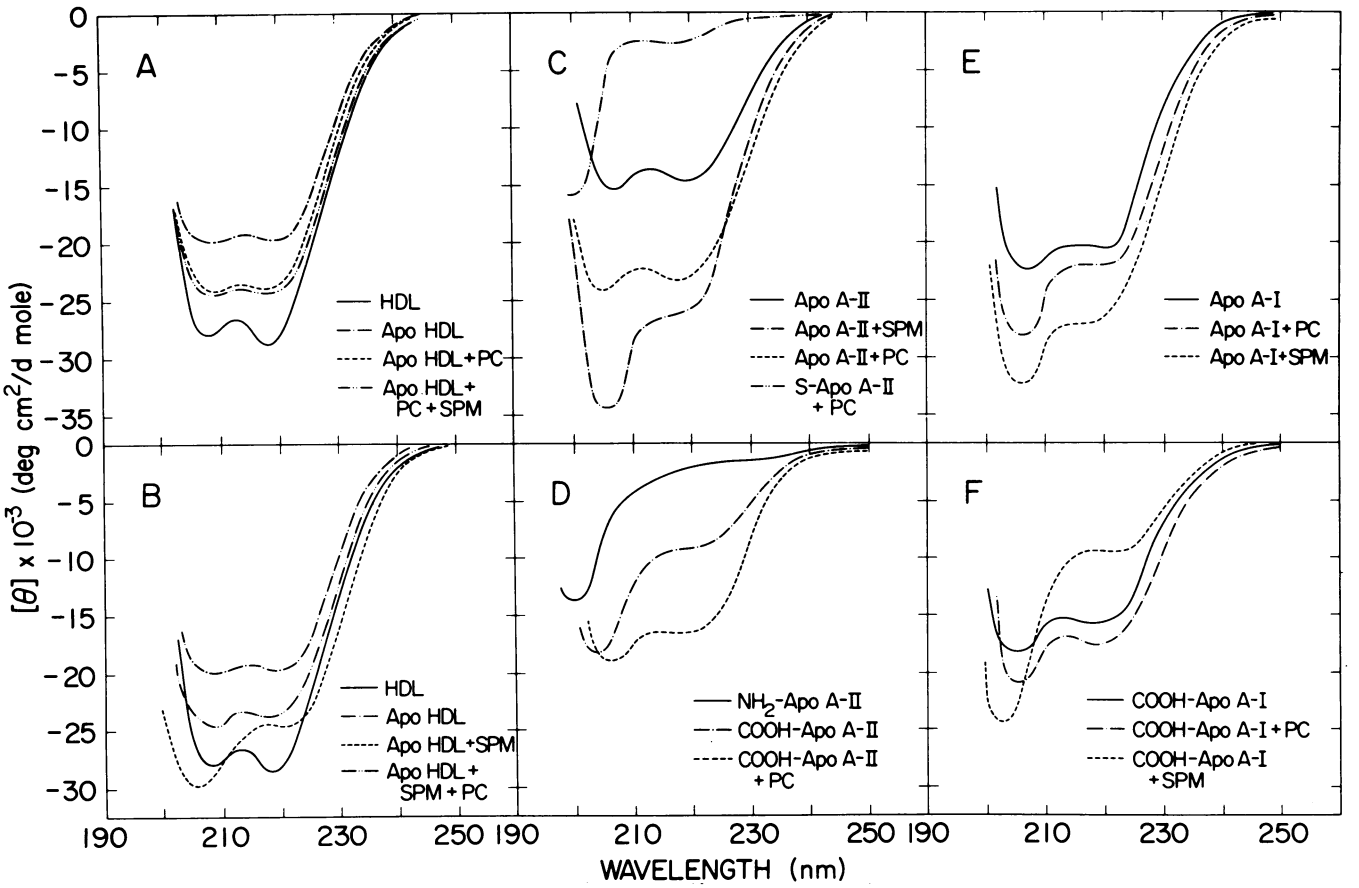

FIG. 2. Circular dichroism spectra of native and recombined HDL lipoproteins and peptide fragments. S-apoA-II = succinylated apoA-II; NH_2 and COOH = amino- and carboxyl- terminal CNBr peptides. The experimental values for [θ] have been multiplied by the indicated factor to obtain the numbers on the ordinates.

COOH-terminal CNBr peptide of apoA-I, and recombined apoprotein–lipid complexes are shown in Fig. 2. The far ultraviolet spectra of HDL, apoHDL, apoA-I, and apoA-II contain double troughs at 208 and 222 nm, characteristic of α helical proteins. These spectra are similar to those reported by other investigators (4, 13, 21, 41).

Delipidation of HDL results in about 20% loss of α helical structure. Relipidation of apoHDL with PC or SPM resulted in a partial restoration of helical structure. Addition of SPM to the PC–protein complex, and PC to the SPM–protein complex produced no significant further change in the CD spectra (Fig. 2*A* and *B*).

