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Chemical Studies on the Structure of Human Serum High-Density Lipoprotein (HDL)

Photochemical Crosslinking of Azido-Labelled Lipids in HDL

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Summary: The lipid classes of native human serum high-density lipoprotein (HDL) were exchanged against phosphatidylcholine, sphingomyelin and cholesteryl ester species substituted with photosensitive fatty acyl residues and against 25-azido-27-norcholesterol. The photosensitive fatty acyl residues were 5- and 16-azidopalmitic, 12-azidooleic and 18-azidolinoleic acid, all labelled with high tritium radioactivity. The lipid exchange method previously described was used.

After UV irradiation and delipidation the apoproteins AI and AII, photocrosslinked with the radioactive lipids, were separated. The yield of covalently crosslinked lipid molecules amounted to 30% of the photosensitive lipid molecules incorporated into the HDL particle.

ApoAI and apoAII were labelled by each of the photosensitive lipid classes, integrated in the HDL particle although in very different stoichiometry. The regiospecific photochemical labelling of the lipopolypeptides AI and AII was established by cyanogen bromide cleavage and the separation of the four apoAI and two apoAII CNBr fragments. The analytical data prove the close steric relation and interaction of defined protein regions with hydrophobic regions of the lipid molecules by chemical means.

These results are discussed with respect of the HDL structure.

Chemische Untersuchungen über die Struktur des menschlichen Serum-High-Density-Lipoproteins (HDL)
Photochemische Quervernetzung von azidomarkierten Lipiden im HDL

Zusammenfassung: Die Lipidklassen des nativen High-Density-Lipoproteins aus menschlichem Serum (HDL) werden gegen Phosphatidylcholin-, Sphingomyelin- und Cholesterinester-Spezies, die mit photosensitiven Sonden tragenden Fettsäureresten substituiert sind, sowie 25-Azido-27-nor-

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Enzymes:
Lecithin-cholesterol acyltransferase, lecithin:cholesterol acyltransferase (EC 2.3.1.43); Phospholipase A_2, phosphatidate 2-acylhydrolase (EC 3.1.1.4).

Abbreviations:
HDL = human serum high density lipoprotein (d = 1.063 – 1.210 g × cm^{-3}); apoAI = apolipoprotein AI; apoAII = apolipoprotein AII; apoAII/2 = apoAII reductively carboxymethylated; apoHDL = high density apolipoprotein; PC = phosphatidylcholine; C = cholesterol; CE = cholesterylester; N_3 = azido.

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cholesterin ausgetauscht. Die photosensitiven Fettsäurereste waren 5- und 16-Azidopalmitin-, 12-Azidoöl- und 18-Azidolinolsäure, alle mit hoher Tritium-Radioaktivität markiert. Die früher beschriebene Lipid Austausch-Methode wurde angewendet.

Nach Bestrahlung in langwelligem UV-Licht und Delipidierung wurden die mit den radioaktiven Lipiden quervernetzten Apoproteine AI und AII präparativ getrennt. Die Ausbeute der kovalent gebundenen Lipidmoleküle betrug 30% der in die HDL-Partikel durch Austausch eingeführten photochemischen Sonden.

ApoAI und apoAII wurden durch alle verwendeten photosensitiven Lipidklassen, wenn auch mit sehr verschiedener Stöchiometrie, markiert. Durch Bromcyanspaltung der beiden Lipopolypeptide AI und AII und Trennung der vier Fragmente des apoAI und zwei Fragmente des apoAII wurde die Regiospezifität der photochemischen Markierung bestimmt. Damit wurde räumliche Nachbarschaft und Wechselwirkung von bestimmten Proteinregionen und hydrophoben Ketten der Lipide chemisch bewiesen. Diese Befunde werden im Hinblick auf die HDL-Struktur diskutiert.

Key words: Exchange of HDL lipid classes against photosensitive species, photochemical reaction and yield, separation of crosslinked lipopolypeptides, CNBr fragmentation and distribution of crosslinks.

Human serum high-density lipoprotein (HDL) is a major lipoprotein class, characterized by its spherical shape of 80–120 Å diameter, d = 1.063–1.21 g x cm^{-3}, characteristic apoprotein /lipid mass ratio of about 1 and composition, apoprotein AI with molecular mass of 28 kDa and 243 amino acid residues[1] and apoprotein AII with 18 kDa and 154 amino acid residues[2]. Apoproteins CI–CIII are present in minute amounts. The apopolypeptides are associated with phospholipids and cholesterol, and the cholesteryl esters are believed to form the hydrophobic nucleus of the HDL particle. Chemical evidence has been presented for the surface location of the apoproteins[5–7].

^{31}P- and ^{13}C-NMR spectroscopy suggested that all polar head groups of phospholipids are oriented as a monolayer to the aqueous interphase and that hydrophobic interactions of their fatty acyl chains interact with the apoproteins[5–7].

