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Covalent Cross-linking of Photosensitive Phospholipids to Human Serum High Density Apolipoproteins (apoHDL)

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Summary: Human serum high density apolipoproteins were reassociated with three different lecithin species substituted with radioactively labelled photosensitive azido fatty acids, bis([^{3}H]-16-azidopalmitoyl)-, bis([^{3}H]12-azidooleoyl)- and bis([^{3}H]18-azidolinoleoyl)glycerophosphocholine.

The lipoprotein particles were reconstituted from a mixture of azido-labelled phosphatidylcholine and non-labelled dioleoylglycerophosphocholine (1 : 9). Excess lipid was separated from the homogeneous particles by Bio-Gel A-5m.

The molecular weight, stoichiometry, fluorescence and circular dichroism of the reconstituted particles were determined before and after photoactivation with covalent cross-linking of the phospholipids with the apoproteins.

The physical parameters of the reconstituted lipoproteins remained unperturbed by the cross-linking reaction between the generated nitrenes and apolipoprotein A-I and A-II. Thus the hydrophobic interactions of the phospholipid molecules with the apoproteins have been proved for the first time by a chemical method.

Kovalente Quervernetzung von photosensitiven Phospholipiden mit menschlichem Serum-High-density-Apolipoprotein (apoHDL)

Zusammenfassung: Menschliches Serum-high-density-lipoprotein wurde mit drei verschiedenen Lecithin-Species, die mit radioaktiv markierten photosensitiven Azidofettsäuren substituiert waren, nämlich Bis([^{3}H]16-azidopalmitoyl)-, Bis([^{3}H]12-azidooleoyl)- und Bis([^{3}H]18-azidolinoleoyl)glycerophosphocholin rekombiniert.

Zur Rekonstitution der Lipoproteinpartikel wurden die azidomarkierten Phosphatidylcholine mit nicht markiertem Dioleoylglycerophosphocholin im Verhältnis 1 : 9 gemischt. Überschüssiges Lipid wurde durch Chromatographie über Bio-Gel A-5m entfernt.

Molekulargewicht, Stiochiometrie, Fluoreszenz und Circulardichroismus der rekonstituierten Partikel wurden vor und nach der kovalenten Quervernetzung der Phospholipide mit den Apolipoproteinen bestimmt.

Die Quervernetzungsreaktion zwischen den durch Photolyse gebildeten aliphatischen Nitrenen und

Enzyme:
Phospholipase A_2, phosphatide 2-acylhydrolase (EC 3.4.4.1).
Abbreviations:
HDL = high density lipoprotein; apoHDL = high density apolipoprotein.

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den Apolipoproteinen A-I und A-II führte zu keiner Veränderung der physikalischen Parameter der rekonstituierten Partikel.

Hydrophobe Wechselwirkungen der Phospholipidmoleküle mit den Apoproteinen in den HDL-Partikeln konnten somit erstmals mit einer chemischen Methode erfaßt werden.

Key words: Radioactive azido-labelled lecithin species; recombination with HDL apolipoproteins; photoactivation and cross-linking; isolation of lipopolypeptides; lipid-protein interactions in HDL particle.

Serum lipoproteins, particularly high density lipoproteins (HDL) of density 1.063–1.21 g/cm^3 are spherical particles about 100 Å in diameter. Their apoprotein to total lipid ratio is 1 on a weight basis. The apoproteins A-I and A-II together with phospholipids render water soluble the two other lipid classes cholesterol and cholesterol ester and permit their transport to the site of their catabolism. In addition, HDL is also a suitable particle for studies on lipid-apoprotein interactions. Lipids of the native particles can be removed completely with organic solvents and the lipid-free apoproteins can be recombined with zwitterionic phospholipids with or without cholesterol. Considerable insight into the molecular interactions between lipids and apopolypeptide chains has been obtained from phospholipids labelled with physical probes e.g. fatty acids enriched with ^{13}C in specific positions or $^{13}CH_3$-groups in the choline moiety of lecithin species. ^{13}C-NMR spin lattice relaxation time studies lend an insight into the interaction of the hydrocarbon chains of the fatty acyl residues and make the binding of the zwitterionic polar head groups of lecithins to the apoproteins A-I and A-II highly unlikely[1–3].

