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Chemical Proof of Lipid-Protein Interactions by Crosslinking Photosensitive Lipids to Apoproteins

Intermolecular Cross-Linkage between High-Density Apolipoprotein A-I and Lecithins and Sphingomyelins

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Summary: The molecular interactions and spatial arrangements of phospholipids and apoproteins of human high-density lipoprotein were studied by a chemical approach.

Phosphatidylcholines and sphingomyelins substituted with fatty acyl residues of high specific radioactivity and labelled with the photosensitive azido group in specific positions were prepared by chemical synthesis. They were recombined with apolipoprotein A-I of human serum high density lipoprotein. The lipoprotein complexes containing either azido lecithins or azidosphingomyelins were purified by agarose chromatography from excess lipids. The irradiation was performed under conditions which prohibit the interference with the apoprotein structure as proven by circular dichroism, fluorescence spectroscopy, immunodiffusion test and disc electrophoresis. Non-covalently bound lipid molecules were removed by Sephadex LH 20 chromatography. Mild alkaline treatment liberated radioactive fatty acids which were not directly linked to the polypeptide chain, but rather via neighbouring phospholipid molecules.

The lipoprotein appeared as a single radioactive band in dodecylsulfate polyacrylamide gel electrophoresis as seen by radioscanning, which further proved the covalent linkage of the fatty acyl residues to the polypeptide chain. In the immunodiffusion test, there is no difference between covalently crosslinked phospholipid-apoLp A-I complex and the non-photolytically treated complex.

This is the first chemical proof of the spatial relationship of the hydrophobic side chains of the lipid and polypeptide chains in a lipoprotein complex.

Chemischer Beweis der Lipid-Protein-Wechselwirkungen durch Quervernetzung von photosensibilisierbaren Lipiden mit Proteinen: Intermolekulare Quervernetzung zwischen High-Density-Apolipoprotein A-I und Lecithinen und Sphingomyelinen

Zusammenfassung: Die molekularen Wechselwirkungen zwischen Phospholipiden und Apoproteinen wurden auf chemischem Wege untersucht. Phosphatidylcholine und Sphingomyeline wurden synthetisiert, deren Fettsäuren mit der photosensitiven Azidogruppe substituiert sind und eine hohe spezifische Radioaktivität besitzen. Sie wurden mit dem Apolipoprotein A-I (apoLp A-I) des menschlichen High-Density-Lipoproteins (HDL) rekombiniert. Die Lipoproteinkomplexe, die entweder Azidolecithine oder Azidosphingomyeline enthielten, wurden durch Agarose-Chromatographie von überschüssigem Lipid gereinigt. Nach der Photolyse, die unter Bedingungen durchge-

führt wurde, die zu keiner meßbaren Änderung der Apoproteinstruktur (CD, Fluoreszenzspektroskopie, Immundiffusionstest und Dodecylsulfat-Disc-Elektrophorese) führten, erfolgte die Abtrennung von nicht kovalent gebundenen Lipidmolekülen durch Chromatographie an Sephadex LH-20. Milde alkalische Behandlung setzt radioaktive Fettsäureketten frei, die nicht direkt, sondern über benachbarte Phospholipidmoleküle an die Polypeptidkette gebunden sind.

Die Radioaktivitätsverteilung und die Radiographie in der Dodecylsulfat-Polyacrylamidgel-Elektrophorese zeigen, daß das quervernetzte Lipopolypeptid als einheitliche Proteinbande wandert. Im Immundiffusionstest zeigten sich keine Unterschiede der Präzipitationslinien zum nicht photolytisch quervernetzten Lipoproteinkomplex.

Dies ist der erste chemische Beweis der räumlichen Anordnung der hydrophoben Ketten des Lipids und des Polypeptids durch kovalente Quervernetzung der hydrophoben Alkanketten der Phospholipide zu den Apolipoproteinpolypeptidketten in einem Lipoproteinkomplex. Hierdurch wird die Bestimmung des nächsten Lipidnachbarn zu Polypeptidkettensegmenten ermöglicht.

Key words: Photoaffinity-labelled phospholipids, phospholipid-apoprotein recombination, photolytic cross-linking, purification of covalently linked lipopolypeptide complexes.

