Hoppe-Seyler's Z. Physiol. Chem.
Bd. 360, S. 691–707, Juni 1979

Conformational Analysis of Serum Apolipoprotein AII in Lipoprotein Complexes with Bifunctional Crosslinking Reagents

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(Received 19 March 1979)

Summary: Apolipoprotein AII isolated from human serum high density lipoproteins was recombined with phosphatidylcholine to yield homogeneous particles of 80–120 Å diameter. The radioactive bifunctional crosslinkers dimethyl [1,1′-^{14}C]suberimidate and dimethyl 4,4′-dithiobis([1-^{14}C]butyrimidate) were reacted with these particles. The kinetics of the reactions and the localisation of the crosslinked lysines of the polypeptide chains were determined. Thermolysin hydrolysis followed by two-dimensional separation of the peptides and isolation of the mono- and bifunctionally modified peptides allowed the assignment of the crosslinked peptides of the apoprotein AII sequence. The crosslinking pattern indicates a close-neighbour relationship (13–15 Å) of the peptide chains between amino acid residues 3, 23, 46 and 55 of the symmetrical halves of the apo AII molecule. A reconstruction of the secondary structure of Apo AII in the lipoprotein complex on the basis of theoretical calculations is given and correlated with the chemical data.

Konformationsanalyse des Serum-Apolipoproteins AII in Lipoproteinkomplexen mit bifunktionellen Quervernetzungsreagenzien

Zusammenfassung: Apolipoprotein AII aus menschlichem Serum-High-Density-Lipoprotein wurde mit Phosphatidylcholin zu homogenen Partikeln von 80–120 Å Durchmesser rekombiniert. Die radioaktiven bifunktionellen Quervernetzer Dimethyl-[1,1′-^{14}C]suberimidat ($SbiMe_2$) und Dimethyl-4,4′-dithiobis([1-^{14}C]butyrimidat) ($(SBtMe)_2$) wurden mit diesen Partikeln zur Reaktion gebracht. Die Kinetik der Reaktionen und die Lokalisation der quervernetzten Lysine in den Polypeptidketten wurden bestimmt. Hierzu wurden Thermolysin-Spaltungen mit anschließender zweidimensionaler Peptidtrennung und Isolierung der mono- und bifunktionell modifizierten Pep-

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Enzymes:
Phospholipase A_2, lysolecithin acylhydrolase (EC 3.1.1.4); thermolysin (EC 3.4.24.4).

Abbreviations:
HDL = human high density lipoproteins (d = 1.065 – 1.21 g/cm^3); Apo AII = apolipoprotein AII of human serum high density lipoprotein; $SbiMe_2$ = dimethyl suberimidate; $(SBtMe)_2$ = dimethyl 4,4′-dithiobisbutyrimidate, PPO = 2,5-diphenyloxazol.

Trivial name:
Polyenephosphatidylcholine, soy-bean phosphatidylcholine with more than 70% linoleic acid residues among its fatty acids.

0018-4888/79/0360-0691$02.00

tide durchgeführt. Die quervernetzten Peptide wurden nach Spaltung $(SBtMe)_2$ bzw. Abspaltung $(SbiMe_2)$ des Quervernetzers getrennt und dann die Stöchiometrie der Aminosäuren in den Peptiden durch Aminosäureanalyse nach Totalhydrolyse bestimmt. Das Quervernetzungsmuster läßt auf eine enge Nachbarschaft (13–15 Å) der Polypeptidketten zwischen den Lysinresten 3 über 23 und 46 zu 55 der symmetrischen Molekülteile des Apo AII schließen. Auf der Grundlage des Quervernetzungsmusters wurde die Sekundärstruktur rekonstruiert. Diese Daten wurden mit theoretischen Berechnungen zur Sekundärstruktur korreliert.

Key words: Apolipoprotein AII – phosphatidylcholine complex, bifunctional crosslinkers, proteolytic cleavage, peptide analysis, sequence assignment and apo AII topography.

Structural studies concerned with the arrangement of the lipid components and the apoproteins in human serum high density lipoprotein particles have been based mainly on physical techniques such as ^{13}C and ^{31}P NMR spectroscopy, which at most allow the study of overall and averaged interactions of lipids and apoproteins and their possible overall arrangement[1–4].

Since X-ray crystallography of the HDL particle is at the moment out of the question, we turned our efforts to chemical approaches to study the nearest neighbour relationship of lipids and apoproteins and the topography of polypeptide chains in a lipoprotein particle.

The photoaffinity technique, in which the covalent crosslinking of phospholipids substituted with azido-labelled fatty acids of high specific activity are used to determine which polypeptide regions of apo AI and AII interact with the hydrophobic part of the phospholipid molecules, is one approach[5,6].

In this paper we describe another strategy which should reveal the folding of the polypeptide chains in a lipoprotein complex. A prerequisite of the approach is the knowledge of the primary structure of the apolipoprotein.

Bifunctional crosslinkers such as dimethyl suberimidate and dimethyl dithiobisbutyrimidate, which have previously been applied only to study the subunit structure of ribosomal proteins[7] and the protein arrangements of erythrocyte membranes[8–10], mitochondria[11] and bacterial membranes[12], were reacted with intact lipoprotein particles.

Apo AII-lecithin lipoprotein particles were reconstituted and treated with the radioactive bifunctional crosslinkers dimethyl suberimidate $(SbiMe_2)$ and dimethyl dithiobisbutyrimidate $(SBtMe)_2$. The delipidated apoproteins, the polypeptide chains of which were linked by the radioactive crosslinking molecules, were then fragmented by proteolytic enzymes. The radioactive peptides were isolated by the fingerprint technique. Either they were radioactive due to monosubstitution by one of the bifunctional crosslinking reagents, and yielded the same peptide after cleavage of the crosslinker, or the crosslinker carried two peptides at its terminal ends. These were the sequences of the apoprotein separated from one another by the length of the crosslinker (13 Å for $SbiMe_2$ and 16 Å for $(SBtMe)_2$).

The cleavage of the crosslinker followed by the separation of the two peptides by two-dimensional electrophoresis/chromatography and amino acid analysis of the two peptides allows the assignment of neighbouring polypeptide sequences.

The spatial arrangement of the apoprotein in the apo AII-lecithin lipoprotein complex has been deduced from the chemical modification and degradative studies.

In the preceding paper[13], we demonstrated that extended sequences of apo AII cover the surface of the reconstituted particle, as proven by the crosslinking to derivatized imidoester of polystyrene resin beads.

Materials and Methods

Synthesis of ^{14}C-labelled crosslinkers

Dimethyl [1,1'-^{14}C]suberimidate hydrochloride: A solution of 1.6 g *dibromohexane* in 2.5 ml ethanol and 0.4 g (0.3 mCi) *$Na^{14}CN$* in 1 ml water was refluxed for 2 h.

The solvent was evaporated under vacuum and the oily residue distilled. Yield: 0.5 g (87% of theoret.) 1,6-dicyanohexane, spec. act. 2.35×10^5 dpm/μmol; 0.1 μCi/μmol.