One drawback of spectroscopic studies such as NMR, fluorescence and ESR spectroscopy on lipid protein interactions is that the processes probed by the proton or carbon 13 isotope, fluorophors or nitroxide radicals, probe random processes. Specific lipid-interaction sites on apopolypeptides or proteins in the membrane structures interacting with lipid classes cannot be localized by these techniques. However the use of lipids labelled with the photosensitive azido group in the fatty acyl chains of complex lipids offers the possibility of studying specific sites in polypeptide sequences that interact with these lipids in lipoproteins[4] or membranes[5,6]. The azido group attached to long chain fatty acyl chains of phospholipids causes little perturbation of lipid mono- and bilayers as demonstrated by Corey-Pauling-Koltun models. On UV-irradiation above 300 nm the resulting nitrene formed reacts immediately with the nearest neighbour by insertion into C-H groups or addition to double bonds. Due to the very short lifetime of aliphatic nitrenes ($\sim 10^{-11}$ s) the lateral diffusion of the lipid moiety during this interval can be neglected. In a preliminary study azido fatty acyl substituted lysolecithin was cross-linked to apolipoprotein A-I and the linkage was shown to be covalent by delipidation, cyanogen bromide cleavage and sodium dodecylsulfate-polyacrylamide gel electrophoresis of its fragments. In this paper apoHDL was recombined with three azido-labelled phosphatidylcholine species, bis([^{3}H]16-azidopalmitoyl)-, bis([^{3}H]12-azidooleoyl)-, and bis([^{3}H]18-azidolinoleoyl)glycerophosphocholine. UV-irradiation led to cross-linking of these phospholipids to apolipoprotein A-I and A-II with no conformational change of the reconstituted HDL particles. An approach to the molecular analysis of the lipopolypeptides is described.

Materials and Methods

Materials

1,2-Bis([9,10-$^3H_2$]12-azidooleoyl)-*sn*-glycero-3-phosphocholine (specif. radioactiv. 37 μCi/μmol), 1,2-bis([9,10-,12,13-$^3H_4$]18-azidolinoleoyl)-*sn*-glycero-3-phosphocholine (specif. radioactiv. 14 μCi/μmol) and 1,2-bis([9,-10-$^3H_2$]16-azidopalmitoyl)-*sn*-glycero-3-phosphocholine (specif. radioactiv. 40 μCi/μmol) were synthesized in this laboratory*. Human high density apolipoproteins were prepared by delipidation of the human serum high density lipoprotein fraction (ρ = 1.063–1.21 g/cm^3) which was homogeneous in agarose electrophoresis.

* Stoffel, W., in preparation.

Recombinations

HDL apolipoproteins were recombined according to the procedure elaborated in this laboratory[7]. 13 mg (0.13 μmol) apoproteins were preincubated for 2 h with 12 μmol hydrogenated lysolecithin (obtained by phospholipase A_2 treatment of soya lecithin) in 40 ml recombination buffer containing 8M deionized urea. Vesicles from 25 μmol dioleoylglycerophosphocholine and 2.5 μmol of the radioactive, azido-labelled phosphatidylcholine species were prepared by ultrasonication in 10 ml recombination buffer for 20 min in a nitrogen atmosphere. The vesicles were added to the apoprotein-lysolecithin solution and stirred for 3 h at room temperature; they were then dialysed overnight against water, pH 8.0, concentrated by ultrafiltration (Diaflow filter UM 2) to 2–5 ml and passed over a Bio-Gel A-5m column, bed volume: 2 cm Ø x 90 cm. V_0 = 112 cm^3. The eluant was 0.1M Tris buffer pH 8.2, and 2-ml fractions were collected. Fractions containing the recombined HDL were pooled, dialysed against water pH 8.2 and concentrated by ultrafiltration to a volume of about 3 ml. The sample was then irradiated at $\lambda > 300$ nm for 40 min as described previously[4].

Phospholipase A_2 hydrolysis

0.1 mg protease-free phospholipase A_2 from *Crotalus adamanteus* (Boehringer, Mannheim) per 10 mg apoHDL protein in 1 ml buffer pH 7.0 (0.02mM histidine, 0.1mM KCl, 5mM $CaCl_2$) were stirred for 5 h at room temperature. Then 2 ml 2-butanol/acetic acid 4 : 1 was added, the mixture stirred for 2 h, and then chromatographed on Sephadex LH-20.