The structure of high density lipoprotein from human serum and of artificial liposomes and simple natural membranes such as the envelope of lipid containing viruses (VSV*) is being studied in this laboratory[1-8]. In these investigations, polar lipids containing either a carbon-13 nucleus or a fluorescent group, such as the anthryl residue, positioned either in the polar head group or defined carbon atoms along the fatty acyl chains, are used to probe the hydrophilic and hydrophobic region of the molecule.

Spin-lattice relaxation time of the ^{13}C probe and polarisation of the chromophore were studied in relation to the lipid matrix and membrane proteins.

The NMR and fluorescence techniques proved to be very effective in probing the mobility of lipid molecules or segments of these amphiphiles, allowing conclusions to be drawn regarding the dynamics of the lipid-lipid and lipid-protein interactions. It appears, however, that a more thorough understanding of the molecular basis of these interactions can be gained at this time only by a chemical approach.

Using methods similar to the insertion of ^{13}C probes, we have introduced azido groups along the phospholipid molecules by chemical syntheses, up to the ω-carbon atom of long chain fatty acids, and have used these azido fatty acids for the acylation of the phospholipids and sphingolipids. Azido groups are photolysed by UV irradiation to nitrenes as primary products. Nitrenes may react among others by insertion into the C-H bond or by addition to double bonds, including those of aromatic rings[9,10]. In lipid-protein complexes they may form cross links either to lipid molecules or polypeptide chains in closest proximity to the reactive groups.

The analytical and chemical characterization of these cross-linking products should then allow a "regio-analysis" or a topography of the region around the azido fatty acid.

In this communication we report the first successful cross-linking of phosphatidylcholine and sphingomyelin, both substituted with 18-azido-[9,10,12,13-$^3H_4$]linoleic acid, with the apolipoprotein A-I (apoLp A-I) of human high density lipoprotein. The high specific radioactivity facilitated the further characterization of the cross-linked lipopolypeptide. The localization of the azido group in phosphatidylcholine and sphingomyelin far away from the hydrophilic moiety of these amphiphilic lipids and the easy cross-linking with the apoprotein is conclusive chemical evidence for the hydrophobic interactions in the phospholipid-apoLp A-I complexes formed. These chemical results are in full support of our conclusions drawn from previous ^{13}C-NMR relaxation studies[4,11].

* Vesicular stomatitis virus.

Methods and Materials

ω-Azido-[^{3}H]linoleic acid was synthesized via the following intermediates: 1-bromo-8-chlorooct-2-yne + dec-9-ynoic acid→*18-chlorooctadeca-9,12-diynoic acid*→18-chloro-[9,10,12,13-$^3H_4$]octadeca-*all-cis*-9,12-dienoic acid→18-azido-[9,10,12,13-$^3H_4$]linoleic acid. This synthesis and that of phosphatidylcholine and sphingomyelin substituted with this acid will be described in detail elsewhere*. The specific radioactivity of 18-N_3-[9,10,12,13-$^3H_4$]linoleic acid *is 1.8 × 10^8 dpm/μmol* (90 μCi/μmol).

Apolipoprotein A-I from human high density lipoprotein was purified to electrophoretic and immunological homogeneity by standard procedures: delipidation with chloroform/methanol, DEAE-cellulose chromatography *in 8M urea in a Tris/HCl gradient* and *final* purification by Sephadex G-200 chromatography[12-15]. Disc electrophoresis in 6M urea[16] and sodium dodecylsulfate gel electrophoresis[17] were used for control of purity. The immunodiffusion technique of Ouchterlony[18] was applied for comparison of the recombined phospho*lipid-apoLp A-I complex*, before and after irradiation, to the apoprotein.

The recombination procedure elaborated for the [^{13}C]-lipid-containing lipoproteins[11,19] and described before was adapted to these azido-labelled lipids. The lipoprotein complexes were separated *from the excess* liposomes by agarose chromatography (see legends to Fig. 1). The fractions containing the complex were concentrated by ultrafiltration using an Amicon cell and Diaflo ultrafilter UM 2. The UV irradiation (HPK 125 W lamp) was carried out at 10 °C for 30 min with careful control of the tryptophan fluorescence and the circular dichroism of the samples. Fluorescence intensity was measured with a Perkin Elmer fluorescence spectrophotometer, model MPF-3. CD spectra were recorded with a Jasco Instrument, model 41 A in order to measure the α-helix content.

