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Covalent Binding of Photosensitive 1-(12-Azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine (Lysolecithin) to Human Serum High Density Apolipoproteins

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Summary: Human serum high density apoproteins were complexed with increasing concentrations of 1-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine up to the saturation concentration (72 mol lysolecithin per mol apo HDL). Ultraviolet irradiation generated the nitrene which led to crosslinking with the two main apolipoproteins AI and AII. Methods are described for the removal of excess, unbound lipid and the column chromatographic separation of the lipopolypeptides AI and AII.

Kovalente Bindung von photosensitivem 1-(12-Azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholin (Lysolecithin) an die High-Density-Apolipoproteine des menschlichen Serums

Zusammenfassung: Menschliche Serum-High-Density-Apolipoproteine wurden mit 1-(12-Azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholin in steigenden Konzentrationen bis zur Sättigung (72 mol Lysolecithin pro mol apoHDL) komplexiert. Ultraviolette Bestrahlung schuf Nitrene aus der Azidogruppe, die zur Quervernetzung des Lysolecithins mit den beiden Haupt-Apoproteinen AI und AII führte. Es werden Methoden zur Entfernung überschüssigen und nicht kovalent gebundenen Lipids sowie die säulenchromatographische Trennung der Lipopolypeptide AI und AII beschrieben.

Key words: Radioactive 1-(12-azidooleoyl)glycero-3-phosphocholine-apo HDL, azidolysolecithin complexes, photoactivation, separation of covalently crosslinked lysolecithin-apo AI and -AII, analysis of lipopolypeptides.

Previous ^{13}C-NMR spin lattice relaxation time studies in this laboratory have demonstrated that lysolecithin binds to the main apoprotein AI of human serum high density lipoprotein

Enzyme:
Phospholipase A_2, phosphatide 2-acylhydrolase (EC 3.1.1.4).

Abbreviations:
Apo AI and AII = apoproteins AI and AII of human serum high density lipoprotein; HDL = high density lipoprotein; lysolecithin = 1-acylglycero-3-phosphocholine.

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(HDL) with the monoacylgroup but not with the zwitterionic polar head group[1]. This monoacylphospholipid, like the anionic detergent sodium dodecyl sulfate and the cationic detergent tetradecyltrimethylammonium bromide[2,3] binds to the apoproteins and prohibits their strong aggregation due to protein interactions. The saturation concentration reaches about 70 molecules lysolecithin per apo HDL ($M_r \approx 100000$).

The reconstitution method described before[4,5] utilizes saturated lysolecithin, not only for the disaggregation, but it is assumed that the monoacyl phospholipid is substituted by the more hydrophobic diacylphospholipid which, in addition, allows lipid-lipid interactions and therefore the formation of larger lipoprotein complexes.

The ^{13}C-NMR approach to the study of lipid-protein interactions has been recently extended by a chemical method aiming at a more detailed insight into lipid or apoprotein environment.

Phospholipids substituted with photosensitive 16-azidopalmitic, 12-azidooleic and 18-azidolinoleic acid of high specific radioactivity were synthesized and introduced in reconstitution experiments[6]. Due to the short life time of the nitrenes generated from the azido group upon UV irradiation, crosslinks to the nearest neighbouring lipid or protein molecules occurs.

In this report, chemical proof is given for the hydrophobic interaction of the fatty acyl chain of 1-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine with apo AI and apo AII of HDL.

Methods are described to overcome separation problems of photocrosslinked lipid-apoproteins such as the separation of excess lipids from the lipopolypeptides and the separation of the latter, which is required for their chemical characterization. Furthermore, analytical data on lysolecithin-apo AI and lysolecithin-apo AII are reported.

Materials and Methods

1-(12-Azido-[9,12-$^3H_2$]oleoyl)glycero-3-phosphocholine, spec. act. 1.5 Ci/mol, was prepared by phospholipase A_2 (Boehringer Mannheim GmbH) hydrolysis from 1,2-di-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine.

Human serum high density lipoprotein (HDL) was isolated by the sedimentation and flotation centrifugation method[7]. Apo HDL was obtained after delipidation with chloroform/methanol[8].