Delipidation by Sephadex LH-20 chromatography

Bed volume: 1.5 cm Ø x 150 cm, solvent system: 2-butanol/water/acetic acid 4 : 5 : 1 (v/v)[8]. The lipopolypeptide-containing fractions were dialysed against one charge of water/acetic acid 9 : 1 and then against water.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

This gel electrophoresis was carried out on 15% acrylamide gels according to Lämmli[9]. The lipid-polypeptide cross-linked samples for electrophoresis were first treated with phospholipase A_2, followed by separation of noncovalently linked excess lipids by chromatography on Sephadex LH-20.

Fluorescence spectroscopy

Fluorescence spectroscopy was performed with a Perkin Elmer spectrofluorimeter, model MPF 3. Emission spectra were recorded at an excitation wavelength of 290 nm. The path length was 1 cm.

Measurement of circular dichroism

The circular dichroism spectra were recorded in a Durrum-Jasco J-41 A spectropolarimeter equipped with a data processor. The path length was 0.1 cm. The results are reported in terms of [Θ], the mean residue ellipticity in units of degree cm^2 $decimol^{-1}$. The instrument was calibrated with epiandrosterone in dioxane (Θ_{304} = 10925 degree x cm^2 x $decimol^{-1}$). The content of α-helix, β-structure and "random" structure was computed according to Saxena and Wetlaufer[10], Chen et al.[11] and Greenfield and Fasman[12].

All samples containing photosensitive azido-phospholipids were protected from light.

Molecular weight determination

Molecular weights of the recombined HDL-complexes were determined by chromatography on a calibrated agarose column Bio-Gel A-5m, 200–300 mesh, 1.5 cm Ø x 90 cm.

For stoichiometric calculations protein was determined according to Lowry et al.[13], phospholipids by determination of microphosphorus[14], and the azido-labelled lecithin from the specific radioactivity.

Results

Recombination of apoHDL with radioactive azido-labelled lecithin

The recombination procedure, using 8M urea for the unfolding of the apoproteins and hydrogenated lysolecithin as mediator for the binding of phosphatidylcholine species was performed as described for similar recombinations with ^{13}C-labelled lecithins. Dioleoyllecithin and one of the three radioactive azido-labelled lecithins, bis([9,-10-$^3H_2$]12-azidooleoyl)-, bis([9,10,12,13-$^3H_4$]-18-azidolinoleoyl-, and bis([9,10-$^3H_2$]16-azidopalmitoyl)glycerophosphocholine were mixed in a molar ratio of 9 : 1, and liposomes were formed by ultrasonication. These were used for recombination with the unfolded apolipoprotein-lysolecithin complex. Excess lipids were separated from the reconstituted HDL-particles by chromatography on Bio Gel A-5m. The three complexes with different azido-labelled lecithin species eluted in the same volume (V_e) Fig. 1, whereas the excess lipids separated with the void volume (V_0). The apparent molecular weight of the particles was determined by chromatography on a Bio-Gel A-5m column, calibrated with test proteins. A molecular weight of about 200000 similar to that of native HDL, was found (Fig. 2).

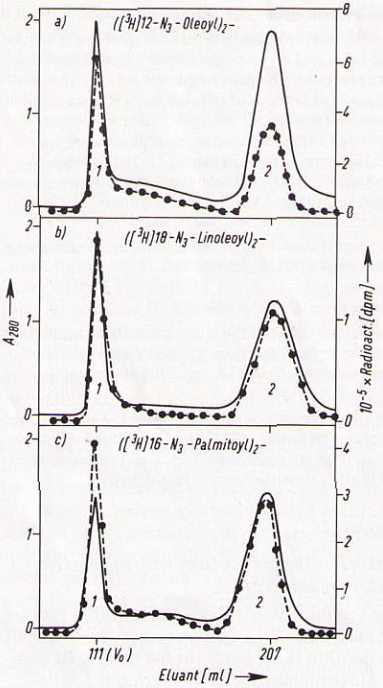

Fig. 1. Gel filtration of reconstituted HDL-particles. Liposomes of dioleoyllecithin together with a) bis(12-azidooleoyl)glycerophosphocholine, b) bis(18-azido-linoleoyl)glycerophosphocholine and c) bis(16-azido-palmitoyl)glycerophosphocholine were used for the reconstitution. Chromatography was performed on a Bio Gel A-5m column, bed volume 2 cm Ø × 90 cm. The excess lipid (1) eluted in the void volume V_0 (111 ml). V_e was the same for all the lipoproteins (207 ml). 0.1M Tris buffer pH 8.2 was used as eluant.