0.500 g *1,6-dicyanohexane* was dissolved in 2 ml ice-cold methanol and 15 ml absolute ether. Dry gaseous hydrochloric acid was introduced for 1/2 h and the mixture was left at 4 °C for 24 h. More dry ether (10 ml) was added. The white crystalline precipitate was isolated by centrifugation and washed intensively with dry ether. Yield: 830 mg (83% of theoret.). The crystalline product dimethyl [1,1'-^{14}C]suberimidate hydrochloride was characterized by mass spectroscopy. Typical fragments were: m/e = 200 ($M^{\oplus}$); m/e = 169 ($M^{\oplus}-OCH_3$); m/e = 128 ($M^{\oplus}-CH_2-C(=NH)OCH_3$).

4,4'-Dithiobis([1-^{14}C]butyronitrile) was made by dissolving 0.63 g (4 mmol) *1-bromo-3-chloropropane* and 0.196 g (4 mmol) *Na^{14}CN* (2 mCi) in 1.5 ml ethanol and 0.4 ml water and refluxing for 2 h. 0.988 g (6 mmol) *sodium thiosulfate* dissolved in 0.8 ml water and 0.3 ml ethanol were added and heated under reflux. Iodine was added to the hot solution with stirring until the brown colour persisted[14]. The reaction mixture was extracted with 2.5 ml methylene chloride. The product was purified by column chromatography on Kieselgel G and eluted with increasing concentrations of ether in light petroleum (6:4 v/v). Yield: 170 mg, (0.805 mmol 4,4'-dithiobis-([1-^{14}C]butyronitrile) (43% of theoret.), spec. act. 2.2×10^6 dpm/μmol; 1 Ci/mol.

Dimethyl 4,4'-dithiobis([1-^{14}C]butyrimidate) hydrochloride: 50 mg (0.25 mmol) *dithiobis([1-^{14}C]butyronitrile* was dissolved in 3 ml dry dioxane, 100 μl dry methanol was added, and gaseous HCl introduced with ice cooling for a period of 20 min. The reaction was complete after 3–4 days at 4 °C. Cold absolute ether was added and the precipitated hydrochloride isolated by centrifugation and several washings with dry ether. The imidate was stored over P_2O_5. Yield: 81 mg (0.24 mmol) (95% of theoret.); spec. act. 2.2×10^6 dpm/μmol.

Mass spectroscopy revealed the following characteristic fragments: m/e = 264 ($M^{\oplus}$); m/e = 233 ($M^{\oplus}-OCH_3$); m/e = 200 [$M^{\oplus}-(2CH_3OH)$]; m/e = 132 ($M^{\oplus}/2$).

Isolation of plasma apolipoproteins

Human HDL was prepared from plasma obtained from healthy donors by ultracentrifugation in a 60 Ti rotor at 60000 rpm by the sedimentation/flotation procedure[15]. The apolipoproteins were obtained by delipidation with chloroform/methanol (2:1 v/v), separated by DEAE-cellulose chromatography in 8M urea and an increasing Tris/HCl buffer gradient (0.04–0.09M)[16]. Apo AI and AII were subsequently rechromatographed on Sephadex G-100 (6M urea, 0.1M Tris/HCl, pH 8.3). Apo AII, which was homogeneous in polyacrylamide gel electrophoresis [17,18], was recombined with polyenephosphatidylcholine* (about 70% linoleic acid[19]).

Lysophosphatidylcholine prepared from polyenephosphatidylcholine by phospholipase A_2 hydrolysis was catalytically hydrogenated[20].

Reconstituted lipoproteins were chromatographed on Bio-Gel A-5m as described in the preceding paper[13]. Molecular weights of particles were determined on the same column, using test proteins for calibration, and their density was estimated by continuous CsCl-density-gradient centrifugation (11–20.5%) at 38000 rpm for 72 h in a Beckman SW 41 rotor. Protein was determined according to Lowry et al.[21] Polyenephosphatidylcholine* was labelled in the choline group with $^{14}CH_3$ as described before[22] (spec. act. 1 Ci/mol). Lysophosphatidylcholine obtained by phospholipase A_2 (Boehringer Mannheim GmbH) treatment of polyenephosphatidylcholine was hydrogenated with PtO_2. The lipid content and stoichiometry was calculated from the total phosphorous determination[23] and the radioactivity of polyenephosphatidylcholine.

Lipoprotein electrophoresis was carried out according to Masket[24].

Crosslinking procedure with imidoesters

The amounts of imidoester given in the legends of the figures were dissolved in 0.2M triethanolamine buffer, pH 8.9 and added to the lipoprotein (0.3 mg/ml) in the same buffer. The reaction mixture was maintained at room temperature for 1 h, then the pH adjusted to 5–6 with 6N HCl and dialysed for 48 h. After delipidation with chloroform/methanol (2:1 v/v), the apoprotein crosslinked with $SbiMe_2$ was chromatographed on a Sephadex G-75 column equilibrated with 0.1M Tris/HCl, 0.02% NaN_3, 0.01% EDTA, 0.1% mercaptoethanol and 6M urea, and the $(SBtMe)_2$-crosslinked products on a Sephadex G-100 column equilibrated with 0.1M Tris/HCl, 0.02% NaN_3, 0.01% EDTA and 6M urea, pH 8.3.

The degree of crosslinking was determined with trinitrobenzene sulfonic acid according to Habeeb[25]. 0.3–0.7 mg protein in 2 ml 4% $NaHCO_3$, pH 8.5, was incubated for 2 h at 40 °C, 0.5 ml 1N HCl and 1 ml 10% sodium dodecylsulfate were then added, and the extinction was measured at 335 nm.

When $(SBtMe)_2$ was used as crosslinker, the degree of crosslinking was determined in the following way: one of two micro-columns (0.5 × 10 cm) of Sephadex G-50 (fine) was equilibrated with 0.1M Tris buffer, pH 8.3, 6M urea, and the other with this buffer plus 0.1% mercaptoethanol. Equal portions were passed over these

* Kindly provided by *Fa. Nattermann & Cie*, Köln.

columns and the degree of crosslinking calculated from the radioactivity under the protein peaks. Protein samples were proteolytically hydrolyzed with thermolysin at an enzyme/protein ratio of 1:100, in 0.05M ammonium hydrogencarbonate buffer, pH 8, for 16 to 20 h. Samples were lyophylized and, for peptide mapping, dissolved in the electrophoresis buffer, pH 4.4 (pyridine/acetic acid/acetone/water 1:2:8:40). The two-dimensional peptide separation[26], electrophoresis at 400 V for 2–2.5 h in the first dimension and chromatography (butanol/acetic acid/pyridine/water 75:15:50:60) in the second for 8–9 h was performed on cellulose-coated thin-layer plates (20 × 20 cm) (Schleicher & Schüll) with dansylarginine as internal marker[27,28]. Peptide spots were visualized with ninhydrin[29] and incubation of the plates at room temperature for 1–2 days in the dark.

11% (w/v) 2,5-diphenyloxazol (PPO) solution in dimethylsulfoxide was sprayed on the plates to locate radioactive spots by autoradiography (fluorography). The plates were incubated in contact with Kodak No. screen X-ray film at – 90 °C for 7 to 10 days.

Ninhydrin-positive or radioactive spots were scraped from the glass plate with a sharp spatula and sucked into a pasteur pipette plugged with a glass-fiber membrane filter (Sartorius SM 13400). Ninhydrin or PPO was washed out with 0.5 m*l* acetone and the peptides with 50% acetic acid or 6N HCl, depending on their further processing (end group determination, Edman degradation or amino acid analysis).