Apo-HDL-lysolecithin complexes

10 mg apo HDL was stirred with different molar amounts of photosensitive lysolecithin (apo HDL, M_r 100000): 1-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine 1:18; 1:36; 1:54 and 1:72) (see Table 1) in 2 ml Tris buffer, pH 8.2, as described by Rosseneu et al.[9].

The apoprotein-lysolecithin micellar solution was irradiated for 40 min at 4 °C with an HPK 125 W lamp in a gas-tight Sovirel glass tube, thoroughly flushed with argon gas to make the solution oxygen free. The Sovirel glass served as cut-off filter at 310 nm.

Samples were taken before and after photolysis for circular dichroism measurements with a Jasco-41 A spectropolarimeter, and tryptophan fluorescence was recorded with a Perkin Elmer fluorimeter, model MPF-3.

Non-covalently linked azidolysolecithin was removed by Sephadex LH 20 gel filtration chromatography of the apo HDL-lysolecithin solution. Excess lysolecithin separated well from the covalently linked lipid apoprotein complex, column (160 × 2 cm), butanol/acetic acid/water 4:1:5 as solvent[10]. For sodium dodecyl sulfate polyacrylamide gel electrophoresis, 15% acrylamide cylindrical or slab gels and the Laemmli system were used[11,12].

Cylindrical gels were cut into 2-mm slices. These were digested with a H_2O_2/70% $HClO_4$ 1:1 (v/v) mixture at 60 °C overnight and dissolved in Soluene scintillator for counting.

Crosslinked lysolecithin-apo AI and -apo AII were separated on a Sephadex G-150 column (120 × 1.5 cm). Two buffer/anionic detergent systems were used: A) Tris/sodium dodecyl sulfate buffer, pH 8.3, consisting of 0.01M Tris, 0.02M NaCl, 0.02% NaN_3, 0.02M EDTA, 1% (w/v) in sodium dodecyl sulfate, 0.001M 2-mercaptoethanol and B) 0.01M Tris, 0.02% NaN_3, 0.01M EDTA, 1% sodium cholate, 0.01M 2-mercaptoethanol (thioglycol), 8M urea, pH 8.3. 10-mg samples were incubated in 2 ml of buffer B for 1 h at 70 °C and then applied to the column. The fractions containing apo AI crosslinked with lysolecithin were rechromatographed on the Sephadex G-150 column with buffer A as eluting solvent, the combined apo AII-lysolecithin fractions after concentration by ultrafiltration (Amicon filter UM 2) over a Sephadex G-100 column (1.5 × 120 cm) with buffer A as eluting buffer, however, without mercaptoethanol.

The distribution of the radioactivity in samples of the fractions of either the Sephadex LH 20 or the Sephadex G-150 column chromatographic separation was determined with a Packard Tricarb Scintillation counter, modell 544.

Protein was determined according to Lowry et al.[13].

Results

Human serum high density apolipoproteins obtained by delipidation of HDL have been complexed with increasing molar amounts of 1-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine, Table 1.

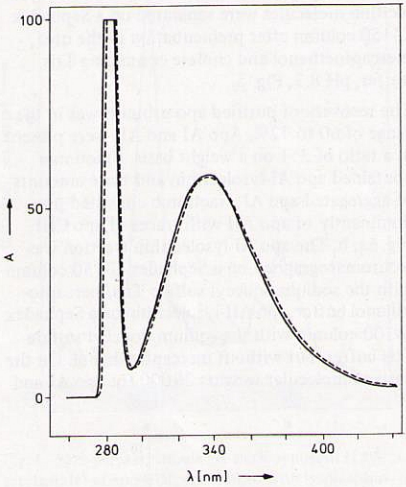

Fig. 1. Fluorescence emission spectra of apo HDL-1-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine complex (micelles)
—— before; – – – – – – after irradiation at λ = 310 nm. Excitation wave length: 290 nm.