The model of HDL derived from these data and from similar studies with artificial lipoprotein complexes (apoAI-phosphatidylcholine, lysophosphatidylcholine and sphingomyelin recombinants[5,6]) represents a strong structural basis for the important function of HDL regarding cholesterol uptake and esterification by the serum enzyme lecithin-cholesterol acyltransferase[7] and the lipid transport in general.

Besides our NMR studies and the chemical approach with bifunctional reagents such as dimethyl suberimidate and dimethyl 4,4′-dithiobisbutyrimidate[4], we have attempted to demonstrate by chemical means the lipid-protein interactions. Photoactivatable lipids with an azido group in different positions of the hydrophobic portions of lysophosphatidylcholines, phosphatidylcholines, sphingomyelins, 27-norcholesterol and cholesteryl esters, which on UV irradiation form highly reactive nitrenes, were synthesized as compounds with high specific radioactivity. In previous communications[8,9] we have given evidence for the crosslinking of nitrenes from azido fatty acids in lysophosphatidylcholine-apoAI micelles and artificial lipoprotein complexes between apoAI and photoactivatable phosphatidylcholines.

These photoactivatable probes have been exchanged against the respective lipid classes of native HDL particles by the cholate procedure described previously[10]. The separation of the labelled crosslinked apoproteins and their degradation by cyanogen bromide cleavage strongly support the asymmetric labelling of the CNBr fragments of apoAI and AII. These data will be discussed with regard to the HDL structure.

Materials and Methods

The syntheses of 5-azido-[8,9-$^3H_2$]- and 16-azido-[9,10-$^3H_2$]palmitic, 12-azido-[9,10-$^3H_2$]oleic and 18-azido-[9,10,12,13-$^3H_4$]linoleic acids with specific activity of 100 Ci/mol and improved syntheses of phosphatidylchol-

ine and sphingomyelin species, of cholesteryl esters and esters of 25-azido-[25-^{3}H]-27-norcholesterol have been described in preceding communications[11–13].

The HDL fraction of human serum was isolated by the sedimentation-flotation technique in the ultracentrifuge between density 1.063 and 1.210 g × cm^{-3}[14–16]. Apoproteins were obtained after delipidation[17] and separated by DEAE-cellulose chromatography in 8M urea and an increasing Tris buffer gradient[18]. Apo AI and AII proved to be homogenous in dodecyl sulfate polyacrylamide gel electrophoresis[19] and urea polyacrylamide gel electrophoresis[20].

Lipid-exchange procedure

Native HDL and a micellar solution of sodium cholate, azido-labelled phosphatidylcholine, sphingomyelin, cholesterol and cholesteryl ester in molar ratios of 230:80:16:24:53 were incubated as described before [10]. Cholate was separated by Sephadex G-25 and the HDL fraction by Bio-Gel A-5m chromatography in a yield of 60–80%.

The amount of micellar lipids was equal to that of the HDL. 44% of phosphatidylcholine, 58% of cholesterol and 15% of cholesteryl esters were exchanged when the equilibrium was reached after about 6 h. All lipoproteins appeared as spherical particles in electronmicroscopy after negative staining (buffered 2% phosphotungstic acid). A Philips EM 300 was used at 80 kV. Standard procedures were applied to the quantitative determination of phosphate[21], total cholesterol[22] and protein[23].

Measurement of circular dichroism

Circular dichroism was measured in a Durrum-Jasco J-41 A spectropolarimeter. The samples were dissolved either in water or 1mM Tris, 1mM EDTA, 0.15mM KF. Molar ellipticity θ was calculated according to

$$\theta = \psi \times MRW \times 10^{-2} \quad [deg \times cm^2 \times dmol^{-1}]$$
$$\psi = \theta/(c \times cl)$$

c = protein concentration (g × cm^{-3}), l = light path (in dm), MRW (mean residue weight) apo AI 116.5; apo AII 112.1; apo HDL 114.7.

UV irradiation

UV irradiation of azido-lipid-containing particles was carried out in Sovirel tubes, carefully degassed and oxygenfree with a Philips HPK 125 W lamp at 4 °C. The Pyrex glass serves as a cut-off filter between 300 and 310 nm.

Fluorescence spectroscopy

Fluorescence emission spectra were recorded with the spectrofluorimeter model MPF-3, Perkin Elmer, excitation wavelength 285 nm.

Irradiated lipoproteins were delipidated by Sephadex LH-20 chromatography with solvent system 2-butanol/acetic acid/water 4:1:5[24].

Cyanogen bromide cleavage[1] and *reductive carboxymethylation* was carried out as described[25].

Phospholipase A_2 hydrolysis

10 mg photocrosslinked apoprotein was treated with 0.1 mg protease-free phospholipase A_2 (Boehringer, Mannheim) at room temperature for 15 h in 2 ml buffer (0.02M histidine, 0.1M KCl, 5mM $CaCl_2$, pH 7.0). 2 ml 2-butanol/acetic acid 4:1 was added and the protein delipidated on a Sephadex LH-20 column (120 × 2.5 cm).

