Hoppe-Seyler's Z. Physiol. Chem.
Bd. 364, S. 1631–1640, November 1983

A Serum Proteinase Converts Proapolipoprotein AI Secreted by Rat Hepatocytes to the Mature Apolipoprotein

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(Received 28 July 1983)

Summary: Apolipoprotein AI integrated into the high-density lipoprotein particle in serum was synthesized in the rat hepatocyte in the presence of radiolabelled amino acids and isolated from the cells in primary culture (suspension) as its proform, with the N-terminus extended by a hexapeptide segment. The primary secretion product is this proform which is only further proteolytically processed in the presence of the serum fraction with density higher than 1.21 g/m*l*. The secretion product and the proteolytically converted product were characterized by Edman degradation of their respective amino-acid sequences after radiolabelling with [^{3}H]valine and [^{3}H]phenylalanine, the positions of which are well established in the preproform and in the N-terminus of mature rat apolipoprotein AI. The products from the lysed cells and their culture medium were purified by immunoprecipitation, sodium dodecyl sulfate gradient gel electrophoresis and subsequent electroelution of the apo AI band.
The proform sediments associated with a particle of density 1.16–1.20 g/m*l*.
The serum proteinase which is inhibited by phenylmethanesulfonyl fluoride, but not by sulfhydryl reagents, is presumably a serine proteinase.

Proteolytische Spaltung der Pro-Form zum Apolipoprotein AI durch eine Serumproteinase

Zusammenfassung: Apolipoprotein AI, das mit dem High-Density-Lipoprotein-Partikel im Serum integriert vorliegt, wurde in Rattenhepatozyten-Suspensionen in Gegenwart von radioaktiven Aminosäuren synthetisiert und sowohl die zelluläre Form als auch das sezernierte Apoprotein isoliert. Beide liegen als Proform, die ein Hexapeptidsegment am N-Terminus trägt, vor. Die sezernierte Proform wird nur in Gegenwart der Serumfraktion $d > 1.21$ g/m*l* weiter proteolytisch in die reife apo-AI-Form konvertiert. Sekretions- und konvertierte Form wurden durch Edman-Abbau nach Markierung mit [^{3}H]Valin und [^{3}H]Phenylalanin, deren Positionen in der Praepro-, Pro- und in der reifen Polypeptidsequenz des Apolipoprotein AI genau bekannt sind, charakterisiert. Hierzu wurden die Produkte durch Immunpräzipitation aus den lysierten Zellen und dem Medium isoliert und durch Elektroelution nach Trennung durch Natriumdodecylsulfat-Polyacrylamid-Gradienten-Gelelektrophorese in radiochemisch reiner Form erhalten.

Abbreviations:
Apo AI = Mature apolipoprotein AI; Apo AII = mature apolipoprotein AII; HDL = high-density lipoproteins; KIE = kallikrein inhibitor units; LPDS = lipoprotein deficient serum; (d = 1.21 g/m*l* infranatant).

Die Serumproteinase, die durch Phenylmethansulfonylfluorid gehemmt wird, nicht hingegen durch Sulfhydryl-Reagentien, ist vermutlich eine Serinproteinase.

Key words: Intracellular processing of preapolipoprotein AI, secretion of proapolipoprotein AI by rat hepatocytes, extracellular processing of proapolipoprotein AI.

High-density lipoproteins represent a major class of particles responsible for the transport of free cholesterol and its esters. They are also involved in the esterification reaction catalysed by the serum enzyme lecithin-cholesterol acyltransferase.

These particles are characterized by two main apolipoproteins apo AI and apo AII in man, whereas apo AI is the main HDL apolipoprotein in the rat. These polypeptides cover the surface of HDL with amphipathic helices which interact with their hydrophobic faces with the hydrophobic core of the HDL particle. This topography largely came from NMR and chemical cross-linking studies[1–5].

The assembly process of the apoproteins and lipids to HDL is still obscure. We initiated studies directed toward this problem and unravelled the structure of the primary translation product of apo AI mRNA from rat liver and of apo AI and apo AII mRNA from liver of man[6,7]. In simultaneous and independant studies it has been demonstrated that the primary translocation product of rat liver and intestinal apolipoprotein AI mRNA carries an N-terminal sequence which is processed cotranslationally[6,8,9]. The presegment of apo AI, which is released by the signal peptidase, consists of 18 largely hydrophobic amino-acid residues. The product translocated into the luminal compartment of the endoplasmic reticulum differs from the mature apo AI by a prosegment of six amino-acid residues terminating with Gln-Gln. Human and rat prepro- and pro-apo AI sequences exhibit extensive homologies.

The preprosequence of human apo AII, however, differs by a 17 amino-acid residues long presegment from human prepro-apo AI. The prosegment of apo AII is also a hexapeptide. The partial amino-acid sequences of the signal peptide and the prosegment have been determined and again revealed the strong hydrophobicity of the presegment obviously required for membrane interaction (penetration). The hexapeptide prosegments of rat and human apo AI and human apo AII all end with glutamine[7,9], followed by an acidic sequence (Asp-Glu) of the mature apoprotein.