The UV-irradiated phospholipid-apoLp A-I complexes were chromatographed on Sephadex LH-20 (2.5 × 90 cm), unbound lipids thereby separating from the covalently bound phospholipid-apoLp A-I particles. Water/2-butanol/acetic acid 5:4:1 was used as eluent[20]. The combined *fractions* were dialysed against water/acetic acid mix*tures (9:1) with decreasing acetic acid concentration* and concentrated by ultrafiltration. Sodium dodecylsulfate gels with samples of the radioactive cross-linked phospholipid-apoLp A-I complexes were run under the conditions given in the legend of Fig. 7 and scanned directly with a Berthold radio thin-layer chromatogram scanner, model LB, 2723 after staining with Coomassie Brilliant Blue.

Phosphate was determined *according to Bartlett*[21] and protein according to Lowry et al.[22].

* Stoffel, W., Salm, K. P. & Körkemeier, U., manuscript in preparation.

Results

1) Recombination of apoLp A-I with azido-labelled lecithin and sphingomyelin

In a previous report from this laboratory[11] we have described a procedure for the recombination of lipid-free purified apolipoproteins, e.g. apoLp A-I with single lipid species or mixtures of lipid classes with reproducible stoichiometry. This procedure consists of the unfolding of the polypeptide in 6M urea, substitution of the hydrophobic site with lysolecithin and recombination of this apoLp A-I-lysolecithin complex with the lipid species present as liposomes. In this last step lysolecithin is competitively substituted by the *binding phospholipid* under the conditions of dialysis.

This method was applied to the recombination of apoLp A-I with phosphatidylcholine and sphingomyelin containing 18-azidolinoleoyl residues. ApoLp A-I-*phospholipid complexes* were obtained which did not differ in their chromatographic behaviour, stoichiometry or properties from those prepared with the corresponding ^{13}C or unlabelled phospholipids. The lipoprotein complexes eluted from Bio-Gel A-5m columns separated well from the excess liposomes, the protein peak and that of the radioactive azidophospholipids coinciding exactly. Fig. 1a and 1b represent the purification of apoLp A-I-phospholipid complexes by Bio-Gel A-5m chromatography. It should be mentioned *that all chroma*tographic procedures, concentrations of fractions and storage were carried out with protection from UV light in order to avoid the decomposition and thereby premature reactions of the *azido group*. The lipoprotein complexes containing either the lecithin or sphingomyelin retain the antigenic sites exposed for the reaction with Lp A-I antibodies (Fig. 5).

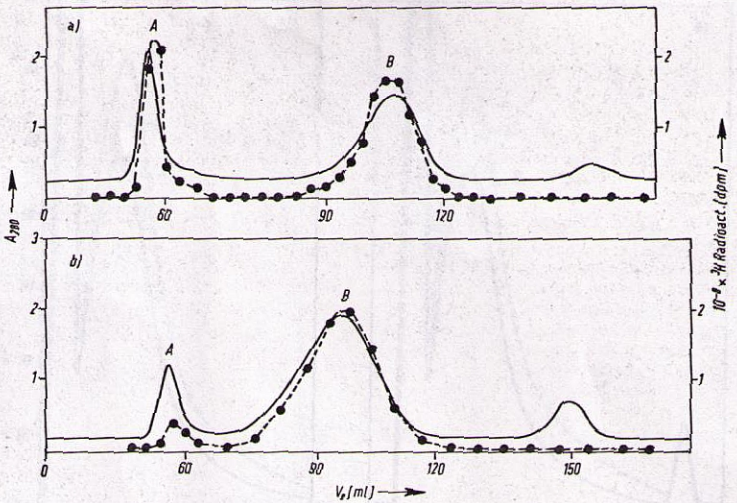

Fig. 1. Bio-Gel A-5m chromatography of the complexes of apolipoprotein A-I with a) phosphatidylcholine and b) sphingomyelin, both substituted with 18-azido-[9,10,12,13-$^3H_4$]linoleic acid, before photo-cross-linking. Elution buffer: 0.1 M Tris/HCl, pH 8.2, 150 mM NaCl, 1 mM EDTA and 0.02% NaN_3; column: 90 × 1.5 cm. A: excess liposomes consisting of lysolecithin and 1,2-bis(18-azido-[9,10,12,13-$^3H_4$]linoleoyl)-*sn*-glycero-3-phosphocholine; B: recombined *apoLp A-I* azidolecithin complex. Solid line: absorbance at 280 nm; broken line: radioactivity.