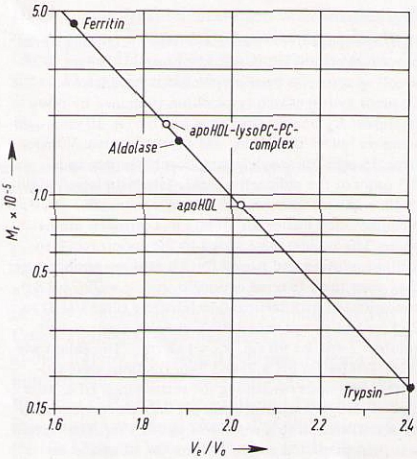

Fig. 2. Determination of apparent molecular weight of reconstituted HDL-particles by Bio-Gel A-5m chromatography on a 2 cm Ø × 90 cm column.
Test proteins used for calibration are indicated in the figure. PC = phosphatidylcholine.

Microanalyses (protein, microphosphorus and radioactivity) yield the stoichiometry summarized in Table 1. A total of about 140 to 150 phospholipid molecules per particle, one third of which were lysolecithin molecules, were complexed with the apolipoproteins. A protein to lipid ratio of 1 : 1 on a weight basis similar to that of native HDL was obtained. A density of 1.14–1.16 g/cm³ for the three particles was determined by CsCl-gradient (11–20.5%) centrifugation[3].

Photoactivation and isolation of apolipoproteins cross-linked with lipids

The suspensions of reconstituted particles consisting of apolipoprotein A-I and A-II, and the azido-phosphatidylcholine species together with dioleoylglycerophosphocholine and hydrogenated lysolecithin were irradiated with UV-light above 300 nm as described previously. Tryptophan and tyrosine fluorescence and circular dichroism were measured before and after the photoactivation and cross-linking process (Fig. 3 and 4).

It is quite obvious that there has been no change in these spectroscopic properties. We regard this as a rather sensitive indication for the preservation of secondary and tertiary structures. Table 2 summarizes the corrected ellipticities and degrees of α-helicity before and after irradiation of lipid-protein complexes, consisting of solely apoHDL

Table 1. Molar ratios of apoHDL ($M_r \sim 100000$): dioleoylglycerophosphocholine + azido-labelled lecithin species (in parenthesis): lysolecithin in reconstituted HDL-particles purified by agarose chromatography.

Dioleoylglycerophosphocholine and the azido-lecithin species are used in a 9:1 molar ratio.

Azido-labelled lecithin species	ApoHDL : Lecithin$_{Total}$: Lysolecithin	Weight [%]	
		Protein	Lipid
(12-N_3-oleoyl)$_2$-	1 : 102 (9) : 39	50	50
(18-N_3-linoleoyl)$_2$-	1 : 112 (10) : 37	48	52
(16-N_3-palmitoyl)$_2$-	1 : 114 (10) : 42	47	53

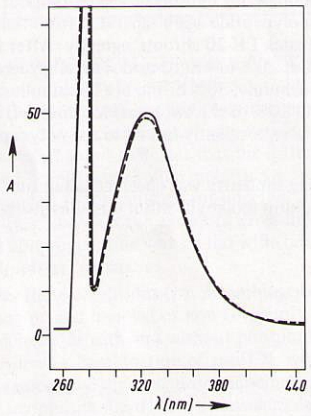

Fig. 3. Fluorescence spectra of HDL reconstituted with dioleoylglycerophosphocholine containing bis(12-azido-oleoyl)glycerophosphocholine.
—— Before, – – – after irradiation.