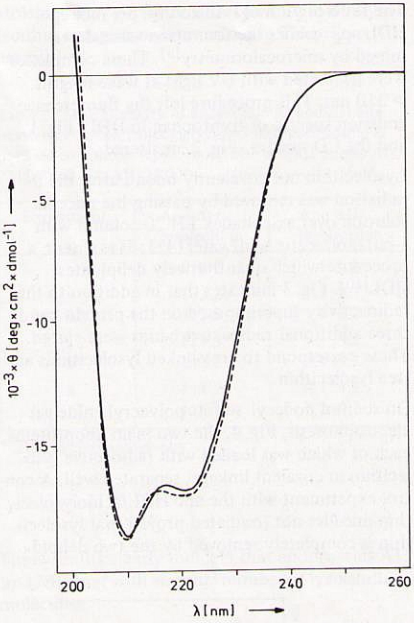

Fig. 2. Circular dichroic spectra of apo HDL-1-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine complex (micelles)
—— before; – – – – – – after irradiation at λ = 310 nm. Θ_{222} corresponds to -1.75×10^4 (deg × dmol^{-1}) or $f_H = 0.50$.

Table 1. Stoichiometry of apo HDL-1-(12-azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine and yield of photocrosslinking.

Experiment	Molar ratio of apo HDL/1-(12-azido-[9,10-$^3H_2$]oleoyl)-glycero-3-phosphocholine		
	Before irradiation	After irradiation and delipidation	% crosslinked lysolecithin
1	1:18	1:3.2	18
2	1:36	1:7.2	20
3	1:54	1:11.4	21
4	1:72	1:14	20

* M_r Apo HDL 100000.

The ratio of 72 mol lysolecithin per mol apo HDL approaches the saturation value determined by microcalorimetry[9]. These complexes were irradiated with UV light at wave-lengths > 310 nm. This procedure left the fluorescence emission spectra of tryptophan in HDL, Fig. 1, and the CD spectra, Fig. 2, unaltered.

Lysolecithin not covalently bound after the irradiation was removed by passing the micellar solution over a Sephadex LH 20 column with 1-butanol/acetic acid/water (4:1:5) as eluent, a procedure which quantitatively delipidates HDL[10]. Fig. 3 indicates that in addition to the radioactivity superimposed on the protein band, three additional radioactive bands were eluted. These correspond to crosslinked lysolecithins and free lysolecithin.

On sodium dodecyl sulfate polyacrylamide gel electrophoresis, Fig. 4, the two main apoproteins, each of which was loaded with radioactive lysolecithin in covalent linkage, separated well. A control experiment with the apo HDL/azidolysolecithin micelles not irradiated proved that lysolecithin is completely removed by the two delipidation procedures, Sephadex LH 20 chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The hump on the ascending radioactivity curve of apo AII (B) in Fig. 4 corresponds to C-apoproteins present in very minute amounts in this preparation. The HDL apoproteins with their crosslinked lysolecithin molecules were separated on a Sephadex G-150 column after preincubation in the urea, mercaptoethanol and cholate containing Tris buffer, pH 8.3, Fig. 5.

The recovery of purified apo proteins was in the range of 60 to 72%. Apo AI and AII were present in a ratio of 3:1 on a weight basis. Fraction a contained apo AI-lysolecithin and trace amounts of aggregated apo AI, fraction b consisted predominantly of apo AII with traces of apo CIII, Fig. 6a, b. The apo AI-lysolecithin fraction was rechromatographed on a Sephadex G-150 column with the sodium dodecyl sulfate Tris/mercaptoethanol buffer, apo AII-lysolecithin on a Sephadex G-100 column with the sodium dodecyl sulfate Tris buffer, but without mercaptoethanol. On the basis of molecular weights 28000 for apo AI and

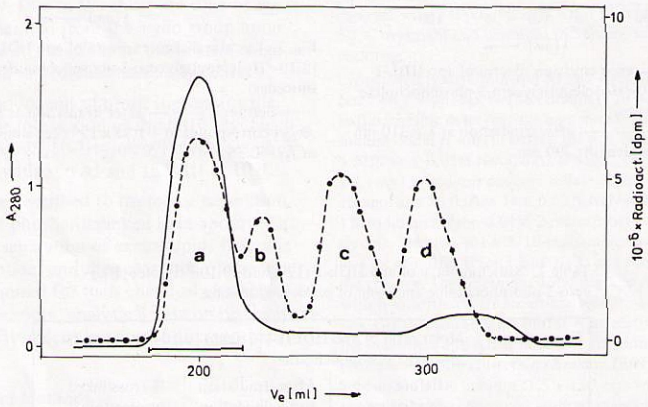

Fig. 3. Removal of non-covalently linked lysolecithin from apo-HDL-lysolecithin micelles after photocrosslinking by means of Sephadex LH-20 column chromatography. Solvent: 1-butanol/acetic acid/water 4:5:1. —— absorbance at 280 nm; –·–·– radioactivity.
a) Covalently bound apo HDL-lysolecithin, b) and c) polymeric lysolecithin, d) lysolecithin.