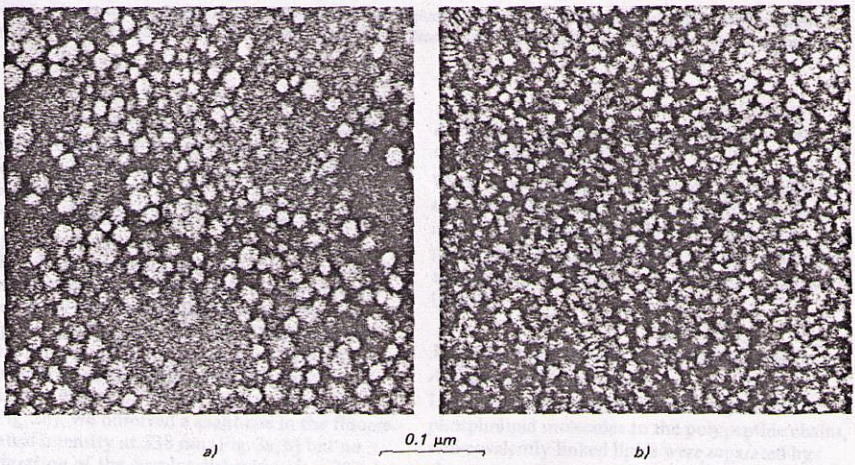

Fig. 2. Electron microscopy of the complex of apolipoprotein *A-I with sphingomyelin* containing the 18-azido-linoleoyl residue a) before and b) after photolysis. Magnification 1:180000.

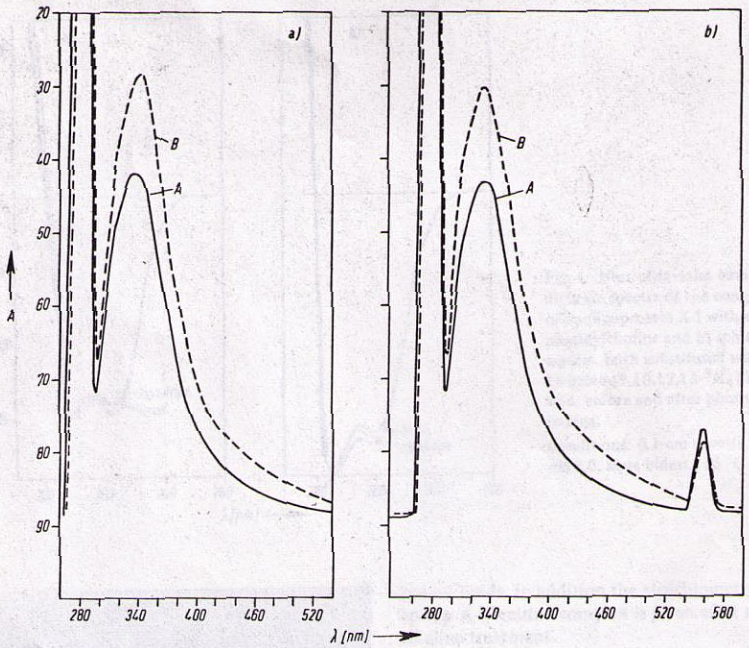

Fig. 3. Fluorescence spectra of the complexes of apolipoprotein A-I with a) phosphatidylcholine and b) sphingomyelin, both substituted with 18-azido-[9,10,12,13-$^3H_4$]linoleic acid, before (A) and after (B) photo-cross-linking.

2) Cross-linking of apoLp A-I and radioactive phosphatidylcholine and sphingomyelin containing ω-azidolinoleoyl residues by UV irradiation

The purified lipoprotein complexes, which also appear homogeneous in electron microscopy (Fig. 2a), were then submitted to UV irradiation in order to generate the highly reactive nitrene group for the cross-linking with neighbouring lipid or polypeptide segments. This irradiation was carried out in oxygen-free medium with careful control of the tryptophan fluorescence, of circular dichroism and electron microscopy (Fig. 2b). We observed a slight rise in the fluorescence intensity at 338 nm (Fig. 3a, b) but no alteration of the circular dichroism after 30 min of irradiation. The apoLp A-I-lecithin and apoLp A-I-sphingomyelin complex showed unaltered ellipticities, Fig. 4a, b.