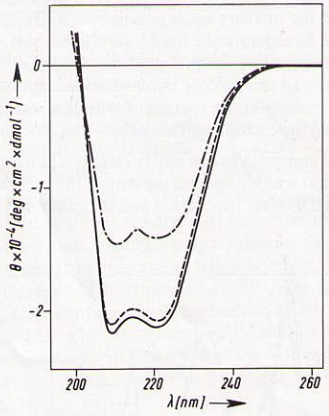

Fig. 4. Circular dichroic spectra of –·–·– apoHDL, reconstituted HDL particle containing bis(12-azidooleoyl)-glycerophosphocholine —— before and – – – after UV-irradiation.

Table 2. Molecular ellipticities (Θcorr.) and α-helicity (f_H) of apolipoproteins in HDL complexes reconstituted with dioleoylglycerophosphocholine-azido-labelled lecithin species (molar ratio 9:1), native high density lipoprotein (HDL) and delipidated HDL (apoHDL) before and after photoactivation of azidolipids.

Azido-labelled lecithin species in dioleoylglycero-phosphocholine	Before irradiation		After irradiation	
	$\Theta_{222}^{corr.} \times 10^{-4}$	f_H	$\Theta_{222}^{corr.} \times 10^{-4}$	f_H
(12-N_3-oleoyl)$_2$-	– 2.20	0.64	– 2.10	0.62
(18-N_3-linoleoyl)$_2$-	– 2.10	0.62	– 2.05	0.60
(16-N_3-palmitoyl)$_2$-	– 2.20	0.64	– 2.10	0.62
HDL (native)	– 2.20	0.64	–	–
apoHDL	– 1.40	0.38	–	–

and of native HDL. It can be seen that on recombination with these lipids the apolipoproteins regain the secondary structure of the native HDL particle with a considerable increase in α-helix structure.

The analysis of the cross-linked lipopolypeptides was considerably facilitated by removal of non-covalently bound lipid on a Sephadex LH-20 column with 2-butanol/acetic acid/water (4:1:5) as eluant. In order to remove as much of the attached lecithin molecules as possible the lecithin-apolipoprotein was treated with phospholipase A_2 and the mixture again passed over a LH-20 column to separate the lipid-hydrolyzed components from the lipopolypeptides. Figs. 5a and b represent an example of two procedures applied to the cross-linking experiment with bis(16-azidopalmitoyl)glycerophosphocholine.

Table 3 summarizes the molar ratios of radioactive azido lecithin species present in the recombined HDL after irradiation and delipidation by Sephadex LH-20 chromatography, and finally after phospholipase A_2 treatment and removal of non-covalently linked lipid.

The results given in Table 3 indicate that about 40% of the radioactive bis-azidolabelled phospholipid species are covalently linked to apolipoprotein A-I and A-II. These radioactive lipid molecules could either be attached to the polypeptide chains or to neighbouring lipid molecules which are cross-linked to the apoproteins. To eliminate the lipids that are not immediately attached, a phospholipase A_2 hydrolysis was carried out and the lipopolypeptide again separated from the lipid by Sephadex LH-20 chromatography. After this step about 20% of bis(12-azidooleoyl)glycerophosphocholine, 30% of the bis(18-azidolinoleoyl)- and 20% of the bis(16-azidopalmitoyl) compound were covalently linked to the polypeptide chains.

A striking similarity was observed when the "matrix" lecithin dioleoyllecithin was substituted by

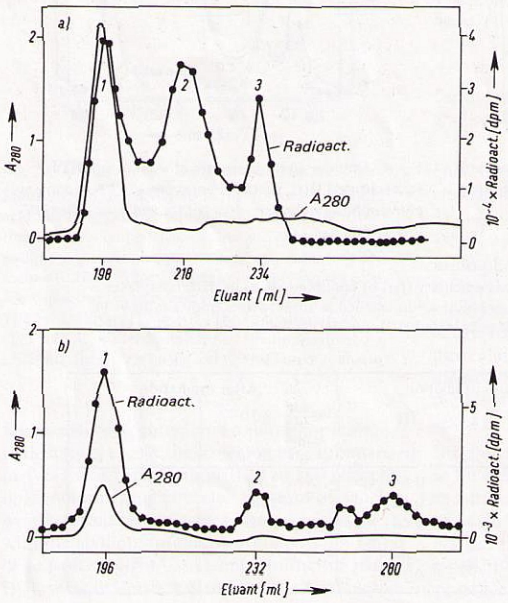

Fig. 5. a) Separation of covalently linked apoHDL-phospholipid from free lipid by Sephadex LH-20 chromatography (bed volume: 1.5 cm Ø x 150 cm), solvent system: 2 butanol/acetic acid/water 4:1:5.