17000 for apo AII and the radioactivity of 1-(12-azido[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine covalently linked to the apoproteins, the stoichiometry of the crosslinked apoprotein-lysolecithin complexes summarized in Table 2 was established.

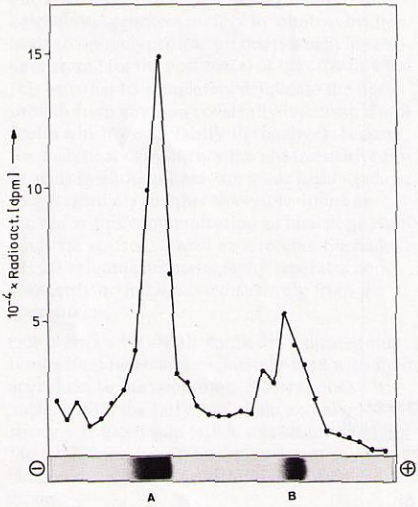

Fig. 4. SDS-polyacrylamide gel electrophoresis (15% acrylamide) of apo HDL crosslinked with lysolecithin by photoactivation of 1-(12-azido-[9,10-$^3H_2$]oleoyl)-glycero-3-phosphocholine.

The gel was cut into 2-mm slices, which were digested with $HClO_4/H_2O_2$ and counted for ^3H radioactivity. A) apo AI, B) apo AII.

Table 2. Stoichiometry of apoprotein AI and AII and *covalently* linked 1-oleoylglycero-3-phosphocholine in purified complexes (appr. molecular weights of apo AI, 28000 and apo AII, 17000).

Experiment	Apo AI/ *lysolecithin*	Apo AII/ *lysolecithin*
1	1:0.9	1:1.0
2 a	1:2.2	1:2.3
2 b*	1:2.5	1:2.2
3 a	1:3.3	1:3.2
3 b*	1:3.5	1:3.3
4 a	1:4.2	1:4.3
4 b*	1:4.1	1:4.3

* after rechromatography.

These results clearly indicate that apoproteins AI and AII react with an equal number of lysolecithin molecules.

This finding is in agreement with the idea that the *photosensitive lysolecithin molecules form micelles* with the single apoprotein molecules like detergents. Apo AI and apo AII should therefore *possess very* closely equal numbers of binding sites for this amphiphilic lysophospholipid.

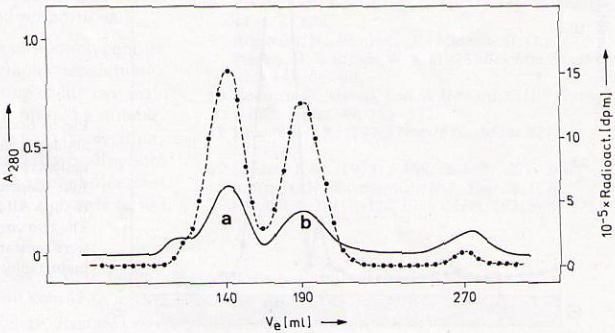

Fig. 5. Sephadex G-150 column chromatographic separation of apo AI-lysolecithin (a) and apo AII-lysolecithin (b) lipopolypeptides.

For elution buffer and details see Methods.