For cyanogen bromide cleavage[30], end group determination[27] and the manual Edman degradation[31,32], established procedures were used.

Amino acid analyses were performed on a home-made analyzer with the one-column method (0.32 × 25 cm, Durrum DC-4A resin, citrate buffers pH 3.25, 3.95, 4.75) and detection of amino acids after their reaction with *o*-phthalaldehyde (Fluropa, Durrum)[33–35] and quantification in a fluorescence detector (Knauer, Berlin). Between 100 and 500 pmol amino acid gave satisfactory analytical results.

Amidine linkages between crosslinker and bridged peptides were hydrolyzed by an 8–10 h incubation in conc. NH_4OH/formic acid 15:1 at room temperature with subsequent lyophylization for the "finger print" analysis. Protein and peptide hydrolysis were carried out in 0.5–1 m*l* 6N HCl for 24 h at 110 °C or in 12N HCl/propionic acid (1:1; v/v) for 20 min at 160 °C[36].

Mass spectra were recorded with a Varian MAT CH5 mass spectrometer, emission current 300 μA at 70 eV and an acceleration voltage of 3 kV. The temperature of the ion source was 250 °C.

A Jasco 41 A circular dichrometer was used for recording CD spectra of lipoprotein complexes. The clear preparations (0.2 mg/m*l* in 1mM Tris buffer, pH 8.0, 0.15mM KF and 1mM EDTA) were measured in a 0.1 cm cuvette between 260 and 200 nm. The molar ellipticity [Θ] (deg × cm^2 × $dmol^{-1}$) was determined at 222 nm and f_H calculated according to Chen and Yang[37].

The distribution of radioactivity in the gels was analyzed by the digestion of 2-mm gel discs with 0.2 m*l* H_2O_2/0.4 m*l* $HClO_4$ (60%) in counting vials for 2 h at 60 °C or in 1 m*l* Lumagel (Packard). 10 m*l* toluene scintillator was added.

For fluorography, gels were sliced longitudinally and incubated for 30 min in dimethylsulfoxide and 2 h more in 11% PPO in dimethylsulfoxide. Gel strips were rinsed with water for 20 h, laid on filter paper, dried under vacuum and incubated with Kodak film at – 90 °C for 7–10 days.

Results

Reassociation of apo AII, polyenephosphatidylcholine and hydrogenated lysophosphatidylcholine

The procedure described before was used for the formation of these lipoprotein particles. With a molar ratio of apo AII:polyenephosphatidylcholine:lysophosphatidylcholine = 1:90:15, particles homogeneous in electron microscopy with the following properties were obtained: recombinants had a diameter of 80–120 Å, their apparent molecular weight, determined by calibrated Bio-Gel A-5m column chromatography, amounted to 170000–190000. Four to five apo AII were associated with 100 polyenephosphatidylcholine and 35 lysophosphatidylcholine molecules. The data of 18 well reproducible recombination experiments were averaged. In CsCl-density-gradient centrifugation, the lipoprotein had an average density of 1.175 g/m*l*.

Conditions of the crosslinking reaction

Apo AII-recombinants were crosslinked with $(SBtMe)_2$ in pilot experiments between pH 6 and 11. A ratio of 1:2 $(SBtMe)_2$ to lysine was chosen. The yield of covalently bound labelled $(SBtMe)_2$ per apo AII was determined after exhaustive dialysis. Fig. 1. summarizes these results and indicates that an optimal crosslinking occured between pH 9 and 11. In all further experiments, the pH of the reaction mixture was 8.9–9.2.

The kinetics of the hydrolysis of $SbiMe_2$ and $(SBtMe)_2$ were determined to discover the optimal

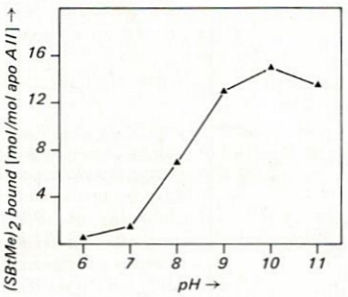

Fig. 1. pH-dependence of crosslinking reaction between apo AII-phosphatidylcholine lipoprotein complexes and [^{14}C](SBtMe)$_2$.

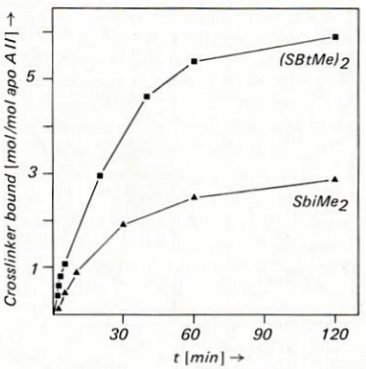

Fig. 2. Kinetics of the crosslinking reaction of apo AII-phosphatidylcholine recombinants with SbiMe$_2$ and (SbtMe)$_2$.

2 nmol apo AII dissolved in 1 ml 0.2M triethanolamine buffer, pH 8.9, and 18 nmol (SBtMe)$_2$ (0.5 mol/mol lysine) in 200 µl of the same buffer were reacted for 1 h at room temperature, at pH of 5–6 adjusted with 6N HCl and dialysed against 0.01N ammonium hydrogencarbonate buffer for 48 h. Samples of the solution were taken for radioactivity determination.

reaction times. [^{14}C]SbiMe$_2$ and [^{14}C](SBtMe)$_2$ were dissolved in 0.2M triethanolamine buffer, pH 8.9. Samples were taken at intervals and added to excess bovine serum albumin; the radioactivity bound to albumin was then determined under the conditions described in the legend to Fig. 2. Half the SbiMe$_2$ was hydrolyzed after 50–60 min, but the half-life of (SBtMe)$_2$ was 5 h. Therefore the hydrolysis constants appear to be 0.53 h^{-1} and 0.09 h^{-1}, respectively.

In order to achieve a predominantly bifunctional reaction of the crosslinker molecules, a ratio of 3 crosslinkers to 1 lysine residue was used, and the reaction stopped after 1 h by acidification with 6N HCl. Fig. 2 demonstrates the kinetics of crosslinking apo AII-PC recombinants with SbiMe$_2$ and (SBtMe)$_2$. Ratio of crosslinker to lysine 3:1.

The degree of crosslinking was determined by reacting the remaining free ϵ-amino groups of lysine with trinitrobenzene sulfonic acid[25]. Whereas in the case of SbiMe$_2$ as crosslinker, only the number of crosslinker molecules incorporated could be determined, with (SBtMe)$_2$ the degree of mono- and bifunctional crosslinking could be measured. Because of the symmetric radiolabelling, treatment with mercaptoethanol released one half of the monosubstituted (SBtMe)$_2$. The degree of bifunctional substitution was then calculated from the protein-bound radioactivity before and after mercaptoethanol treatment. Equal portions were passed over two Sephadex G-50 columns (0.5 × 10 cm), one of which was equilibrated with mercaptoethanol (for details see Materials and Methods). After 60 min about 60% of the lysine residues were crosslinked when a 3:1 ratio crosslinker/lysine was applied. Circular dicroic spectra of the aqueous solution of apo AII (Fig. 3a), the apo AII-phosphatidylcholine-complex (Fig. 3b), and of the complex after the crosslinking reaction (Fig. 3c) were recorded. It is obvious that the molar ellipticity of the lipoprotein remains unaltered by the crosslinking reaction.