b) apoHDL-phospholipid from band 1 of Fig. 5a after phospholipase A_2 treatment and repeated chromatography under the same conditions. 1 = Cross-linked apoHDL-radioactive lipid (phospholipid or fatty acid); 2 = high molecular weight cross-linked lipids; 3 = low molecular weight lipid.

Table 3. Azidophospholipid-protein ratio (M_r apoHDL ~ 100000) in reconstituted HDL particles (after agarose chromatography and CsCl-gradient), UV-irradiation and removal of non-covalently linked phosphatidylcholine (reconstituted HDL recombinant after irradiation, Sephadex LH-20 chromatography, phospholipase A_2 treatment and repeated LH-20 chromatography).

Azido-labelled lecithin species	Molar ratio azido-lecithin:apoHDL			
	Agarose	CsCl-grad.	Irradiation, Seph. LH-20	Irradiation, Seph. LH-20, phospholipase A_2, Seph. LH-20
(12-N_3-oleoyl)$_2$-	9 : 1	9 : 1	4 : 1	2 : 1
(18-N_3-linoleoyl)$_2$-	10 : 1	9 : 1	4 : 1	3 : 1
(16-N_3-palmitoyl)$_2$-	10 : 1	11 : 1	4 : 1	2 : 1

soya lecithin (EPL Nattermann & Cie., Köln, this lecithin contains about 70% linoleic acid) and bis(12-azidooleoyl)- and bis(16-azidopalmitoyl)-glycerophosphocholine were admixed in a molar ratio 9 : 1. Table 4 indicates that the different azido phosphatidylcholine molecules were incorporated to the same extent into the "HDL-particles;" i.e. that the extent of cross-linkage with apopolypeptide was similar with both phospholipids as "matrix".

Under these conditions (i.e. recombination, photoactivation and removal of non-covalently bound phospholipids with and without phospholipase A_2 treatment) recombination of apoHDL with bis-(12-azidooleoyl)glycerophosphocholine as the only diacylphospholipid led to a considerable increase in the number of lipid to apoprotein cross-links (Table 4, Section II).

Labelling patterns of apolipoproteins A-I and A-II by cross-linked ^{3}H-labelled fatty acids (phospholipids)

The distribution of ^{3}H-radioactivity bound to the apopolypeptides A-I and A-II was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis after exhaustive delipidation by successive Sephadex LH-20 chromatography, phospholipase A_2 treatment and again Sephadex LH-20 chromatography. Figs. 6a–c combine protein patterns of the Coomassie Blue stained gels with the distribution of ^{3}H-radioactivity in the dodecylsulfate-polyacrylamide gel.

Table 4. Azido-labelled phospholipids in reconstituted "HDL-particles" before and after photoactivation and removal of non-covalently linked lipids by chromatography and phospholipase A_2 hydrolysis followed by another chromatography.
Section I: Soya lecithin as matrix phospholipid of bis(12-azidooleoyl)lecithin and bis(16-azidopalmitoyl)glycerophosphocholine;
Section II: No matrix phospholipid was used.
Conditions were those outlined under Table 3.

Azido-labelled lecithin species	Molar ratio of azido-lecithin:apoHDL		
	Without irradiation	Irradiation, Seph. LH-20	Irradiation, Seph. LH-20, phospholipase A_2, Seph. LH-20
I. (12-N_3-oleoyl)$_2$- + soya-PC	11 : 1	5 : 1	3 : 1
(16-N_3-palmitoyl)$_2$- + soya-PC	13 : 1	4 : 1	2 : 1
II. (12-N_3-oleoyl)$_2$-	84 : 1	40 : 1	15 : 1