Our results on the structure of the primary translation product of human liver apo AI mRNA and its processing to the proform and similar studies reported recently[10] are in full agreement. These results, together with the characterization of the secretion product as pro-apo AI into the plasma, provide the molecular basis for the apolipoprotein AI isoproteins described by Zannis et al.[11,12], which makes this nomenclature obsolete. In a recent communication these authors come to the same conclusion[13]. The determination of the nucleotide sequence of the apo AI gen also revealed the pre- and prosegment in the coding sequence[14].

In the preceding publication we reported on the synthesis, identification and characterization of the intracellular apo AI precursor in rat hepatocytes and the secreted product which was isolated by immunoprecipitation from the culture medium[15]. We characterized the secretion product as the proform. It is identical with the product of the signal peptidase reaction, synthesized in the cell-free reticulocyte system in the presence of dog microsomal membranes.

Here, we report that rat apo AI, secreted with its prosegment, is processed by a serum proteinase present to mature apo AI. Preliminary inhibition experiments refer to a serine proteinase. The radioactive pro-apo AI is associated with a particle of density 1.16–1.20 g/m*l*.

Materials and Methods

Materials

Collagenase, hyaluronidase, pepstatin and leupeptin were purchased from Boehringer Mannheim GmbH (D-6800 Mannheim), protein A-Sepharose CL-4B from Pharmacia Fine Chemicals (D-7800 Freiburg), Trasylol

from Bayer (D-5090 Leverkusen) and benzamidine from Sigma Chemical Corp., St. Louis.

The following labelled amino acids were obtained from Amersham-Buchler: L-[2,3,4,5,6-^{3}H]phenylalanine, spec. act. 115 Ci/mmol, L-[3,4-^{3}H]valine, spec. act. 32 Ci/mmol and L-[^{35}S]methionine, spec. act. 800 Ci/mmol.

Rat apo AI was isolated from rat high-density lipoprotein fraction (1.063–1.21 g/m*l*) as described before[4].

Antibodies

Monospecific antibodies against homogeneous rat apolipoprotein AI were raised in rabbits. The IgG fraction was partially purified by ammonium sulfate precipitation[16].

Rat serum and lipoprotein deficient serum (LPDS)

Anesthesized rats were exsanguinated by puncture of the abdominal aorta. The density of the serum was adjusted to 1.21 g/m*l* with potassium bromide. Ultracentrifugation, performed in a Beckman Ti 60 rotor for 20 h at 60000 rpm yielded a floating layer of lipoproteins which was removed with a syringe. The sample was filled up to its original volume with 0.9% sodium chloride, dialysed extensively against NaCl/P_i buffer (containing in mM: 750 sucrose, 1.0 EDTA, 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.47 KH_2PO_4; pH 7.4) and used immediately as an enzyme source in the incubations.

Preparation and incubation of hepatocytes

The isolation procedure of rat hepatocytes and their incubation with radioactive precursor amino acids applied in these studies were those described in the previous communication[15]. Cells were separated from the culture medium by a short centrifugation at 100 × *g* for 30 s in a Christ-table top labfuge and a subsequent centrifugation at 10000 × *g* for 15 min in a Sorvall centrifuge at 4 °C. The clear supernatant (medium) was carefully removed from the pellet with a Pasteur pipette.

Incubation of culture media with serum and lipoprotein deficient serum (LPDS)

Culture medium containing the radiolabelled secretion products of the hepatocytes was mixed with serum or LPDS to a final serum concentration of 16 to 60% (v/v). After 2 h of incubation at 37 °C in a shaking water bath, the mixture was filtered through a Millipore filter (0.45 μm), diluted with NaCl/P_i buffer-proteinase inhibitor solution (2mM EDTA, 5mM iodoacetamide, 100 KIE Trasylol, 1mM phenylmethanesulfonyl fluoride, 0.1mM *p*-chloromercuriphenylsulfonate and 10mM pepstatin) and applied to the affinity column.

Affinity isolation and purification of apolipoprotein AI

100 mg Protein of rabbit IgG fraction containing monospecific apo AI antibodies was coupled to 20 m*l* Sepharose CL-4B analogous to the procedure described previously[17]. 80% of the protein was covalently attached to the Sepharose. Newly secreted apo AI was adsorbed to the affinity-matrix by recycling the medium, containing NaCl/P_i buffer, the proteinase inhibitor mixture and 1% Triton X-100 for about 5 h. Unbound radioactive proteins were washed out with 400 m*l* NaCl/P_i buffer. The bound apoprotein was desorbed with 0.1M glycine/HCl buffer pH 2.8 and fractionated. Radioactive fractions were pooled, neutralized with 1M NaOH and dialysed against 5mM Tris/HCl pH 8, lyophilized and dissolved in electrophoresis sample buffer (3.5% sodium dodecyl sulfate, 10% glycerol, 80mM Tris/HCl pH 8.5) for sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis.

The radioactive band, visualized by autoradiography[18], was excised and the protein isolated by electroelution as described before[15].