Localisation of the crosslinked positions in the polypeptide chains

Thermolysin was used as endopeptidase. The peptide mixtures of the hydrolysates were separated by two-dimensional fingerprint peptide mapping on cellulose-coated thin-layer plates (20 × 20 cm), electrophoresis with pyridine/acetic acid/acetone/water 1:2:8:40, pH 4.4, in the first dimension and chromatography in n-butanol/acetic acid/pyridine/water 75:15:50:60 in the

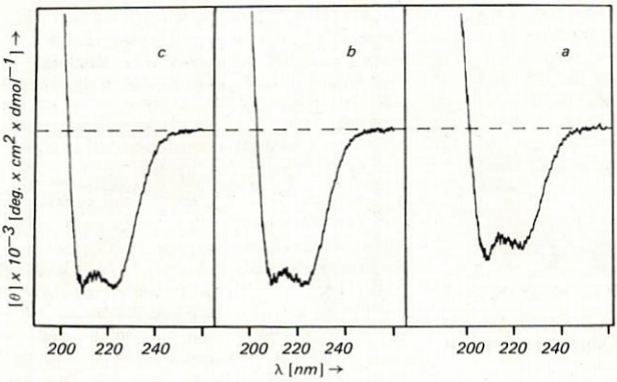

Fig. 3. Circular dichroic spectra of a) apo A II, b) apo AII-phosphatidylcholine complex c) complex after treatment with $(SBtMe)_2$. Apo AII and its lipoprotein complex were dissolved in 1mM Tris, 1mM EDTA and 1.5mM KF (0.2 mg protein/ml), 0.1 cm cuvette. Spectra were recorded between 260 and 200 nm.

second dimension[26]. Between 100 and 500 μg of the hydrolysate could be separated rapidly and effectively.

Radioactive peptide spots were visualized by autoradiography or two-dimensional scanning of the thin-layer plates. They were scraped off and the peptides eluted. Cleavage of the amidine linkages between lysine residues and mono- or bifunctionally substituted $SbiMe_2$ with conc. NH_4OH/HCOOH, 1:15, or reductive cleavage with mercaptoethanol of $(SBtMe)_2$-peptide crosslinks released two peptides in the case of bifunctional crosslinking, and one when a monofunctional substitution had occurred. Two-dimensional peptide mapping disclosed this. The peptide spots were again eluted, acid hydrolyzed and the amino acid stoichiometry determined by amino acid analysis using the sensitive fluorometric method with *o*-phthalaldehyde as reagent[33–35].

Peptides of the established amino acid stoichiometry around lysine were assigned to the known sequence of apo AII.

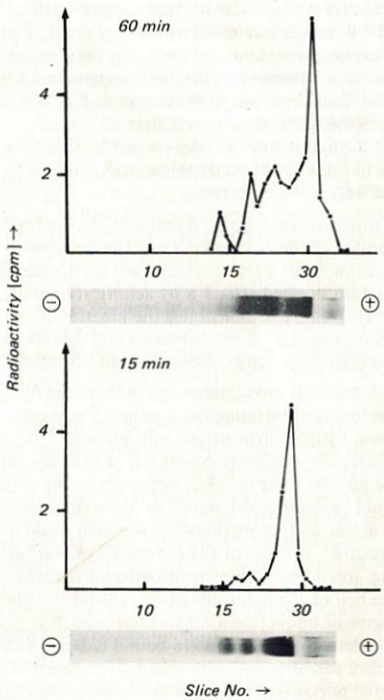

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of samples of apo AII-phosphatidylcholine complexes crosslinked with $[^{14}C]SbiMe_2$ in the presence of mercaptoethanol (11% acrylamide[18]).

Reaction of apo AII-polyenephosphatidylcholine-lipoprotein complex with dimethyl [^{14}C]-suberimidate

The apo AII-phosphatidylcholine particles were reacted with [^{14}C]SbiMe$_2$ under conditions analogous to those described in the legend of Fig. 2 and samples were taken for dodecyl sulfate polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol at defined time intervals. Fig. 4 represents the gels (stained with Coomassie Brilliant Blue) of samples after 15 and 60 min reaction time.

Within the first 10 min after the addition of the reagent, only crosslinks within each symmetric half of apo AII are formed. Bands with molecular weights of 18000 (2 × AII/2) and 27000 (3 × AII/2) appear on extension of the reaction time. The distribution of the radioactivity in the band corresponding to apo AII/2 (M_r = 9000) remains constant after 10 min reaction time. The protein bands were separated after delipidation of the lipoprotein particle with chloroform/methanol (2:1 v/v) and ether on a Sephadex G-75 column in the presence of mercaptoethanol. The elution profile together with the stained dodecyl sulfate polyacrylamide gels of the peak fractions are demonstrated in Fig. 5. The solid line represents the protein absorption, and this profile coincides with the distribution of the radioactivity. Two crosslinked products corresponding to apo AII (fractions 41–46) and apo AII/2 (fractions 53–62) were isolated in homogeneous form (fractions 44 and 56). Autoradiograms of the gels together with the total mixture of crosslinking products are shown in Fig. 6. These fractions were most strongly labelled with SbiMe$_2$.

Apo AII contains two methionine residues in the symmetrical positions 26. Cyanogen bromide cleavage of the crosslinked apo AII and apo AII/2, however, produces no change in the molecular weights of the two products, which indicates that crosslinking between lysines of the amino and carboxy terminus must have occurred.

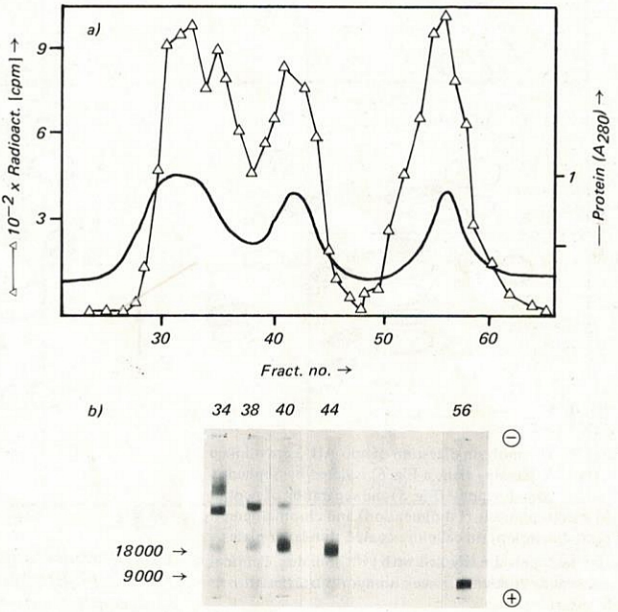

Fig. 5.
a) Sephadex G-75 elution profile of [^{14}C]SbiMe$_2$ crosslinked proteins from AII-polyenephosphatidylcholine-lysophosphatidylcholine complex after delipidation in the presence of mercaptoethanol.
b) Sodium dodecyl sulfate polyacrylamide (15%) gel electrophoresis in the presence of mercaptoethanol of samples of the fractions indicated on top of the gels. Protein was stained with Coomassie Brilliant Blue.

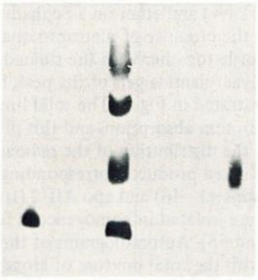

Fig. 6. Radioautographic pattern of polyacrylamide gels (15%) of apo AII fractions isolated from reconstituted apo AII-polyenephosphatidylcholine-lysophosphatidylcholine particles crosslinked with [^{14}C]$SbiMe_2$ after delipidation.