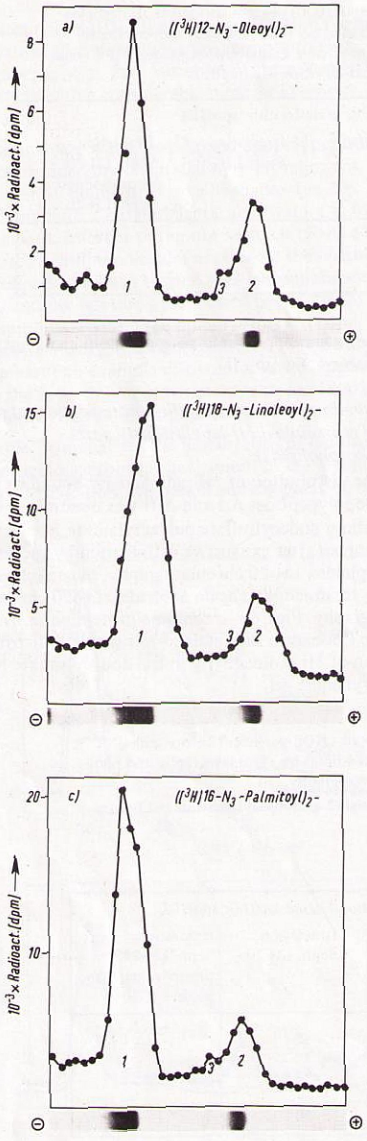

Fig. 6. Sodium dodecylsulfate-polyacrylamide gel (15%) electrophoresis of HDL apolipoproteins cross-linked with ^{3}H-labelled phospholipids after removal of non-covalently linked lipid and phospholipase A_2 treatment.

1 = apoAI-lipid; 2 = apoAII/2-lipid; 3 = C proteins. Upper diagram: distribution of radioactivity in gel. Lower diagram: gel stained with Coomassie Blue. a) Cross-linking with bis([^{3}H]12-azidooleoyl)glycerophosphocholine; b) cross-linking with bis([^{3}H]18-azidolinoleoyl)glycerophosphocholine; c) cross-linking with bis([^{3}H]16-azidopalmitoyl)glycerophosphocholine.

Protein bands coincide with the radioactive bands. The quantitation indicates that a rather equal distribution of cross-linking occurs between the three phospholipids substituted with azido-labelled fatty acids of rather different degrees of unsaturation. Also the two apolipoproteins A-I and A-II bind numbers of azido-labelled phospholipids proportional to their number in the "HDL"-particle.

Discussion

The elucidation of lipid protein interactions, structure-function relationship (lipid dependency) and topography of membrane constituents urgently demand more potent methods than the physical probe methods so far applied. Only X-ray crystallographic analysis and those chemical methods which allow the analysis of the nearest neighbourhood-relationship of macromolecular constituents without serious perturbation, promise advances in the understanding of the molecular array of a lipoprotein or a membrane. Since X-ray analysis is still waiting for a breakthrough in this field, a new chemical approach is being successfully applied to the problems mentioned above. We attached the photosensitive azido group to fatty acyl chains of the most common fatty acids of phospholipids. Different positions along the chain were chosen. These fatty acids chemically incorporated into the lecithin structure physically (Langmuir monolayer force/area isotherms) behave like the normal unsubstituted phospholipid.

Photoactivation at $\lambda > 300$ nm proceeds with a rapid generation of the highly reactive nitrene

(–$\bar{N}$) group, which momentarily either cross-links to neighbouring molecules by insertion or addition or reacts intramolecularly. Since the lifetime of aliphatic nitrenes is extremely short ($\sim 10^{-11}$ s) the immediate neighbour is the partner in the cross-linking reaction. We have applied this photoaffinity labelling technique, using azido-labelled lysolecithins of high specific radioactivity, to prove the hydrophobic binding of this lipid class to human serum high density apolipoprotein A-I.

Like the unsaturated fatty acid, the azido fatty acids are utilized in biosynthetic reactions. Thus, these acids have been successfully used for pre-labelling the membrane lipids of host cells, for example, the lipid-containing envelope of viruses such as vesicular stomatitis virus. Due to the different positions of the reactive nitrene group along the acyl chain these compounds serve as chemical "rulers" of the membrane. The radioactive cross-linking products[6] permit certain deductions as to whether a protein is intrinsic, and the depth to which integral membrane proteins enter or penetrate the lipid bilayer.