Results

HDL-lipid exchange

The photosensitive lipid probes were incorporated into native human serum high-density lipoprotein by the lipid exchange method elaborated in this laboratory[10]. Phosphatidylcholine, sphingomyelin, cholesterol and cholesteryl ester, one of which was radio- and azido-labelled, were present in cholate micelles in the ratio of these lipids present in HDL (molar ratios of 80:16:24:53). The equilibrated HDL particles were separated from cholate (Sephadex G-25) and excess lipid (Bio-Gel A-5m) as described before[10], Fig. 1.

Table 1 summarizes the stoichiometry of HDL. The figures in brackets indicate the number of azido-labelled lipid molecules. Table 1 indicates that 44% of phosphatidylcholine in HDL was exchanged (7 mol N_3-PC in HDL vs. 16 mol N_3-PC in micellar solution), 58% of cholesterol (14 vs. 24) and 15% of cholesteryl ester (2 vs. 13).

Photocrosslinking reaction

The HDL preparations containing the ^{3}H-labelled photosensitive lipid probes and in addition soya phosphatidyl-[*N*-$^{14}CH_3$]choline to follow the delipidation procedures, were irradiated under the conditions described before[9] for 20 min; 25-azido-27-norcholesterol-containing samples were irradiated for 60 min. Delipidation with chloroform/methanol/ether 2:1:1.5 and by Sephadex LH-20 chromatography[24] had the same result. (More than 98% of ^{14}C-labelled phosphatidylcholine and cholesterol and cholesteryl esters were extracted.) In sodium dodecyl sulfate gel electrophoresis of the delipidated apoproteins no front activity, indicative for non-covalently bound lipid, was detectable. The radioactivity of the

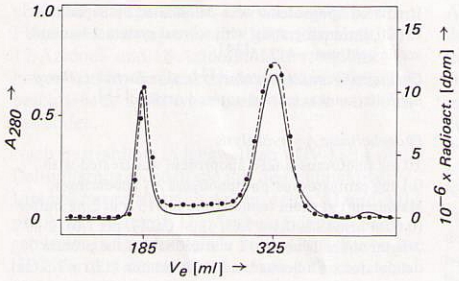


Fig. 1. Separation of lipid vesicles from HDL, in which phosphatidylcholine has been exchanged against 1-acyl-2-(12-azido-[9,10-$^3H_2$]oleoyl)-glycero-3-phosphocholine by Bio-Gel A-5 m column chromatography (2 x 80 cm), Tris/EDTA buffer, pH 8.2
Dotted line: ^{3}H-radioactivity. Peak at V_e = 185 ml excess lipid, V_e = 325 ml HDL-particles. The cholate had been removed before by Sephadex G-25 chromatography.

Table 1. Stoichiometry of lipid-exchanged HDL.
All figures indicate mol of cholate, phosphatidylcholine (PC) or 1-acyl-2-(12-azidooleoyl)glycero-3-phosphocholine (N_3-PC), cholesterol (C) or 25-azido-27-norcholesterol (N_3-C), cholesteryl esters (CE) or cholester-3β-yl 12-azido-oleate (N_3-CE) in micellar solution per mol apoHDL (100 kDa; 2 apoAI + 1 apoAII). The figures in brackets indicate the number of azido-labelled lipid molecules.

In micellar solution				In HDL particles	
Cholate	PC or N_3-PC	C or N_3-C	CE or N_3-CE	Phospholipid N_3-PC	Cholesterol N_3-C, N_3-CE
230	80	24	53 (13)	89	87 (2) [N_3-CE]
230	80	24 (24)	53	93	88 (14) [N_3-CE]
230	80 (16)	24	53	91 (7)	92

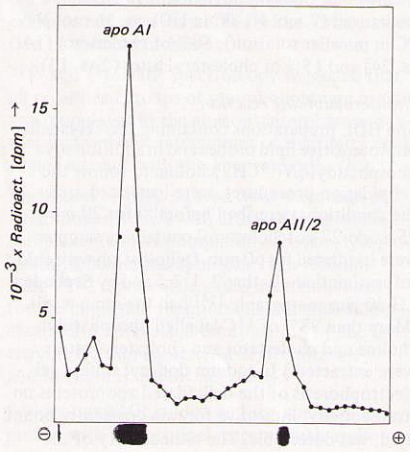


Fig. 2. Dodecyl sulfate polyacrylamide (15%) gel electrophoresis of HDL photocrosslinked with 1-acyl-2-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine. The sample was delipidated and reductively carboxymethylated under standard procedures. The gel was sliced and after digestion the distribution of the radioactivity in the gel determined.

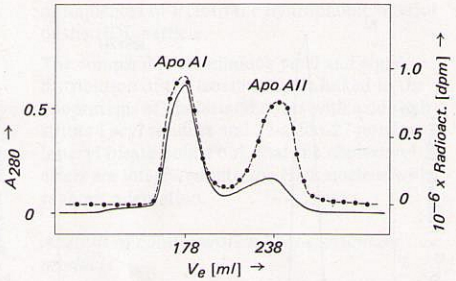


Fig. 3. Separation of apoAI-PC and apoAII-PC lipopolypeptides by Sephadex G-150 chromatography (2 x 120 cm).
Solvent: Tris·HCl pH 8.4, 0.5% sodium dodecyl sulfate, 8M urea. Dotted line: ^{3}H-radioactivity. ApoAI eluted at V_e = 178 ml; apoAII at 238 ml.

