Hoppe-Seyler's Z. Physiol. Chem.
Bd. 360, S. 685–690, Juni 1979

Surface Localization of Apolipoprotein AII in Lipoprotein-Complexes

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(Received 7 February 1979)

Summary: Lipoprotein particles reconstituted from the apolipoprotein AII (apo AII) component of human serum high density lipoprotein, phosphatidylcholine and lysophosphatidylcholine were covalently linked to the imidoester groups of a polystyrene residue. Apo AII was proteolytically digested with thermolysin after delipidation. The covalently bound peptides remaining at the resin were cleaved and separated by combined two-dimensional electrophoresis/chromatography. The peptides were isolated, hydrolyzed and their amino acid composition determined. They were assigned to the apo AII sequence. Since the imidoester groups on the surface of the resin carrier cannot react with buried lysine residues, this method gives strong chemical evidence for the spreading of the apo AII polypeptide chain over the surface of the lipoprotein particle, as far as the sequence carrying lysine residues between residue 22 and 55 of each symmetrical half is concerned.

Lokalisation des Apolipoproteins AII in der Oberfläche von Lipoprotein-Komplexen

Zusammenfassung: Lipoprotein-Partikeln wurden durch Rekombination von Apolipoprotein AII des menschlichen Serum-High-Density-Lipoproteins, Phosphatidylcholin und Lysophosphatidylcholin hergestellt und kovalent an durch Imidoester-Gruppen aktiviertes Harz gebunden. Nach Delipidierung wurde das Apoprotein-AII mit Thermolysin hydrolysiert, die kovalent gebundenen Peptide vom Harz abgespalten, durch kombinierte zweidimensionale Elektrophorese und Chromatographie getrennt, isoliert, ihre Aminosäure-Zusammensetzung bestimmt und die Peptide der Sequenz des Apolipoproteins AII zugeordnet. Die Analysen-Daten, die auf einer Methode beruhen, nach der nur an der Oberfläche gelegene Lysin-Reste des Apoproteins mit der Oberfläche des Imidoester-Harzes reagieren können, geben Evidenz für die Anordnung des Apo-AII-

Enzymes:
Phospholipase A_2, phosphatide 2-ácylhydrolase (EC 3.1.1.4); thermolysin (EC 3.4.24.4).
Abbreviations:
Apo AII = apolipoprotein AII of human serum high density lipoprotein; HDL = human high density lipoprotein.
Trivial names:
Polyenephosphatidylcholine, soy-bean phosphatidylcholine with more than 70% linoleic acid residues among its fatty acids; lysophosphatidylcholine was enzymatically prepared from polyenephosphatidylcholine and then catalytically hydrogenated.

0018-4888/79/0360-0685$02.00

Polypeptids in der Oberfläche der Lipoprotein-Partikel, mindestens soweit dies die Sequenz zwischen den Lysinen 22 und 55 einer jeden der symmetrischen Hälften des Apo AII betrifft.

Key words: Imidoester resin, covalent binding of apoprotein AII, proteolytic cleavage, peptide analysis, assignment to sequence.

Structural studies on human serum high density lipoprotein particles aim at the elucidation of the topological arrangement of the apoproteins and lipid components. On the basis of the stoichiometry and ^{13}C nuclear magnetic resonance studies with apoprotein and ^{13}C-enriched phospholipids[1], and with native HDL preparations in which the lipid classes have been exchanged, by means of the cholate exchange methods[2], against ^{13}C-labelled lecithin species, cholesterol and cholesterol ester, a model of HDL has been proposed in which the apoproteins, together with the polar head groups of the phospholipids, cover the surface[3] of the particle, which is 80 and 120 Å in diameter. Models proposed subsequently by others[4–6] agree well with this general concept. This paper describes an approach to obtain experimental evidence for the surface localization of apoproteins in lipoprotein complexes. A simple model has been chosen for the sake of a convincing analysis. Apolipoprotein AII has been recombined with phosphatidylcholine and the particles (80–100 Å Ø) covalently linked to imidoester-activated polystyrene beads. Due to the size of the beads, chemical crosslinking can only occur with groups on the surface of the HDL particles. Apo AII of this lipoprotein complex binds to the resin with lysine residues distributed between residues 22 and 55 of each identical half of apo AII, each of which consists of 77 amino acid residues[7]. This proves the surface arrangement of large polypeptide sequences of apo AII in the lipoprotein complex and exemplifies its more general applicability for studies of the topography of apolipoproteins.