Lane a, apo AII/2-$SbiMe_2$; lane b, complete protein mixture; lane c, apo AII-$SbiMe_2$, after Sephadex G-75 chromatography.

On the average, apo AII/2 was labelled with 3.5 molecules dimethyl [^{14}C]suberimidate and apo AII with 5 molecules.

a) Analysis of the peptides bridged by $SbiMe_2$ in crosslinked apo AII/2

Apo AII/2 was incubated with thermolysin (100:1), peptides separated by two-dimensional electrophoresis/chromatography and incubated for autoradiography for one week. Fig. 7 is the peptide map, the hatched areas corresponding to the radioactive peptides. These areas were isolated, the radiolabelled peptides eluted, the crosslinker hydrolyzed and the peptide(s) rechromatographed. After the recovery of the separated peptides their amino-terminal end groups were determined by the dansyl method[28], and the amino acid stoichiometry established by amino acid analysis. Table 1 lists the peptides either monosubstituted with [^{14}C]$SbiMe_2$ or crosslinked.

The analyses of these peptides therefore indicate the modification of Lys 23, Lys 39, Lys 44, Lys 46, Lys 54 and Lys 55.

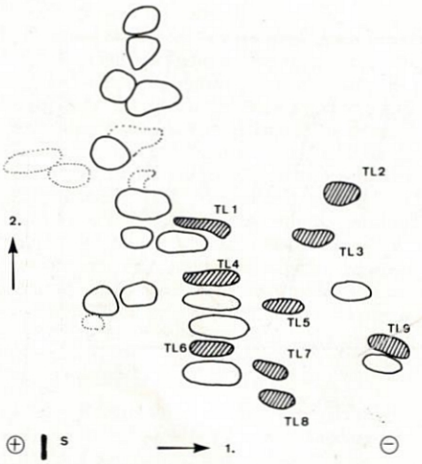

Fig. 7. Thermolysin digestion of apo AII/2 crosslinked with [^{14}C]$SbiMe_2$ (lane a Fig. 6) isolated by Sephadex G-75 chromatography (Fig. 5) and separation of peptides by electrophoresis (1st dimension) and chromatography (2nd dimension) on cellulose-coated thin-layer plates.

Hatched spots are labelled with [^{14}C]$SbiMe_2$. Peptides were made visible by a weak ninhydrin treatment. S = start.

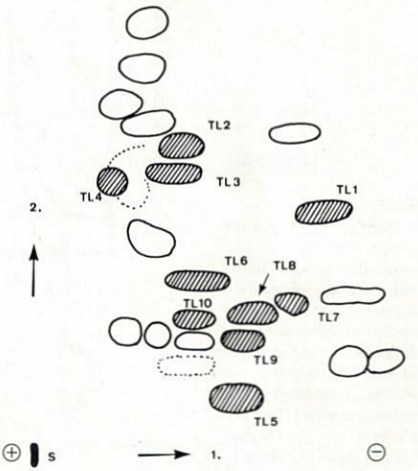

Fig. 8. Thermolysin digestion of apo AII crosslinked with [^{14}C]$SbiMe_2$ (lane c, Fig. 6).

Conditions were those of Fig. 7. S = start.

Table 1. Thermolysin peptides of apo AII/2 crosslinked in the lipoprotein complex with [1,1-^{14}C]SbiMe$_2$.

Peptide	Position in sequence	Sequence	Substituted lysines
TL-1	32–41	Pro-Glu-Leu-Gln-Ala-Gln-Ala-**Lys**-Ser-Tyr	39
TL-2	53–55	Ile-**Lys**-**Lys**	54, 55
TL-3	53–54	Ile-**Lys**	54
TL-4	22–24,	Gly-**Lys**-Asp	23, 46
	45–48	Ser-**Lys**-Glu-Gln	
TL-5	45–52	Ser-**Lys**-Glu-Gln-Leu-Thr-Pro-Leu	46
TL-6	32–48	Pro-Glu-Leu-Gln-Ala-Gln-Ala-**Lys**-Ser-Tyr-Phe-Glu-**Lys**-Ser-**Lys**-Glu-Gln	39, 44, 46
TL-7	42–48	Phe-Glu-**Lys**-Ser-Lys-Glu-Gln	44
TL-8	42–48,	Phe-Glu-**Lys**-Ser-Lys-Glu-Gln	
	53–55	Ile-$\overbrace{\text{Lys-Lys}}$	44, 54 (55)
TL-9	53–55	Ile-**Lys**-**Lys**	54, 55

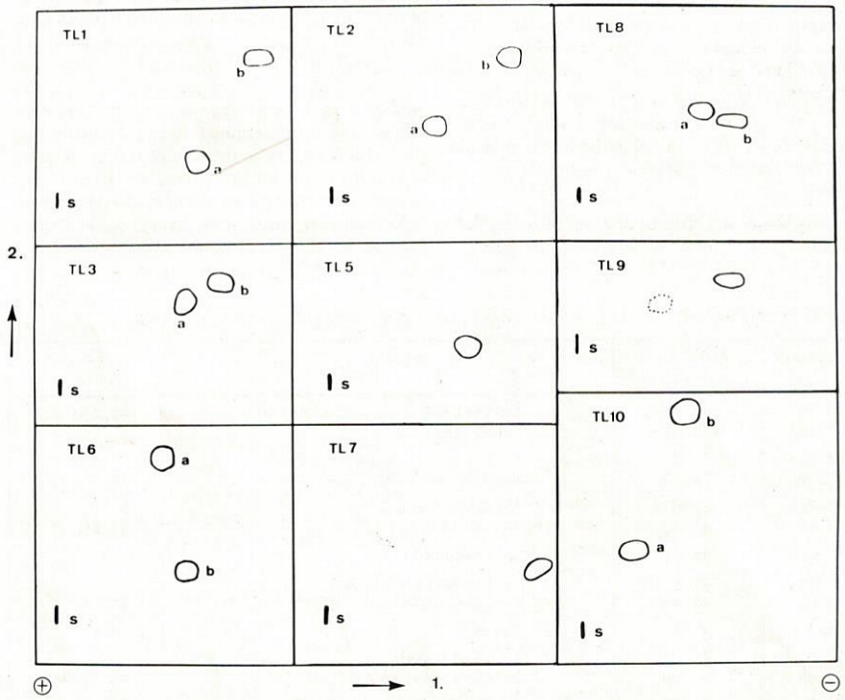

Fig. 9. Two-dimensional separation (electrophoresis/chromatography) of radioactive peptides TL1–TL10 of Fig. 8, isolated from the peptide-mapping thin-layer plate and subsequently freed of the crosslinker with ammonia/formic acid (1:15 v/v).

b) Analysis of thermolysin fragments of crosslinked apo AII

The purified protein band isolated from the apo AII-PC lipoprotein after the treatment with [^{14}C]-$SbiMe_2$ was degraded with thermolysin and the crosslinked peptides analyzed as described for apo AII/2 in the preceding section. The "peptide fingerprint" is given in Fig. 8. The radioactive peptides (hatched spots) were further analyzed by cleavage with conc. NH_4OH/HCOOH, two-dimensional separation, Fig. 9, and amino acid analysis. Table 2 lists the fragments and their positions in the apo AII sequence on the basis of amino acid stoichiometry and amino terminus.