In this communication we report the reconstitution of total apoHDL with azido fatty acid-substituted phosphatidylcholines and dioleoylglycerophosphocholine in a molar ratio of 1 : 9. Lysolecithin served as the mediator detergent. The reconstituted "HDL"-particles which contained about 140 phospholipid molecules, about one third of which is lysolecithin, behave in many respects like native serum HDL particles. Thus the apparent molecular weight is about 200000, their size in electron microscopy is 80–100 Å in diameter after negative staining with phosphotungstic acid.

The considerable conformational changes of the apoHDL due to the binding of the phospholipids become obvious, when the circular dichroic spectra in the UV-range of the reconstituted HDL-particles and native serum HDL are compared. α-Helicity is raised from 38% for delipidated apoHDL to 64% on reconstitution, which is identical with that of native HDL. Apparently 100–150 phospholipid molecules are required for the most stable particle structure. Cholesterol and cholesterol esters, neither of which can recombine with apoHDL, do not contribute to the secondary structure of the HDL-apoproteins and particle stability.

The UV-generation of the reactive nitrene groups in the phospholipid molecules and the lipid-apoprotein cross-linking does not cause any alterations of the circular dichroism of the particles or their intrinsic fluorescence (tyrosine and tryptophan fluorescence).

The photoactivatable azido groups of bis(16-azidopalmitoyl)-, and bis(18-azidolinoleoyl)glycerophosphocholine are inserted in the terminal 16 and 18 position, respectively, and bis(12-azidooleoyl)glycerophosphocholine carries the group in the central part of its molecule. The linkage of these molecules to the apopolypeptide chains therefore proves that the phospholipid molecules in HDL-particles are hydrophobically fixed, or conversely the water-soluble apolipoprotein A-I and A-II act as detergents. This is in full support of ^{13}C-NMR relaxation studies and the proposed model of the serum HDL-particle reported from this laboratory[1]. In addition to the phospholipid-apolipoprotein cross-links, there must also be linkages between phospholipid molecules and between fatty acyl chains of the same lecithin molecule. Strong support for these di- and probably polymeric lipid molecules came from the Sephadex LH-20-chromatographic separation of non-protein bound lipids in which a higher molecular weight radioactive lipid band (Fig. 5) eluted well ahead of a low molecular weight lipid band. It is advisable to detach as much as possible of the lipid components not directly linked to the apoprotein, because this greatly facilitates the subsequent analysis of the lipopolypeptide. For this reason we added hydrolysis with a phospholipase A_2 (protease free), followed by Sephadex LH-20 chromatography to the isolation procedures.

Fig. 7 schematically indicates that either free fatty acid or lysolecithin is cross-linked to apolipoprotein, but some higher cross-linked acyl chain arrangements are also possible with lecithin substituted with azido-fatty acyl chains in both positions.

Depending on the molar ratio of azido-labelled lecithin to unsubstituted matrix lecithin, the number of lipid to apoprotein cross-links can be manipulated. Also changes in the irradiation conditions may serve the same purpose. It is now

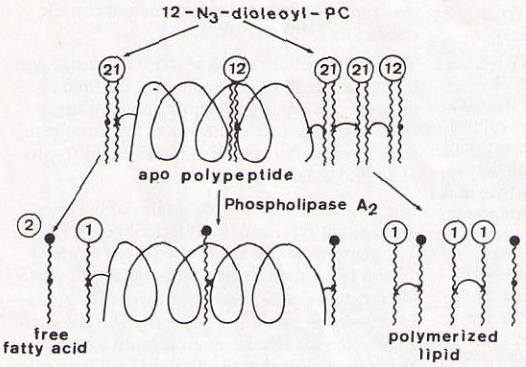

Fig. 7. Schematic presentation of cross-linked lipopolypeptide and polymerized lipid products after phospholipase A_2 hydrolysis of apoHDL-bis(12-azidooleoyl)glycerophosphocholine complex irradiated for activation of photosensitive groups.
1 = Fatty acid in *sn* 1-position; 2 = fatty acid in *sn* 2-position of lecithin.

in the realm of the chemical analysis to determine whether specific polypeptide sequences of the apolipoproteins A-I and A-II (the primary structures are now known) bind the phospholipid molecules and whether there are specific high affinity binding sites on either of the two apolipoproteins.

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