Methods and Materials

HDL was isolated from human serum by the sedimentation-flotation centrifugation procedure in a density range between 1.063 and 1.21 g × cm^{-3}[8]. Apolipoprotein AII (apo AII) was isolated from delipidated HDL apolipoproteins by DEAE-cellulose chromatography in 8M urea and an increasing Tris/HCl buffer gradient (0.04–0.09M)[9]. The apo AII was rechromatographed on Sephadex G-100 (6M urea, 0.1M Tris/HCl, pH 8.3)[10] and obtained in homogeneous form, as proven by polyacrylamide gel electrophoresis[11,12].

Recombination

The recombination of apo AII phosphatidylcholine and lysolecithin was carried out as described before[13]. The separation of the lipoprotein particles from excess lipid was achieved by Bio-Gel A-5m column chromatography (1.6 × 90 cm) with 0.1M Tris/HCl, 0.15M NaCl, 0.001M EDTA, 0.02% NaN_3, pH 8.2 as eluant.

The density of the recombined lipoprotein particles was determined by continuous CsCl density gradient (11–20.5%) centrifugation at 38000 rpm in a Beckman SW 41 rotor for 72 h. 1-ml fractions were collected for measuring the density, protein, phosphorus and radioactivity. Protein was determined according to Lowry et al.[14], phosphorus according to Bartlett[15].

Molecular weights of the recombined lipoprotein particles were determined on a calibrated Bio-Gel A-5m column with an accuracy of ± 7000.

Lysophosphatidylcholine was prepared by phospholipase A_2 hydrolysis of polyenephosphatidylcholine (kindly provided by Nattermann & Cie., Köln) by standard procedures. Peptide mapping was carried out by electrophoresis in the 1st dimension at 400 V and 15–20 mA per plate for 2–2.5 h with water cooling and chromatography (2nd dimension) for 8–9 h. 20 × 20 cm plates coated with cellulose (Schleicher & Schüll, Düren) were used. Dansylated arginine was added as internal fluorescent marker to determine the relative mobility of the peptides. Plates were sprayed with ninhydrin reagent and stored for superficial colour development for 1–2 days at room temperature in the dark. Peptide spots were scraped from the plates and collected on a glass-fiber membrane filter (Sartorius SM 13400) pressed into the bottom of a Pasteur pipette. Excess ninhydrin was eluted with 0.5 ml acetone. Peptides were eluted with either 50% acetic acid or 6N HCl for complete acid hydrolysis in 0.5–1.0 ml 6N HCl (24 h at 110 °C) or 12N HCl/propionic acid 1:1 (v/v) 20 min at 160 °C[16] with subsequent amino acid analysis.

Amino acid analysis was performed on a home-made analyser with the one-column method (0.32 × 25 cm, Durrum DC-4A resin and citrate buffers of pH 3.25, 3.95 and 4.75) and the amino acids detected with *o*-phthalaldehyde[17] in a fluorescence detector (Fa. Knauer, Berlin). 100–500 pmol per amino acid was sufficient for an excellent quantitative detection. The dansyl method was used for end-group determination[18].

[*N*-$^{14}CH_3$]Phosphatidylcholine (soy bean) was prepared by the demethylation-remethylation procedure described before[19].

Imidoester-resin

200 mg chloromethylated polystyrene-divinylbenzene resin (2% crosslink) (Bio Rad Laboratories, Richmond, Calif.) and 150 mg sodium cyanide were suspended in 4 ml ethanol and 1.5 ml water and refluxed for 3 to 4 h. The resin was thoroughly washed with ethanol, dried under vacuum, then suspended in dry dioxane and 500 µl methanol added. Dry gaseous HCl was introduced over 30 min. After a reaction time of 4 days at 4 °C, the derivatized resin was washed with ether and ether/methanol 3:1 (v/v) and stored at − 15 °C in a desiccator.

Titration with a 0.1mM lysine hydrochloride solution suggested a 10% derivatization of chloromethyl groups.

Crosslinking the lipoprotein to the imidoester resin

Imidoester resin (200 mg with 25 µmol imidoester groups) was reacted with apo AII-phosphatidylcholine complex (12 mg apo AII, 0.7 µmol) containing 12.5 µmol lysine in 6 ml 0.1M triethanolamine buffer pH 9 for 10 h at room temperature with stirring. The resin was washed three times with the same buffer and with water, dried in a vacuum desiccator and delipidated with chloroform/methanol 2:1 (v/v) and ether.