Fig. 10 summarizes the crosslinking results schematically.

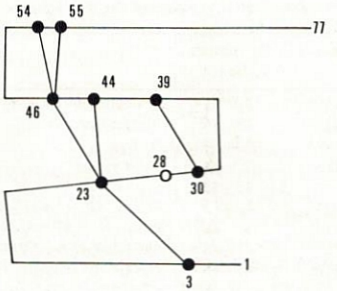

Fig. 10. Schematical presentation of crosslinking results with [^{14}C]$SbiMe_2$ in apo AII-polyenephosphatidylcholine-lysophosphatidylcholine-complexes.
Numbers indicate NH_2-(1) and COOH-terminal end (77), the rest the positions of the nine lysines present in apo AII/2.

Reaction of apo AII-polyenephosphatidylcholine-lipoprotein complex with dimethyl dithiobis-([1-^{14}C]butyrimidate)

The conditions applied in the crosslinking reaction of the apo AII-phosphatidylcholine particle with $SbiMe_2$ were also used in the reaction of the cleavable dithiobisbutyrimidate with a 16 Å spacing.

The kinetics of the crosslinking and oligomer formation were followed by dodecyl sulfate polyacrylamide gel electrophoresis without mercaptoethanol and the determination of the distribution of the radioactivity in the stained protein bands in analogy to the $SbiMe_2$ experiments. $(SBtMe)_2$ also led to a rapid intramolecular apo AII crosslinking and very little dimer, trimer or tetramer formation.

Table 2. Thermolysin peptides of apo AII crosslinked in the lipoprotein complex with [^{14}C]$SbiMe_2$.

Peptide	Position in sequence	Sequence	Substituted lysines
TL-1a	45–48	Ser-Lys-Glu-Gln	46, 54 (55)
TL-1b	53–55	Ile-Lys-Lys	
TL-2a	29–33	Val-Lys-Ser-Pro-Glu	
TL-2b	34–41	Leu-Gln-Ala-Gln-Ala-Lys-Ser-Tyr	30, 39
TL-3a	22–25	Gly-Lys-Asp-Leu	
TL-3b	45–52	Ser-Lys-Glu-Gln-Leu-Thr-Pro-Leu	23, 46
TL-5	45–48	Ser-Lys-Glu-Gln	46
TL-6a	55–60	Lys-Ala-Gly-Thr-Glu	
TL-6b	45–48	Ser-Lys-Glu-Gln	46, 55
TL-7	29–30	Val-Lys	30
TL-8a	22–25	Gly-Lys-Asp-Leu	
TL-8b	42–48	Phe-Glu-Lys-Ser-Lys-Glu-Gln	23, 44 (46)
TL-9	41–48	Tyr-Phe-Glu-Lys-Ser-Lys-Glu-Gln	44, 46
TL-10a	22–24	Gly-Lys-Asp	
TL-10b	1–6	Pca-Ala-Lys-Glu-Pro-Cys	3, 23

The crosslinking reaction of the apo AII-phosphatidylcholine complex with $(SBtMe)_2$ after delipidation and separation of crosslinked products yielded protein fractions of molecular weight 9000, 18000 and 35000. The 9000-dalton protein apparently originates from a disulfide exchange between Cys-Cys in the 6,6′ position of apo AII and $(SBtMe)_2$ which occurred at the pH (8.8–9) required for the crosslinking reaction. No crosslinking between apo AII-phosphatidylcholine particles was observed. This became evident from the elution profile of the particles treated with the crosslinker $(SBtMe)_2$ from the Sepharose column.

Five to six $(SBtMe)_2$ molecules were linked to one apo AII molecule; four of them had reacted bifunctionally. This corresponds to a 65% crosslinking yield. After delipidation, Sephadex G-100 chromatography again separated apo AII/2, apo AII and $(\text{apo AII})_2$ labelled with the $[^{14}C]$-$(SBtMe)_2$ in homogeneous form. Amino acid analyses of these crosslinked species were all identical in their stoichiometry with the untreated apo AII.

a) Analysis of $(SBtMe)_2$-crosslinked apo AII/2

Fig. 11a documents the fingerprint analysis after staining with ninhydrin of the apo AII/2 thermolysin peptides and Fig. 11b, the same after autoradiography.

The assignment of the peptides to the sequence of AII was again possible by end-group determination of every single radioactive peptide, amino acid analysis and the comparison of the electrophoretic mobility of the crosslinked and the thermolysin fragments of untreated apo AII.

Table 3 lists the analytical results and indicates that three bifunctional attacks of $(SBtMe)_2$ have occurred in apo AII/2, namely between Lys 3 and Lys 23, Lys 23 and Lys 30, and Lys 23 and Lys 46.

b) Analysis of $(SBtMe)_2$-crosslinked apo AII and $(\text{apo AII})_2$ fractions

The thermolysin hydrolysates of the apo AII and 2 apo AII crosslinked with $(SBtMe)_2$ and isolated from the apo AII-phosphatidylcholine-lipoprotein yielded peptide fingerprints which were almost identical and also had great similarity to that of apo AII/2.

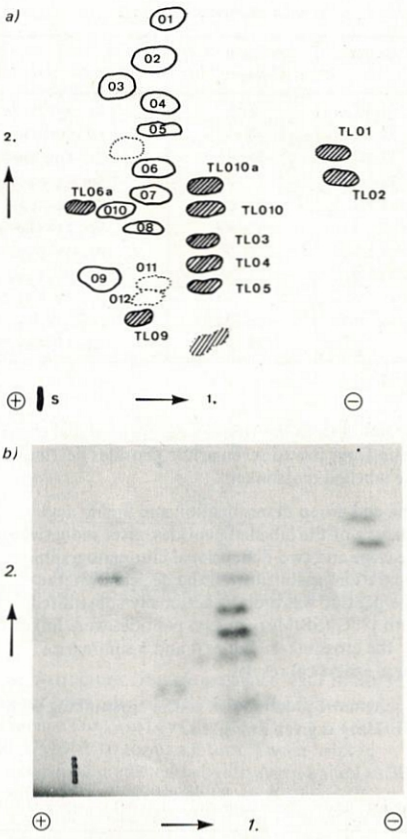

Fig. 11. Fingerprint of peptides released from apo AII/2 crosslinked with $[^{14}C](SBtMe)_2$ in the apo AII-polyene-phosphatidylcholine-lysophosphatidylcholine complex and purified by Sephadex G-75 chromatography.
a) Ninhydrin staining.
b) Radioautographic pattern.
For conditions see Materials and Methods.

Fig. 12a and b displays the peptide mapping of the two crosslinked protein fractions purified by gel chromatography in 6M urea, treated with thermolysin. The radioactive spots were identified by autoradiography and are indicated by the hatched areas. The two basic peptides are well

Table 3. Thermolysin peptides of apo AII/2 crosslinked in the lipoprotein complex with [^{14}C](SBtMe)$_2$.