When both lipoprotein complexes were tested for their antigenicity by the immunodiffusion technique[18], no changes could be observed compared with free apoLp A-I and the non-irradiated apoLp A-I-azido-phosphatidylcholine complex (Fig. 5).

3) Characterization of the cross-linked apoLp A-I/phospholipid complex

In order to prove the covalent linkage of the phospholipid molecules to the polypeptide chains, non-covalently linked lipids were separated by chromatography on Sephadex LH-20 column using a water/2-butanol/acetic acid (5:4:1) mix-

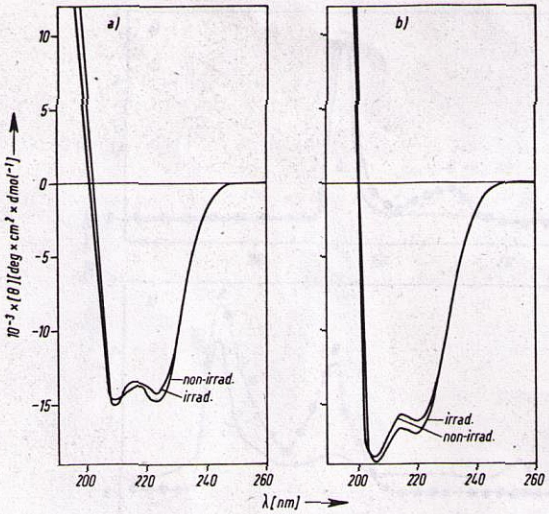

Fig. 4. Near ultraviolet circular dichroic spectra of the complexes of apolipoprotein A-I with a) phosphatidylcholine and b) sphingomyelin, both substituted with 18-azido-[9,10,12,13-$^3H_4$]linoleic acid, before and after photo-cross-linking.

Conditions: 0.1-cm cuvette, pH 8.0, aqua bidest., 25 °C.

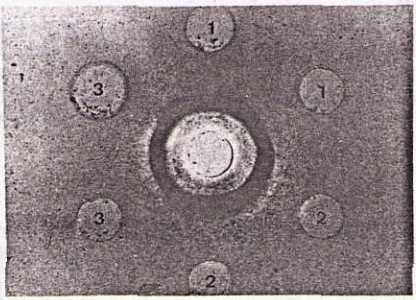

Fig. 5. Immunodiffusion test of anti apoLp A-I against 1) apoLp A-I; 2) complex apoLp A-I/1,2-bis(18-azido-[9,10,12,13-$^3H_4$]linoleoyl)-*sn*-glycero-3-phosphocholine; 3) the same complex after photo-cross-linking procedure.

ture as eluent[20]. The lipid separated well from the lipopolypeptide as shown in Fig. 6a and b.

The table summarizes the chemical analysis of the purified complexes before and after irradiation and after Sephadex LH-20 chromatography which leads to the separation of non-covalently bound lipids. In addition the stoichiometry of the apoLp A-I/lecithin complex is given after mild alkaline treatment.

The pooled fractions with cross-linked products were freed from organic solvent by dialysis against mixtures of acetic acid/water with decreasing acetic acid (10% to 0). The water-soluble covalently bound lipoprotein was then concentrated by ultrafiltration. A mild alkaline treatment (pH 11) for 2 h at 37 °C followed, to hydrolyze all non-covalently-linked fatty acids. The alkali-*treated photoaffinity labelled complexes were* then further characterized by dodecylsulfate gel electrophoresis. The two lipoproteins migrated as homogeneous radioactive bands, which coincided with the protein band, Fig. 7a and b.

Whereas the labelled sphingomyelin/apoLp A-I complex migrated only as one radioactive protein band, the labelled phosphatidylcholine/apoLp A-I sample yielded two radioactive bands, one of which is due to the cross-linked product of the radioactive 18-azidolinoleic acid of lecithin covalently bound to apoLp A-I (peak A), the fastest running band (peak B), however, is due to radioactive fatty acids.