Thermolysin proteolysis

Apo AII covalently linked to the imidoester resin was treated with thermolysin (Boehringer Mannheim GmbH) (50:1 w/w protein/enzyme) in 0.05M ammoniumhydrogencarbonate buffer, pH 8.0, for 16–20 h at 37 °C. The resin with the residual peptides was filtered and washed with the buffer and water. The peptides were cleaved from the resin by incubating the resin with ammonia-formic acid 15:1 (v/v) at room temperature for 8–10 h. The resin was removed by filtration and the peptides obtained by lyophilisation of the filtrate.

Peptide analysis

The peptide mixture was separated under the conditions of the fingerprint analysis[20,21] using the pyridine/acetic acid/acetone/water 1:2:8:40 (v/v) system as electrophoresis buffer in the first dimension and butanol/acetic acid/pyridine/water 75:25:50:60 (v/v) for chromatography in the second dimension.

Results

Preparation and characterisation of apo AII phosphatidylcholine-lysophosphatidylcholine complex

Apolipoprotein AII and hydrogenated lysophosphatidylcholine were combined with phosphatidylcholine vesicles as described before[22].

The reconstituted lipoprotein complex was obtained after dialysis and column chromatography on Bio-Gel A-5m free of excess lipid and non-recombined apolipoprotein AII (10%). The particles formed an electron-microscopically homogeneous population which eluted from a calibrated Bio-Gel A-5m column (1.6 × 90 cm) with an apparent molecular weight of 170000–190000. Masket electrophoresis[23] revealed one single lipoprotein band, the radioscan of which coincided with the band stained for protein and lipid.

The following stoichiometry resulted from protein,phosphorus and radioactivity determination: 1 apo AII molecule was associated with 17.5 molecules of lysolecithin and 80 molecules of either 1-stearoyl-2-linolenoyl-3-*sn*-glycerophosphocholine or soy-bean.

Synthesis of solid-phase-linked imidoester

In order to bind only those lysine residues of apoprotein AII protruding from the surface of the lipoprotein complex, the reactive imidoester groups of the solid phase resin should not extend too far from the solid phase matrix. We used chloromethylated polystyrene crosslinked with divinylbenzene (2%), which is generally applied in the Merrifield synthesis[24]. In a Pinner reaction with sodium cyanide, the corresponding nitrile was prepared, then transformed into the benzylimidate group with HCl/methanol. 0.1–0.12 meq imidoester groups were present per gram resin.

Binding of apo AII-phosphatidylcholine particles to imidoester resin

A suspension of the imidoester resin and the reconstituted apo AII-polyenephosphatidylcholine particles were allowed to react. As a control, the lipoprotein particles were reacted in the same way with the chloromethylated resin. The pelleted products were then delipidated with chloroform/

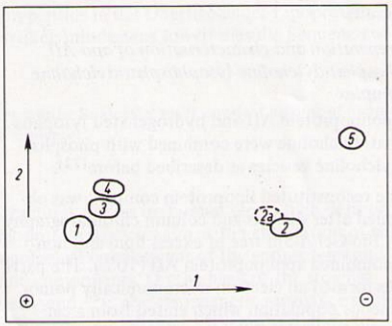

Fig. 1. "Fingerprint" analysis of peptides released from an imidate resin by treatment with conc. ammonia/formic acid 15:1 (v/v).

The apo AII-phosphatidylcholine-lysophosphatidylcholine complex was linked covalently to a resin substituted with imidate groups, then delipidated and treated with thermolysin.

1st dimension: electrophoresis, 2nd dimension: chromatography. For details see Methods and Materials.

methanol[25]. Thermolysin hydrolysis removed large parts of the apo AII sequence, except for those peptides linked to the resin. These peptides were cleaved from the resin and separated by fingerprint analysis, Fig. 1. This revealed three neutral (peptides 1, 3 and 4) and two basic peptides (peptides 2 and 5). The spots of the five peptides were eluted, the peptides hydrolyzed and their amino acid analysis carried out using the *o*-phthalaldehyde method and a fluorescence detector[12].

Table. Amino acid analysis of peptides linked covalently to the imidoester resin and remaining after thermolysin proteolytic treatment of the delipidated apo AII-imidoester resin.

Peptide	Peptide sequence	Position in AII
1	Gly-Lys-Asp	22– 24
2	Ala-Glx-Ala-Lys	36– 39
3	Ser-Lys-Glu-Glx	45– 48
4	Lys-Ala-Gly-Thr-Glu	55– 59
5	Lys	(54–)55

The short imidoester side chains of the resin span crosslinks to lysine residues 23, 39, 46 and 55. The amino acids around these covalently linked lysine residues are determined by amino acid analysis after cleavage of the peptide with conc. ammonia/formic acid 15:1 (v/v). The results are summarized in the table.