Table 2. Stoichiometry of azido-labelled lipid classes and apoproteins in HDL after lipid exchange, photoactivation and delipidation.

Azidolipid	Lipid/ apoHDL after exchange	Lipid/ apoHDL after delipidation	Crosslinks [%]	Lipid/ apoAI *(a)*	Lipid/ apoAII *(b)*	*a/b*
Phosphatidyl-choline						
12-N_3-18:1	30.0:1	5.7:1	19	1.34:1	1.43:1	0.94
	10.0:1	2.4:1	24	0.54:1	0.54:1	1.00
	7.4:1	1.4:1	19	0.38:1	0.40:1	0.95
	3.8:1	0.8:1	21	0.24:1	0.25:1	0.96
18-N_3-18:2	8.0:1	1.6:1	20	0.34:1	0.24:1	1.42
	8.3:1	1.6:1	19	0.36:1	0.26:1	1.39
5-N_3-16:0	12.8:1	1.1:1	9	0.26:1	0.24:1	1.08
	10.8:1	0.8:1	7	0.23:1	0.20:1	1.15
16-N_3-16:0	6.7:1	1.2:1	18	0.37:1	0.24:1	1.54
	7.4:1	1.2:1	16	0.33:1	0.23:1	1.43
Sphingomyelin						
12-N_3-18:1	7.9:1	1.3:1	16	0.28:1	0.30:1	0.93
	6.0:1	0.7:1	12	0.17:1	0.19:1	0.89
18-N_3-18:2	7.5:1	0.8:1	11	0.21:1	0.24:1	0.88
	7.3:1	0.6:1	8	0.15:1	0.16:1	0.94
16-N_3-16:0	12.0:1	1.4:1	12	0.35:1	0.38:1	0.92
	12.0:1	1.3:1	11	0.35:1	0.36:1	0.97
25-N_3-27-Nor-cholesterol	14.2:1	1.4:1	10	0.41:1	0.27:1	1.52
	14.1:1	1.2:1	9	0.33:1	0.25:1	1.32

crosslinked lipid coincided with the two bands assigned to apoAI and apoAII/2, Fig. 2. The two lipopolypeptides were completely separated by Sephadex G-150 chromatography in sodium dodecyl sulfate (0.5%), urea (8M)/Tris buffer, pH 8.5, Fig. 3. The two apoproteins showed no cross contamination, Fig. 4a, b.

Table 2 summarizes the analytical data of photocrosslinking experiments, in which different photosensitive phosphatidylcholine and sphingomyelin species and cholesteryl esters have been exchanged with native HDL lipids. The stoichiometry of the HDL components after the lipid exchange is given together with the stoichiometry of covalently

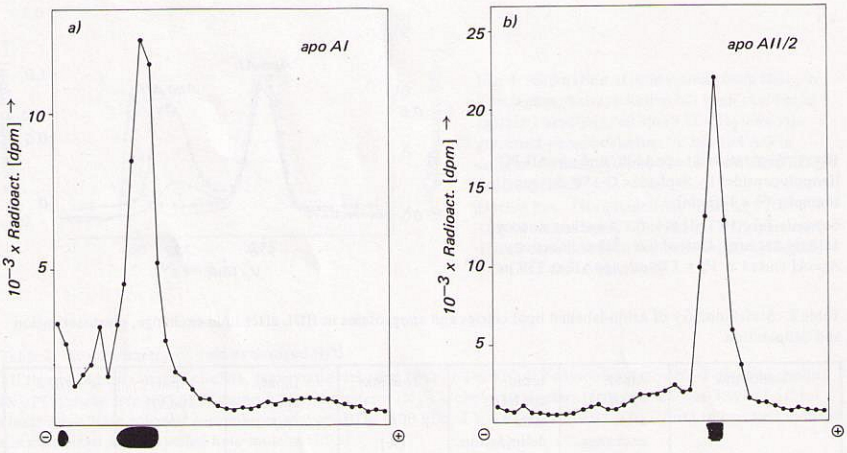


Fig. 4. Dodecyl sulfate polyacrylamide (15%) gel electrophoresis and distribution of ^{3}H-radioactivity in gels a) of apoAI, b) apoAII/2 photocrosslinked in HDL particles with 1-acyl-2-(12-azido-[9,10-$^{3}H_2$]oleoyl)glycero-3-phosphocholine.

The procedure was the same as in Fig. 2.

photocrosslinked lipids per apoHDL, apoAI and apoAII after delipidation, the crosslinking yield and finally the ratio of lipid molecules linked to apoAI and apoAII as an indicator for the preferential binding.