Purified apoA-II contained about 40% α helix (Fig. 2*C*). The helical content of apoA-II increased after recombination with both PC and SPM (Fig. 2*C*). A similar increase in ordered structure was observed after the recombination of the isolated COOH-terminal CNBr peptide of apoA-II with PC (Fig. 2*D*). The NH_2-terminal CNBr fragment of apoA-II did not recombine with lipid and the spectrum of the peptide resembled that of a random coil (Fig. 2*D*) (39).

Succinylation of apoA-II (S-apoA-II) produced a significant loss of ordered structure in the apoprotein. Recombination of S-apoA-II with PC did not produce the characteristic increase in α helix observed with apoA-II (Fig. 2*C*) and the binding efficiency of S-apoA-II was significantly reduced (Table 1).

ApoA-I contained a significant degree of helical structure (about 64%) (Fig. 2*E*), and its recombination with PC and SPM produced a further increase in helical conformation (Fig. 2*E*). The COOH-terminal CNBr peptide of apoA-I was about 50% helical in structure (Fig. 2*F*). This degree of helix would account for about 30% of the helical residues in the intact apoA-I. The contribution of the helical content of the COOH-terminal peptide of apoA-I to the intact apoprotein in these studies is significantly lower than the 82% reported (21). Recombination of the COOH-terminal CNBr peptide of apoA-I with PC resulted in an increase of ordered structure (Fig. 2*F*). Recombination of the isolated peptide with SPM was unusual, however, in that there was a decrease in the ellipticity at 222 nm, and an increase in ellipticity near 200 nm, indicating a loss of helical structure (Fig. 2*F*).

Changes in the CD spectra of the recombined lipid–protein complexes, therefore, indicate that there is an increase in ordered structure of the protein moiety after recombination with lipids. Whether this transformation in secondary structure reflects phospholipid binding or is induced by a more hydrophobic environment is as yet undetermined.

DISCUSSION

These experiments were designed to compare the phospholipid-binding properties of the individual HDL apolipoproteins under identical experimental conditions. This was facilitated by the finding that the formation of lipid–protein complexes was not dependent upon sonication of apolipoproteins in the presence of phospholipid, a method widely employed (2, 4). Instead, isolated apolipoproteins were solubilized in NH_4HCO_3 buffer, pH 8.8, and added to sonicated phospholipid dispersions.

ApoA-II was found to interact strongly with both PC and SPM. The particle sizes of the apoA-II–PC and apoA-II–SPM complexes, as determined by agarose gel chromatography, resembled those of native HDL. Reduction and carboxymethylation of the disulfide bridge of apoA-II did not significantly affect the lipid-binding capacity of the apoprotein. These results support recent observations that chemical modification of the sulfhydryl group using either a spin or fluorescence label did not affect the capacity of the protein to bind PC or the local environment at or near the cysteine residue at position 6 (23).

Cleavage of apoA-II with CNBr at Met 26 produced a 26-amino-acid NH_2-terminal fragment which did not bind PC, and a 51-amino-acid COOH-terminal peptide which recombined. The binding efficiency of the COOH-terminal fragment, however, was significantly less (molar ratio of lipid to protein 7/1) than that of the intact apoprotein (molar ratio of lipid to protein 23–30/1). Succinylation of the ϵ-NH_2 groups of apoA-II significantly reduced its binding capacity for PC. None of the isolated tryptic peptides of apoA-II recombined with PC.

Although it has been reported (21) that both apoA-I and its COOH-terminal CNBr peptide bind PC, quantitative data suitable for comparison with the lipid-binding capacity of other HDL peptides have not been provided. We now have established that the phospholipid binding of apoA-I is almost negligible compared with that of apoA-II. The small amounts of protein recovered in the ultracentrifugal fraction of density less than 1.25 g/ml may be due to protein–lipid aggregation as suggested by the high lipid-to-protein ratio and the gel chromatography elution volume (molecular weight about 1,500,000) of this material. ApoA-I was, however, incorporated into the lipid–protein complex when preincubated with apoA-II or when apoHDL was used in the recombination experiments. These results suggest that protein–protein interactions of apoA-I and apoA-II are of major importance for the integration of apoA-I into reassembled lipoproteins.

Preliminary studies of the interaction of lysolecithin with apoA-I and apoA-II have shown that both apolipoproteins interact with this lipid in micellar solution. The ability of apoA-I to interact with lysolecithin, but only to a limited extent with either lecithin or sphingomyelin, indicates that detailed studies will be required to determine the nature of binding of individual apolipoproteins with specific lipids.

Because of the short sonication times used in these studies, we must assume that the sonicated particles of PC and SPM in the incubation solution are heterogeneous with respect to size, molecular structure, and packing arrangements. The recombined lipid–protein particles, however, appear to be fairly homogeneous, judging from their small elution volume during agarose gel chromatography. Nevertheless, the question of whether the apolipoproteins interacted with the liposomes with or without disturbing their basic bilayer arrangement is as yet unanswered.

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