Peptide	Position in sequence	Sequence	Substituted lysines
TL-01	52–57	Leu-Ile-**Lys**-**Lys**-Ala-Gly	54, 55
TL-02	53–57	Ile-**Lys**-**Lys**-Ala-Gly	54, 55
TL-03	34–41	Leu-Gln-Ala-Gln-Ala-**Lys**-Ser-Tyr	39
TL-04	31–41	Ser-Pro-Glu-Leu-Gln-Ala-Gln-Ala-**Lys**-Ser-Tyr	39
TL-05	22–24, 45–48	Gly-**Lys**-Asp Ser-**Lys**-Glu-Gln	23, 46
TL-06a	1–9	Pca-Ala-**Lys**-Glu-Pro-Cys-Val-Glu-Ser	3
TL-09	1–9, 22–24	Pca-Ala-**Lys**-Glu-Pro-Cys-Val-Glu-Ser Gly-**Lys**-Asp	3, 23
TL-010	22–24, 29–33	Gly-**Lys**-Asp Val-**Lys**-Ser-Pro-Glu	23, 30

separated from the five to seven neutral and these again from five to seven acidic peptides bearing the labelled crosslinker.

The end-group determination and amino acid analysis of the labelled peptides, after reductive cleavage and two-dimensional chromatographic separation, again allowed the decision whether one peptide was monofunctionally substituted with [^{14}C](SBtMe)$_2$ or two peptides were linked by the crosslinker. Tables 4 and 5 summarize these analytical results.

A schematic presentation of the crosslinking with (SBtMe)$_2$ is given in Fig. 13.

Prediction of secondary structure of apo AII

The secondary structure of apo AII was predicted according to the rules of Chou and Fasman[38–40]. Helix (P_α) and β-sheet (P_β) conformational parameters of the amino acids and those for turns were taken from Ref.[40] and Ref.[41].

Fig. 14 summarizes the predicted α-helical, β-sheet and turn regions of apo AII/2 in a linear schematic representation.

The sequence between Val 7 and Ser 12 has a $\langle P_\alpha \rangle = 1.12$. However Leu 10 to Val 18 has a $\langle P_\beta \rangle = 1.27$ and therefore this segment should

Table 4. Thermolysin peptides of apo AII crosslinked in the lipoprotein complex with [^{14}C](SBtMe)$_2$.

Peptide	Position in sequence	Sequence	Substituted lysines
B-101	53–55	Ile-**Lys**-**Lys**	54, 55
B-102	55–56	**Lys**-Ala	55
B-103	42–48	Phe-Glu-**Lys**-Ser-**Lys**-Glu-Gln	44, 46
B-104	42–48, 22–24	Phe-Glu-**Lys**-Ser-**Lys**-Glu-Gln Gly-**Lys**-Asp	23, 46 (44)
B-105	29–33, 22–24	Val-**Lys**-Ser-Pro-Glu Gly-**Lys**-Asp	23, 30
B-106	1–6	Pca-Ala-**Lys**-Glu-Pro-Lys	3
B-107	22–24	Gly-**Lys**-Asp	23
B-108	29–33	Val-**Lys**-Ser-Pro-Glu	30
B-109	45–48	Ser-**Lys**-Glu-Gln	46

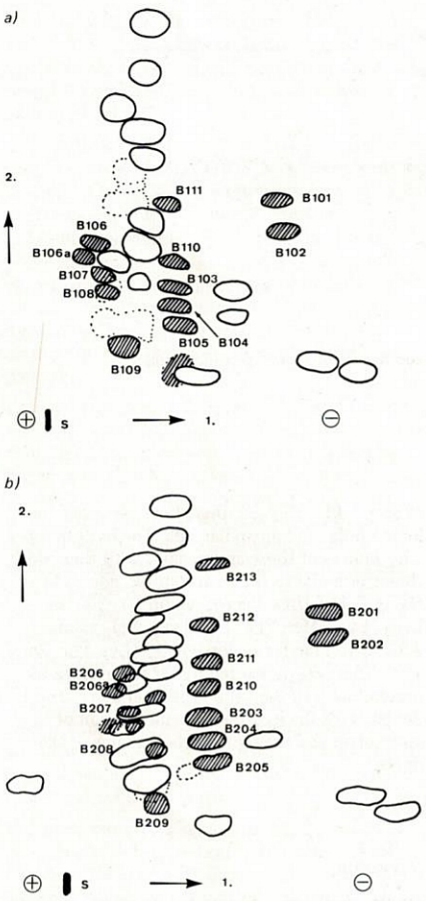

Fig. 12. Fingerprints of peptides released with thermolysin from a) apo AII and b) (apo AII)$_2$ crosslinked with [^{14}C](SBtMe)$_2$.

The hatched areas are the labelled peptides which were isolated and further processed for the sequence assignment.

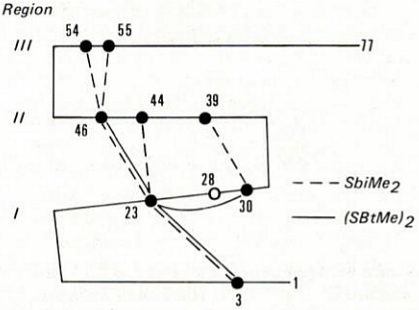

Fig. 13. Schematic presentation of crosslinks between [^{14}C]SbiMe$_2$ and [^{14}C](SBtMe)$_2$ and lysine residues in apo AII.

have β-structure, because sequence Val 7 to Ser 9 cannot form a helix nucleation. The following sequence Thr 19 to Gly 22 with a $\langle P_t \rangle = 1.6 \times 10^{-4}$ and $\langle P_t \rangle > 1.10$ forms a β-turn. Two α-helix regions can be predicted between Asp 24 and Lys 30 and Glu 33 to Lys39 with $\langle P_\alpha \rangle = 1.19$ and $\langle P_\alpha \rangle = 1.31$, respectively. Ser 40 and Tyr 41 with $\langle P_\alpha \rangle < 1$ interrupt the α-helical region, which then continues until residue Leu 49. The chain will have a bend around Thr 50 and Pro 51. $\langle P_\alpha \rangle$ of the se-

Table 5. Thermolysin peptides of 2 apo AII crosslinked in the lipoprotein complex with [^{14}C](SBtMe)$_2$.

Peptide	Position in sequence	Sequence	Substituted lysines
B-205	18–37	Val-Thr-Asp-Tyr-Gly-Lys-Asp-Leu-Met-Glu-Lys-Val-Lys--Ser-Pro-Glu-Leu-Gln-Ala-Gln	23, 30
B-211	22–24, 29–35	Gly-Lys-Asp Val-Lys-Ser-Pro-Glu-Leu-Gln	23, 30

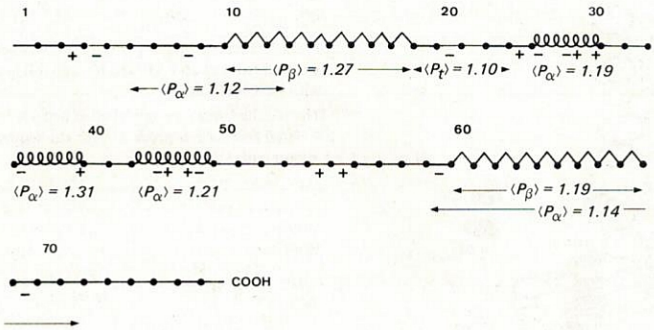

Fig. 14. Prediction of secondary structure of apo AII according to Chou and Fasman [39,40].