The following conclusions can be drawn:

a) 1-Acyl-2-(12-azidooleoyl)glycero-3-phosphocholine in mixed lipid cholate micelles can substitute the lipid of the native HDL particles to 40 to 50%.

b) On the average about 20% of the azido-labelled lipids were crosslinked by photoactivation. The absolute amount of covalently linked azido lipids increases linearly with the amount of azido lipid incorporated by exchange into HDL particles.

c) A comparison of two phosphatidylcholine species differing in the position of the photoactivatable azido function, 1-acyl-2-(5-azidopalmitoyl)- and 1-acyl-2-(16-azidopalmitoyl)-glycero-3-phosphocholine, points out that the phosphatidylcholine with the nitrene in the terminal position of the fatty acyl chain crosslinks to apoAI on a molar basis 40% more than to apoAII. This is also seen with 1-acyl-2-(18-azidolinoleoyl)glycero-3-phosphocholine. 1-Acyl-2-(5-azidopalmitoyl)glycero-3-phosphocholine under these conditions binds only 8% to apoHDL, two and a half times less than the species previously mentioned.

d) The degree of unsaturation of the photoactivatable fatty acid in phosphatidylcholine is without any influence on the crosslinking rate and yield. Therefore apoAI is better accessible for the reactive nitrene groups in the interior of the HDL lipid monolayer shell. On the other hand 1-acyl-2-(5-azido) and 1-acyl-2-(16-azidopalmitoyl)glycero-3-phosphocholine do not differ in the extent of crosslinking to apoAI and apoAII. The two apoproteins are labelled in the same yield.

e) 25-Azido-27-norcholesterol mimicks cholesterol very truly[11]. The nitrene generating azido group is emburried in the hydrophobic region 17 Å distant from the 3-hydroxy group. Therefore it is not surprising that the distribution of the photo

crosslinked norcholesterol in apoAI and apoAII is similar to the terminal-labelled phosphatidylcholine e.g. 1-acyl-2-(18-azidolinoleoyl)glycero-3-phosphocholine.

f) Cholesteryl esters with azido-substituted fatty acyl residues (12-N_3-18:1, 18-N_3-18:2 or 16-N_3-16:0) or the oleic acid ester of 25-azido-27-norcholesterol were incorporated into the native HDL particles. After photocrosslinking, twice the amount of the esters were covalently attached to apoAI as compared to apoAII. The crosslinking yield was reduced to 50% of that of the phosphatidylcholines. The enhanced binding of cholesteryl esters to apoAI is in agreement with the favoured crosslinks between ω-substituted acyl residues of phosphatidylcholines, indicating the deeper penetration of the polypeptide chain or sequences of it, into the hydrophobic interior of the HDL particle.

The comparable crosslinking yield and equal distribution of the labelling lipids linked to the apoproteins of cholesteryl esters with azido-substituted acyl residues and 25-azido-27-norcholesteryl oleate points out, that the cholesteryl esters are integrated into the HDL nucleus with random orientation.

Analysis of apolipoprotein-lipid-crosslinking products

The lipopolypeptides of HDL, thoroughly delipidated, were separated by Sephadex G-150 chromatography in Tris/sodium dodecyl sulfate buffer and obtained in homogenous form, Fig. 3 and 4. Next the apolipopolypeptide AI was cleaved at

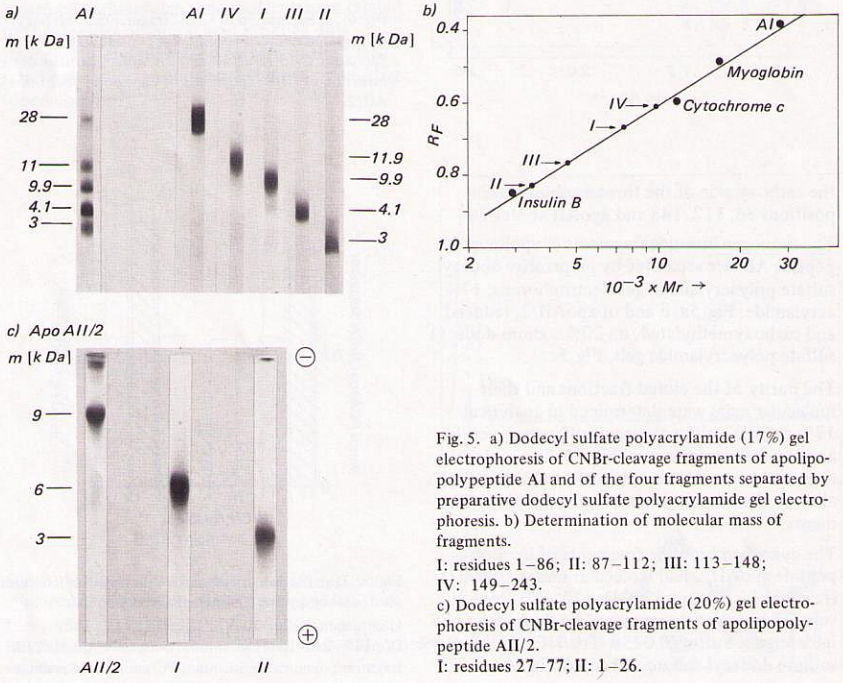


Fig. 5. a) Dodecyl sulfate polyacrylamide (17%) gel electrophoresis of CNBr-cleavage fragments of apolipopolypeptide AI and of the four fragments separated by preparative dodecyl sulfate polyacrylamide gel electrophoresis. b) Determination of molecular mass of fragments.
I: residues 1–86; II: 87–112; III: 113–148; IV: 149–243.
c) Dodecyl sulfate polyacrylamide (20%) gel electrophoresis of CNBr-cleavage fragments of apolipopolypeptide AII/2.
I: residues 27–77; II: 1–26.