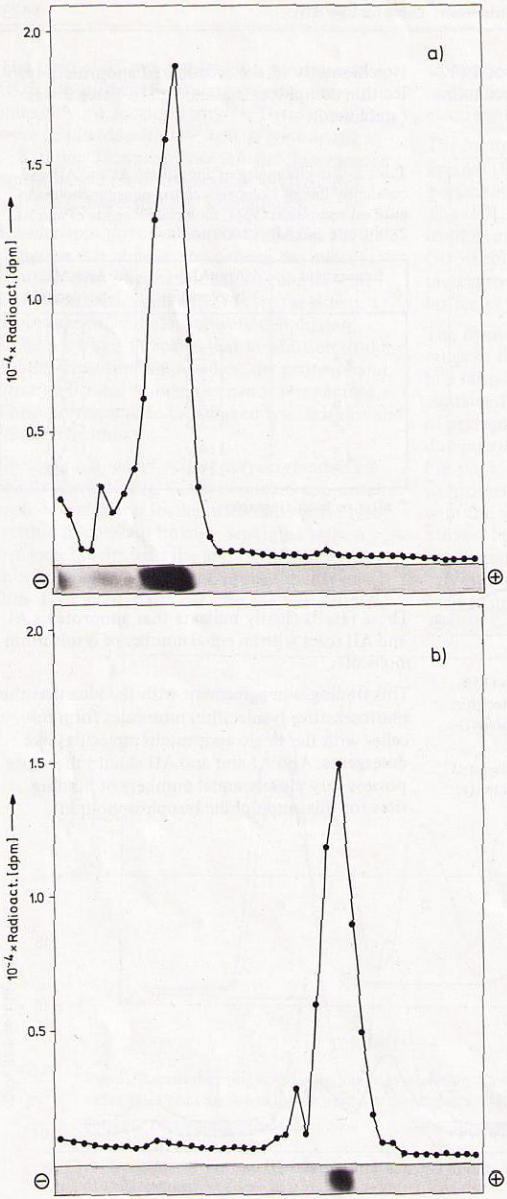

Fig. 6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, distribution of radioactivity and Coomassie Brilliant Blue stained gel, of (a) apo AI-lysolecithin, (b) apo AII-lysolecithin.
The two covalently linked lipopolypeptides were separated by Sephadex G-150 chromatography (see Fig. 5).

Discussion

The chemical approach to study protein-lipid interactions by labelling the lipid components with photosensitive groups and linking them to the neighbouring protein moiety by photoactivation leads to lipopolypeptide products which have to be analyzed for the position(s) of the crossbridges. It is essential to completely delipidate the lipoprotein from any non-covalently linked lipid molecules which would falsify the analyses, because for analytical convenience the photosensitive fatty acids in phospholipids are made highly radioactive. Regardless whether the lysolecithins described in this communication or bisacyl phospholipids are associated with apoproteins, Sephadex LH 20 column chromatography separates non-covalently bound lipids exhaustively from the apoproteins.

Our previous ^{13}C-NMR studies[1] indicated that lysolecithin molecules exclusively bind with their acyl chain to the apoprotein moiety, since ^{13}C-nuclei within the fatty acyl chain exhibited strongly reduced spin lattice relaxation times in the NMR experiment, but this was not so for the choline moiety of the zwitterionic polar headgroup.

The photoaffinity labelling experiments reported here are in full support of the physical studies. 1-(12-Azido-[9,10-$^3H_2$]oleoyl)glycero-3-phosphocholine, which has detergent-like properties, was increased stepwise to its saturation concentration, which is 72 mol per mol apo HDL, and was crosslinked at each of these concentrations to approximately 20% under the conditions chosen. The number of lysolecithin molecules which were linked to apo HDL increased proportionally.

One obstacle in the analysis of lipopolypeptide mixtures is their chromatographic separation. Therefore apo HDL, consisting of the two main components apo AI and AII, offered a suitable model system in conjunction with lysolecithin. The two apoproteins with strongly hydrophobic properties could be separated after photocrosslinking in buffer containing urea/cholate or sodium dodecyl sulfate/Tris buffer on Sephadex G-150 columns. One would expect that apoprotein AI and AII monomers are covered with the surface-active lysolecithin molecules up to their saturation, and therefore protein-protein interactions would be prohibited. Also, a statistical distribution among those azido-lysolecithin molecules activated to nitrenes by photolysis and covalently linked to the apoproteins would be expected. It is remarkable in our observation that the molar ratios of lysolecithin molecules bound to apo AI and AII are close to identical. Further analytical studies are directed towards the determination of the crosslinked sequence within the apopolypeptide chains, to find out whether there are favoured sequences for lipid-protein interactions. The analysis of reconstituted HDL and native HDL particles in which the original lipid species have been substituted by different azido-labelled phospholipids and cholesterol have been made feasible by the separation and purification techniques elaborated with this model system.

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