= β-structure, = α-helix.

quence between Leu60 and Val68 is 1.14 and is exceeded by $\langle P_\beta \rangle = 1.19$. Therefore β-structure in this region might be the favorable conformation. Pro 74 and its environment have a high $\langle P_t \rangle$ and therefore another bend is assumed.

In summary, the prediction rules of Chou and Fasman suggest that the apo AII polypeptide has 29% α-helix, 24% β-structure, 10% β-turn and 37% random coil conformation.

Apo AII in aqueous solution exhibits 27% α-helix structure, and therefore the results of the prediction reflect the conformation of the lipid-free apolipoprotein. The recombination with phospholipids, however, enhances the helix content to 40–50%, and it is reasonable to assume the extension of the α-helix regions to the amino- and the carboxy-terminal ends of the polypeptide.

The hydrophobicity H_Φ of apo AII should affect both the apopolypeptide conformation and its lipid-binding properties, and it should be possible to evaluate this quantity theoretically. On the basis of Nozaki and Tanford's parameters[41], apo AII has an average H_Φ of 4.77 kJ/residue, which is similar to that of many soluble proteins. If, however, the amino acid residues 10 to 30 and 39 to 49 are arranged in α-helical conformation according to the above prediction of the secondary structure, and a Corey-Pauling-Koltun model of apo AII is analyzed, these helical regions exhibit a polar and an apolar side. The hydrophobicity indices of those amino acids with their side chains oriented to the hydrophobic side yield an H_Φ of 7.41 kJ/residue for region 10–30 (Gly, Leu_2, Lys_2, Met, Phe, Thr, Tyr_2, Val_3) and 7.33 kJ/residue for region 39–49 (Lys, Phe, Ser, Tyr). These $H_{\Phi ave}$ exceed by far the average hydrophobicity of the total protein and may form the basis for the hydrophobic interaction of the apoprotein and the hydrocarbon chains of the lipids.

Discussion

Studies on the structure of the human serum high density lipoprotein basically are concerned with the interactions of the three main lipid classes, phosphatidylcholine (sphingomyelin), cholesterol and cholesterol esters, and the two predominant apolipoproteins AI and AII in this spherical particle of roughly 100 Å diameter. ^{13}C and ^{31}P-NMR spectroscopy of native and reassociated HDL particles or artificial particles containing phospholipids and cholesterol and only apoprotein AI or AII have given evidence for the free accessibility of all polar head groups of phospholipids and

cholesterol. However there appear to be strong hydrophobic interactions between the apoproteins and the fatty acyl chains of the phospholipids[1–3,5,6], and also with the side chain of cholesterol[2].

The NMR spectroscopic studies have led us to propose a model of the HDL particle[1] according to which the hydrophilic polar head groups of the zwitterionic phospholipids and the 3-hydroxyl group of cholesterol, together with the hydrophilic sides of the apo AI and AII polypeptide chains form the outer shell. The fatty acyl residues of the phospholipids interacting with the hydrophobic side of the apoproteins and the cholesterol esters form the nucleus of the HDL particle.

Detailed structural information essential for the understanding of the functions, e.g. transport of cholesterol and cholesterol esters and activation of the phosphatidylcholine-cholesterol acyl transferase and of the locus of the reaction, and the dynamics of the lipid and apoprotein exchange must include the spatial arrangement of the components in the HDL particle; particularly of the two main apoproteins AI and AII. On the basis of their primary structures[42,43], the conformation of both apoproteins has been calculated on following the empirical rules of Chou and Fasman[38–40] and compared with their circular dichroic spectra in their free form and associated with phospholipids[3,5]. The prediction of the secondary structure of apo AII is outlined in this communication. A more extensive report will be given in a forthcoming paper*.

The prediction rules agree with the CD measurement, so far as they indicate extended α-helical domains. The α-helicity of apo AII associated with phospholipids exceeds the predicted one. Apparently the α-helix nucleation of the free apoprotein progresses when hydrophobic interaction with the phospholipid acyl chains takes place. In the previous report[13], chemical evidence for the surface alignment of apo AII in a lipoprotein particle with a 55 to 60 Å radius and the composition described in this paper was presented.

We have introduced the bifunctional crosslinkers dimethyl suberimidate and dithiobisbutyrimidate in their radioactive form to establish a subunit structure of the particle. This chemical approach leads to bridges between lysine residues in the polypeptide chains which are distinct distances apart, about 13 Å for $SbiMe_2$ and 16 Å for $(SBtMe)_2$. The polypeptide conformation becomes fixed, but is not altered as far as CD measurements can detect. The subsequent proteolytic cleavage, isolation and characterization of the peptides connected with the labelled crosslinking reagents allowed their assignment to the sequence. We interpret the results as follows: all lysine residues except Lys28 were accessible to the crosslinkers. Except for Lys3, all are concentrated in three regions which, according to the prediction rules for the secondary structure, are separated by β-turns. The experiments reported here clearly indicate that the bifunctional substitution primarily occurred between lysines of region I and those of II, and of region II with lysines of region III of the same half of the apo AII (Fig. 13).

No crosslinks between the symmetrical halves of apo AII could be detected and intermolecular bridges were only introduced on prolonged interaction of the reagents.

Space filling models (Corey-Pauling-Koltun) of apo AII built according to the predicted conformation show the orientation of the lysine residues. The crosslinks which were established can be easily accommodated in this model. The bridge between Lys3 and Lys23 with $(SBtMe)_2$ requires a folding around residue 10, which causes these side chains to approach each other to the required 10–15 Å.

The increase in α-helicity from 27% in free apo AII to 40% in the lipoprotein complex could be the result of the growth of the α-helix region 24 to 31 to the amino terminus. This process would bring together Lys3 and Lys23 to the required distance.

Since no lysine residue is present between position 56 and 77, it is not possible to define the conformation and the relative position of this part of the apoprotein. We assume that it is aligned parallel to the two regions, thus protecting

* Stoffel, W., Preißner, K. & Wittmann-Liebold, B., in preparation.

Lys30 and Lys39 from intermolecular crosslinking.

The crosslinking results also point out that the symmetrical halves of apo AII cannot be arranged as mirror images, but as suggested in Fig. 15. Similar studies with the shorter bifunctional crosslinker tartaroyl diazide with the same lipoprotein particle will be reported elsewhere*.

This chemical approach to conformational problems of proteins of known primary structure might be applied to more complex systems such as native HDL or reconstituted systems with intrinsic membrane proteins.

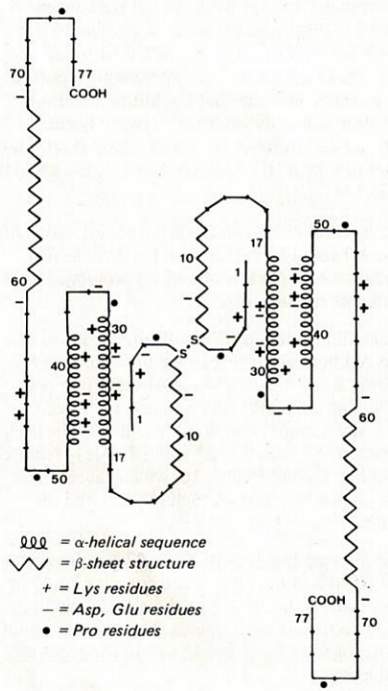

Fig. 15. Suggested conformation and alignment of apo AII on the apo AII-polyenephosphatidylcholine-lysophosphatidylcholine-particle surface.

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