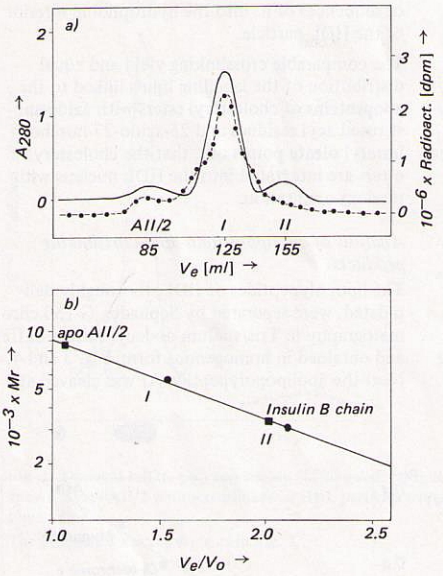


Fig. 6. a) Separation of CNBr fragments of carboxymethylated reduced apolipopolypeptide AII/2 by Sephadex G-75 (2 x 140 cm). b) Molecular mass determination of CNBr fragments of apolipopolypeptide AII/2.

the carboxy side of the three methionines in positions 86, 112, 148 and apoAII at Met 26.

The cyanogen bromide fragments of apolipopolypeptide AI were separated by preparative dodecyl sulfate polyacrylamide gel electrophoresis, 17% acrylamide, Fig. 5a, b and of apoAII/2, reduced and carboxymethylated, on 20% sodium dodecyl sulfate polyacrylamide gels, Fig. 5c.

The purity of the eluted fractions and their molecular mass were determined in analytical 17% or 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 5). The molecular mass and amino acid analysis after acid hydrolysis allowed the unambiguous assignment of the fragments.

The cyanogen bromide fragments of lipopolypeptide apoAII/2 had molecular mass of 3000 Da (residues 1–26) and 5700 Da (27–77) respectively. They were also separated on Sephadex G-75 in detergent buffer (0.025M Tris/HCl pH 8.4, 0.5% sodium dodecyl sulfate, 8M urea), Fig. 6.

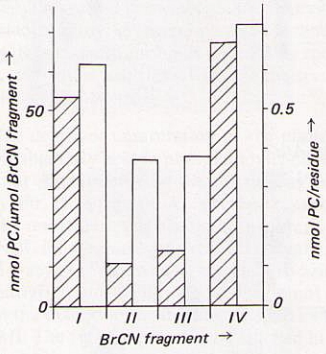


Fig. 7. Distribution of radioactive phosphatidylcholines photocrosslinked to CNBr fragments of apoAI.
I: sequence 1–86; II: 87–112; III: 113–148; IV: 149–243. Hatched columns: nmol PC/μmol CNBr fragment; open columns: nmol PC/amino acid residue.

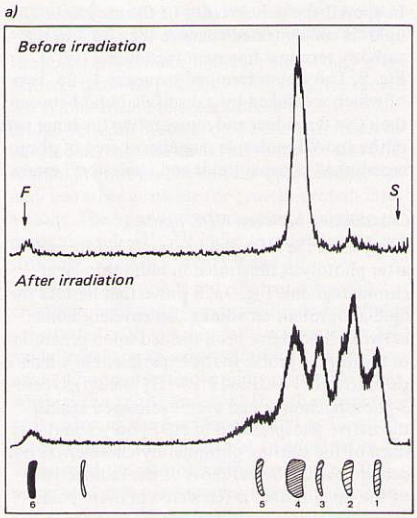
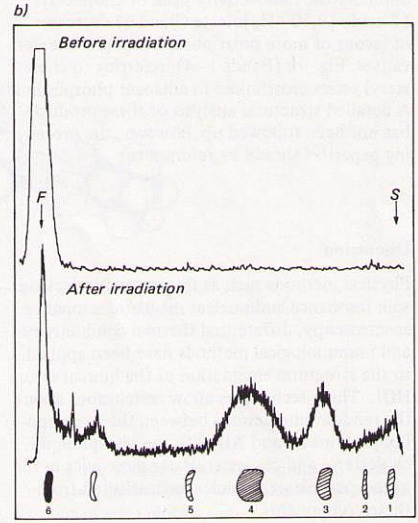


Fig. 9. Radio thin-layer chromatographic analysis of lipid extracts from HDL before (starting material) and after irradiation of a) HDL with 1-acyl-2-(12-azido-[9, 10-$^3H_2$]oleoyl) glycero-3-phosphocholine exchanged against native phosphatidylcholine, b) HDL with exchanged cholesteryl 12-azido-[9,10-$^3H_2$]oleate.
6: Cholesteryl 12-azido-[9,10-$^3H_2$]oleate; 5: undefined: 4: phosphatidylcholine; 1–3: more polar derivatives. Hatched areas: phosphate-positive compounds.
S = start, F = front.