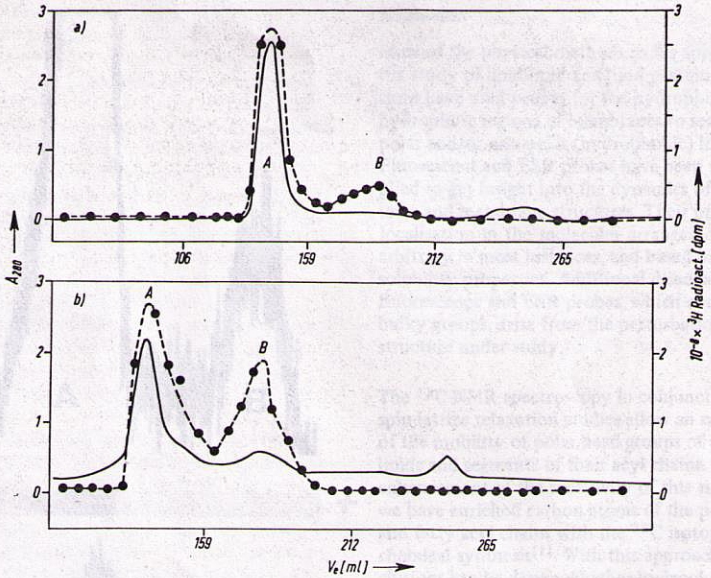

Fig. 6. Chromatography on Sephadex LH-20 column (90 x 2.5 cm) of the complexes of apolipoprotein A-I with a) phosphatidylcholine and b) sphingomyelin, both substituted with 18-azido-[9,10,12,13-$^3H_4$]linoleic acid after photo-cross-linking.

Elution solvent: water/2-butanol/acetic acid 5:4:1. A: lipopolypeptide; B: free phospholipid. Solid line: absorbance at 280 nm; broken line: radioactivity.

Table. Stoichiometry of the complexes of apoLp A-I with 18-azido-[^{3}H]phospholipid complexes before and after photolysis.

	Molar proportions of		
	apoLp A-I	Phosphatidylcholine	Lysolecithin
Before irradiation	1	68	24
After irradiation	1	72	28
After Sephadex LH-20 chromatography	1	29	5
After alkaline treatment	1	17*	0
	apoLp A-I	Sphingomyelin	Lysolecithin
Before irradiation	1	78	33
After irradiation	1	76	37
After Sephadex LH-20 chromatography	1	23	2

* Number of [^{3}H]fatty acids linked to the polypeptide chain. This figure resembles the number of phospholipids which contain only one 18-azido-[^{3}H]linoleic acid.

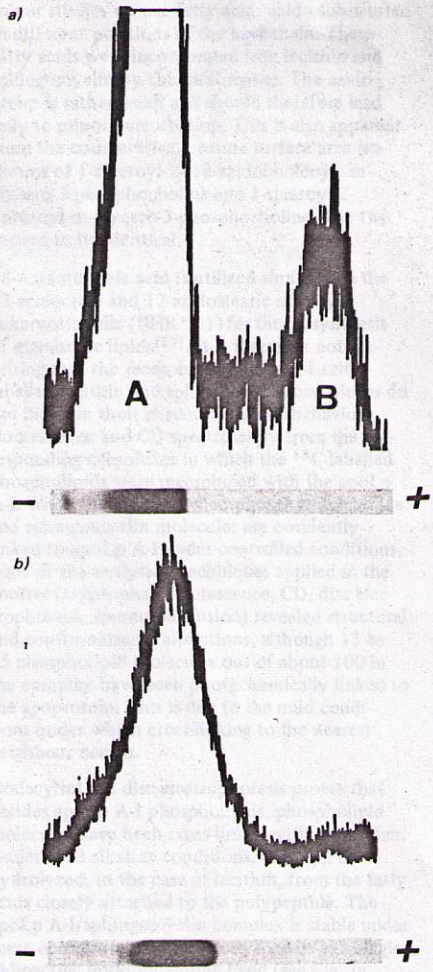

Fig. 7. Dodecylsulfate polyacrylamide gel (10%) electrophoresis of the purified cross-linked complexes of apolipoprotein A-I with a) phosphatidylcholine and b) sphingomyelin, both substituted with 18-azido-[9,10,12,13-$^3H_4$]-linoleic acid.

For experimental details see Methods and Materials. A: apoLp A-I/azidolecithin. B: radioactive lipid, dissociable under the conditions of sample preparation.