Discussion

The present concept of the molecular arrangement of the main HDL apoproteins AI and AII, polar (phosphatidylcholine, sphingomyelin, cholesterol, traces of glycosphingolipid) and apolar lipids (cholesterol esters and traces of triglycerides) commonly present in HDL particles suggests that the apolipoproteins together with the polar lipids form a spherical shell surrounding the apolar nucleus containing the cholesterol esters[1,3–6].

Since X-ray crystallographic studies are out of the question for structural analysis of lipoproteins, we have initiated a chemical approach which aims at the elucidation of the topography of the polypeptide chains of the HDL apoproteins. Bifunctional crosslinkers of defined length were used to crosslink lysine residues within the HDL polypeptide chains. This strategy is feasible because the primary structures of apoproteins AI, AII and the C-proteins of HDL are known. This report presents data which give conclusive evidence for the surface arrangement of apo lipoprotein AII in an apo AII-phosphatidylcholine lipoprotein model particle. It is simultaneously an example of the method with broader applicability. Fig. 2 outlines the analytical steps of our approach.

Imidoester groups linked via methylene groups to the backbone of polystyrene resin beads analogous to the Merrifield resin in solid phase peptide synthesis[24] were reacted with the apo AII-phosphatidylcholine complex. The ϵ-amino groups of those lysine residues which are available for the crosslinking reaction and covalently bound to the resin must protrude from the surface, because the imidoester group of the resin cannot enter the particle, due to the very short methylene arms. Apo AII contains 9 lysine residues in each half of the symmetrical molecule. They are concentrated between residues 23 and 55 (residues 23, 28, 30, 39, 44, 46, 54 and 55). Five peptides were cleaved from the resin after delipidation and thermolysin

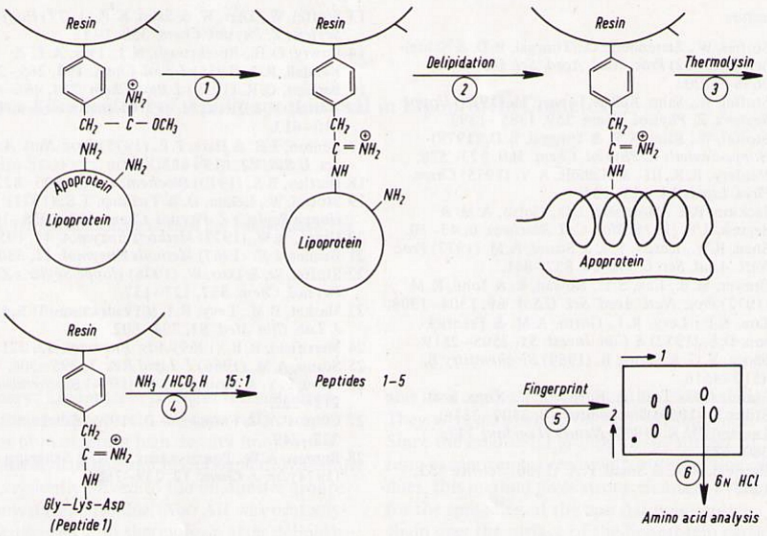

Fig. 2. Schematic presentation of analytical steps.
1) Coupling of lipoprotein complex to imidoester resin.
2) Delipidation.
3) Thermolysin hydrolysis.
4) Cleavage of peptides from resin (exemplified for peptide 1).
5) Thin-layer chromatographic separation of peptides.
6) Amino acid analysis of single peptides.

treatment of the covalently linked complete apo AII polypeptide chain. The crosslinked lysine residue were those in position 23, 39, 46 and 55. Lysine 3 was not attached to the beads, since no peptide sequence around this lysine has been detected. We interprete these results as follows:

1) Only lysine in position 3 is not accessable for the crosslinking imidoester groups of the resin beads.
2) However, most of the polypeptide chains of apo AII must be spread on the surface of the lipoprotein particle.
3) These lysine residues belong to those sequences which form extended α-helical segments when the secondary structure is predicted* from the primary structure according to empirical rules [26–28] and in agreement with circular dichroism measurements*.
4) In these α-helices, most of the lysine side chains are oriented toward the surrounding water environment.

Our finding supports the notion of amphipathic helices in which hydrophilic side chains are oriented to one side, and the hydrophobic residues to the other. In a forthcoming paper, a topographic chemical analysis of the neighbouring polypeptide sequences in this apo AII-phosphatidylcholine complex will be described in which bifunctional reagents served as tools*.

Acknowledgment: We are grateful for the support of our studies by the *Deutsche Forschungsgemeinschaft*.

* Stoffel, W. & Preißner, K., unpublished.

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