The distribution of the radioactive crosslinked phosphatidylcholines among the CNBr fragments of apoAI is summarized in Fig. 7. CNBr-I (residues 1–86) and CNBr-IV (residues 149–243) are labelled four to five times more than CNBr fragments II and III, which represent the internal sequences 87–112 and 113–148 (dashed columns). If, however, the number of covalently linked phosphatidylcholine molecules are related to the number of amino acid residues, this strongly differing labelling pattern of the CNBr fragments, is less pronounced though convincing (open columns).



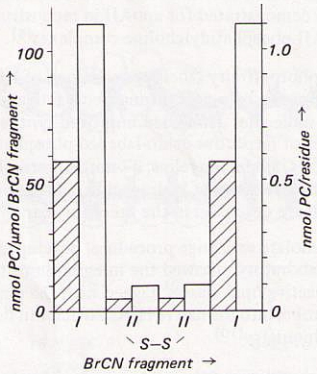


Fig. 8. Distribution of radioactive phosphatidylcholines photocrosslinked to CNBr fragments of apoAII.
I: sequence 27–77; II: 1–26. Hatched column: nmol PC/μmol CNBr fragment; open column: nmol PC/amino acid residue.

In apoAII the radioactivity of the crosslinked lipids is concentrated to more than 90% in the carboxy terminal fragment (residues 27–77), Fig. 8. The amino terminal sequence 1–26, two of which are linked by a disulfide bond between their Cys-6 residues and representing the inner part of the apoAII molecule, are almost free of photocrosslinked phospholipids and cholesteryl esters.

Crosslinking between HDL lipids

Analysis of the HDL lipid extracts before and after photolysis illustrated in radio thin-layer chromatograms, Fig. 9a, b prove that besides the lipid-apoprotein crosslinks also covalent bonds between lipids have been formed upon generation of the nitrene probe. In the experiment in which 1-palmitoyl-2-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine had been exchanged against the native phospholipid of HDL, only about one third of the starting phosphatidylcholine was not decomposed, whereas most of the radioactivity of the lipid extract is recovered in more polar products. Dimerization of two phospholipid molecules via an amino bridge, derived from an insertion reaction, would result in such more polar compounds.

Similarly the radioactivity peak of cholesteryl 12-azido-[9,10-$^3H_2$]oleate (Band 6) decreases in favour of more polar phosphorus-positive derivatives, Fig. 9b (Bands 1–4), referring to cholesteryl esters crosslinked to adjacent phospholipids. A detailed structural analysis of these products has not been followed up; however, the preceding paper[12] should be referred to.

Discussion

Physical methods such as fluorescence, electron spin resonance and nuclear magnetic resonance spectroscopy, differential thermal conductivity and immunological methods have been applied to the structural elucidation of the human serum HDL. These techniques allow conclusions about the random interactions between the main apolipoproteins AI and AII with the phospholipid, cholesterol and cholesterylester molecules in the native particle or particles reconstituted from these components.

We have recently introduced two chemical concepts, new for the elucidation of the lipoprotein structures:

A chemical probe, consisting of a photosensitive azido group in a distinct position of a phospholipid fatty acyl chain, the side chain of 27-norcholesterol or fatty acyl residue of cholesteryl esters and sphingomyelin, will generate the highly reactive nitrene which rapidly leads to crosslinking with its nearest neighbouring lipid or polypeptide molecule. Since we label the photosensitive molecules with high specific radioactivity, these crosslinking products are prone to further analysis. Besides this method, which gives the chemical proof for lipid-protein interaction and adds to our topochemical understanding of lipid-protein complexes of HDL but also of membranes[26], we have elaborated the use of bifunctional crosslinkers of the imidoester type, which specifically bridge lysine side chains at distinct distances (11 Å by dimethyl suberimidate, 13–16 Å by dimethyl dithiobisbutyrimidate). The analysis of the peptides surrounding the attachment point of the bifunctional crosslinker, allows the assignment to the established primary structure and further to reconstruct the topography of the peptide sequences of apoproteins, provided that they are surface located. This has been demonstrated for apoAII in reconstructed apoAII-phosphatidylcholine complexes[3].

The photoaffinity labelling technique of HDL apoproteins by neighbouring photoactivatable lipid molecules demanded improved synthetic routes of respective azido-labelled phosphatidylcholines, sphingomyelins, 27-norcholesterol and cholesteryl esters of high specific radioactivity, which are described in the preceding paper[12].

The cholate exchange procedure, developed in this laboratory, allowed the integration of the radioactive lipid classes, tagged with the chemical probe, into the native HDL particles in defined stoichiometry[10].