Discussion

Most of the physical methods so far applied to the study of lipid-lipid and lipid-protein interactions have used probes for the hydrophobic and hydrophilic regions of membranes to search for polar and/or non-polar (hydrophobic) interactions. Fluorescent and ESR probes have been widely applied to get insight into the dynamics of lipoproteins and membrane structures. Their presumed localisation in the molecular arrangement is arbitrary in most instances, and based on their solubility properties. Additional disadvantages of fluorescence and ESR probes, which consist of bulky groups, arise from the perturbation of the structure under study.

The ^{13}C-NMR spectroscopy in conjunction with spin-lattice relaxation studies allow an estimation of the mobility of polar head groups of phospholipids and segments of their acyl chains. For the enhancement of the sensitivity of this method, we have enriched carbon atoms of the polar group and fatty acyl chains with the ^{13}C isotope by chemical synthesis[1]. With this approach, conclusions can be drawn whether hydrophobic or polar interactions stabilize structures such as lipoproteins[3,4] or simple membranes, e.g. the envelope of lipid-containing viruses (VSV)[5,6].

However, none of these techniques gives insight into the topography of fatty acids and polypeptide chains in hydrophobic interactions. One promising approach is the photoaffinity labelling technique. Photosensitive arylazido groups have been introduced into haptenic groups[23], membrane components have been statistically labelled with 1-azido-4-iodobenzene[24], surface proteins of erythrocytes with *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-taurine)[25] and the ouabain-binding sites with ethyldiazomalonyl-cymarin[26]. The azido group attached to fatty acid chains can be decomposed by UV irradiation to highly reactive intermediate nitrenes, which may immediately react by insertion into C-H bonds with the formation of secondary amines, or by addition to double bonds with aziridin formation, thus leading to cross-links with lipids or polypeptide side chains in the immediate neighbourhood to the nitrene (for review of nitrene reactions see ref.[9,10,27,28]).

In our studies we use fatty acids azido substituted in different positions of the acyl chain. These fatty acids were incorporated into lecithin and sphingomyelin by chemical means. The azido group is rather small and should therefore lead only to minor perturbation. This is also apparent from the comparative pressure surface area isotherms of 1-stearoyl-2-(18-azidolinoleoyl)-*sn*-glycero-3-phosphocholine and 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine. The two proved to be identical.

18-Azidolinoleic acid is utilized similarly to the 12-azidooleic and 12-azidostearic acid by eukaryotic cells (BHK* 21) for the biosynthesis of membrane lipids[29]. It is therefore not surprising that the recombined apoLp A-I azido-labelled lecithin and sphingomyelin complexes do not differ in their chromatographic behaviour, fluorescence and CD spectroscopy from the corresponding complexes in which the ^{13}C-labelled phospholipids were recombined with the apoLp A-I. When the azido-labelled phosphatidylcholine and sphingomyelin molecules are covalently linked to apoLp A-I under controlled conditions, none of the analytical techniques applied in the control (tryptophan fluorescence, CD, disc electrophoresis, immunodiffusion) revealed structural and conformational alterations, although 15 to 25 phospholipid molecules out of about 100 in the complex have been photochemically linked to the apoprotein. This is due to the mild conditions under which cross-linking to the nearest neighbour occurs.

Dodecylsulfate disc electrophoresis proves that besides apoLp A-I phospholipids, phospholipid molecules have been cross-linked with each other. Under mild alkaline conditions, they can be hydrolyzed, in the case of lecithin, from the fatty acids closely attached to the polypeptide. The apoLp A-I/sphingomyelin complex is stable under these conditions and therefore appears as a single radioactive lipopolypeptide band (see Fig. 7a and b).

The results reported in this communication demonstrate at the level of a simple model the method of photoaffinity cross-linking of azido-labelled phospholipids with apolipoprotein polypeptide chains. They give chemical proof for the hydrophobic lipid-protein interactions. The azido group in the 18-position of linoleic acid has no chance to arrange in a hydrophilic region. The method makes the exact location of the cross-links between the fatty acid chains and the side chains of the polypeptide feasible, particularly since the amino acid sequence of this apolipoprotein is well known[30]. These results will be reported in a forthcoming paper.

While this work was in progress, a paper by Chakrabarti and Khorana[31], also describing the synthesis of fatty acids and phospholipids labelled with photosensitive groups, came to our attention.

* Baby hamster kidney.

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