The photolysis of the azido group yielded the crosslinking of the respective lipid molecule with the neighbouring lipid molecules or the peptide sequence of the apolipoproteins, similar to previous reports from this laboratory on model lipoprotein complexes[8,9].

Contrary to reports of other authors[27–30], photolysis under our experimental conditions yielded covalent linkages between the photosensitive lipids and the apoproteins in 10–20% of the integrated probe molecules and in addition the efficient crosslinks between lipid molecules (app. 40–60%), Fig. 9a, b.

This communication preferentially reports on the analysis of the lipid-apoprotein crosslinks and their distribution in the polypeptide chains of apoAI and AII. The two main apolipoproteins, which after irradiation of the HDL particle were labelled by radioactive covalently bound lipids, were separated and purified to homogeneity as demonstrated by dodecyl sulfate polyacrylamide gel electrophoresis. ApoAI with methionine residues in positions 86, 112, 148 and apo AII in position 26, were cleaved with cyanogen bromide. The CNBr fragments could be separated by preparative polyacrylamide gel electrophoresis, Fig. 5, 6.

The distribution of the covalently bound lipid molecules per amino acid residue and per CNBr fragment is delineated in Fig. 7 and 8 in molar stoichiometry. Both presentations indicate a distinct preference of the CNBr fragments at the amino and carboxy terminus of apoAI for the interaction with the phosphatidylcholine molecules.

In apoAII crosslinking lipids are linked only in the carboxy terminus (residues 26–77). Since apoAII is a symmetrical molecule, linked by a disulfide bridge between cysteins 6 of each half, it can be concluded, that the inner parts with the two NH_2-termini are not involved in the lipid-binding but may be rather available for protein-protein interactions. The apoAII conformation according to predictive rules[31–33] indicate extended β sheet structure between residues 9 and 18.

Table 2 clearly points out, that an enhanced crosslinking of apoAI occurs with phospholipids, substituted with ω-azido fatty acids such as 18-azidolinoleic or 16-azidopalmitic acid and 25-azido-27-norcholesterol and its respective esters, whereas the apoAI and apoAII with phospholipids, carrying the 5-azidopalmitoyl or 12-azidooleoyl residues are labelled at an equal ratio. The distance of the photosensitive group from the polar head group is apparent from Fig. 10. The azido group in the ω-position of fatty acyl chains in phosphatidylcholine and in the 25-position of 27-norcholesterol probe the same hydrophobic region. The different labelling pattern of the two apoproteins but also the peptide sequences of

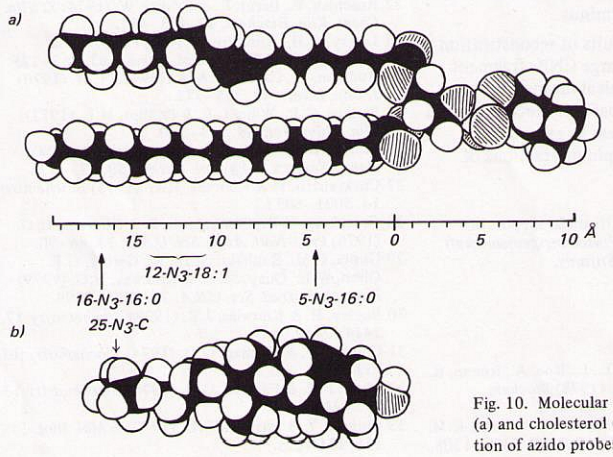


Fig. 10. Molecular model of phosphatidylcholine (a) and cholesterol (b) with indications of localization of azido probes.

the same apoprotein by these chemical rulers therefore point out, that the peptide sequences interact more or less distant to the hydrophilic shell. It is suggested that the apoproteins AI and AII are not uniformly expanded on the surface of the HDL particle, but the sequences mentioned before are folded deeper towards the hydrophobic core of the spherical HDL particle.

The more pronounced labelling of apoAI by photosensitive cholesteryl esters (Table 2) integrated into the HDL particle, is also indicative for a broader exposition of this apoprotein toward the nucleus, which is suggested to store the cholesteryl esters formed in the lecithin-cholesterol acyltransferase reaction. Immunological studies have revealed a reduced accessibility of apoAI antigenic sites in the HDL particle for apoAI antibodies[34–36]. Although this observation is disputable in view of the large antibodies covering many antigenic sites and thus simulate reduced antigen-antibody interaction, they are in agreement with the chemical evidence which makes a completely extended surface location of apoAI unlikely.

On the other hand, apoAII has been shown to be organized in the surface of lipoprotein complexes formed by reconstitution between apoAII and phosphatidylcholine molecules[3,4]. The main interaction with the lipid components of native HDL occurs between the sequence between residue 26 and the carboxy terminus.

Our studies support the results of reconstitution experiments between this large CNBr fragment and phospholipids by chemical means[37]. Further studies with this photoaffinity technique and other methods are in progress to extend our knowledge about protein-lipid interactions of apoAI and AII sequences.

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