Sequence analysis of the radiolabelled products by automated Edman degradation has been described before[6].

Affinity chromatography of plasma HDL and nascent apo AI-containing particles

Rat serum HDL, isolated by ultracentrifugation in the density range 1.063 to 1.21 g/m*l* by flotation-sedimentation, was adsorbed to the anti-apo AI Sepharose CL-4B column as described above. The HDL particles were desorbed after extensive washing of the column as described for the affinity chromatography of apo AI, except that Triton X-100 was omitted. The eluate was neutralized with 1M NaOH and dialysed against 5mM Tris/HCl pH 8 for negative staining and electron microscopy.

Nascent apo AI-containing particles secreted by rat hepatocytes (1.2 × 10^9 cells), pulse labelled with [^{35}S]methionine (0.5 mCi, 120 min), were adsorbed to the anti-apo AI Sepharose CL-4B column from the culture medium in a similar way. Triton X-100 was omitted. The radioactive fractions in the eluent were pooled, neutralized with 1M NaOH and dialysed against water, the pH of which was adjusted to 8 with NH_4OH. The sample was vacuum concentrated to 150 μ*l* in a Speed-ovac centrifuge.

CsCl-gradient centrifugation

The cell free culture medium of hepatocytes, prelabelled with [^{35}S]methionine as described above, was centrifuged for 30 min at 100000 × *g* in a Beckman ultracentrifuge, rotor Ti 60.

1.5 m*l* of the supernatant was mixed with 1.5 m*l* 0.9% NaCl and 9 m*l* 22% CsCl solution and subjected to a

92 h ultracentrifugation in a Beckman SW 41 rotor at 35000 rpm. 0.5-*ml* fractions were collected and their density and radioactivity measured. Fractions between densities 1.069 to 1.118 g/*ml*, 1.127 to 1.145, 1.157 to 1.198 and 1.220 to 1.270 and 1.306 to 1.326 were pooled, dialysed against NaCl/P_i buffer and apolipoprotein AI, immunoprecipitable with monospecific antibodies, isolated for sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis and subsequent fluorography as described before[18].

Isoelectric focusing

Isoelectric focusing was performed according to an established procedure[19,20].

Apolipoprotein standards were dissolved in a 5mM Tris/HCl buffer pH 7.4 containing 10% sucrose, 2% ampholines and 1% sodium dodecyl sulfate. Immunoprecipitates were lyophilized and dissolved in 2% ampholines (solution C), 8M urea and 1% sodium dodecyl sulfate. Polyacrylamide slab gels were formed from the following stock solutions: A, acrylamide solution: 30 g acrylamide, 0.8 g bis(acrylamido)methane, 36 g urea made up to 100 m*l* with water; B, Temed solution: 1 m*l* *N,N,N',N'*-tetramethylethylenediamine, 36 g urea made up to 100 m*l*; C, ampholines: 2 m*l* Servalyt 4–6 and 2 m*l* 6–8 (40%); D, ammonium peroxodisulfate solution: 36 mg ammonium peroxodisulfate in 3 m*l* 8M urea. All solutions were treated before use with the mixed bed ion exchanger V, Merck, Darmstadt.

13.5 m*l* A, 4 m*l* B, 3 m*l* C, were mixed with 33 m*l* 8M urea, degassed for 15 min, ammonium peroxodisulfate added and the solution filled into the vertical chamber.

Upper cathode compartment: 0.02M NaOH, anode compartment: 0.01M phosphoric acid. Focusing was performed at 200 V and 9 W for 15 h and additional 90 min at 600 V.

The gel was incubated for 30 min at 60 °C in a solution of 14.5% trichloroacetic acid and 4.4% 5-sulfosalicylic acid. The gel was stained at 60 °C in a solution of 50% (v/v) methanol, 10% acetic acid and 0.1% Coomassie blue. The gel was destained at 60 °C in methanol/acetic acid/water 50:10:40 (v/v).

For autoradiography the gel was further treated as described before[6].

Results

Rat hepatocytes in suspension were obtained by the collagenase perfusion technique. The suspensions of more than 98% viable hepatocytes were incubated with the radioactive amino acids [^{35}S]-methionine, [^{3}H]valine and [^{3}H]phenylalanine to label the intracellular and the secreted form of the primary translation product of apo AI mRNA.

We have demonstrated that the intracellular form of apo AI in hepatocytes is the proform, with the hexapeptide extension at the N-terminus. This proform is secreted and found in the cell suspension medium as proform, which is isolated by immunoprecipitation and electrophoretic separation and identified by automated Edman degradation. The extracellular processing of the secretion product was studied by using Dulbecco's medium alone or supplemented with rat serum and lipoprotein-deficient rat serum and the apo AI examined by amino-acid sequence determination of the N-terminus labelled with [^{3}H]phenylalanine and [^{3}H]valine, respectively, in specific positions.

Fig. 1 demonstrates that the secretion product in the absence of any serum fraction is the proform, which characteristically migrates on isoelectric focusing further towards the cathode, due to ex-

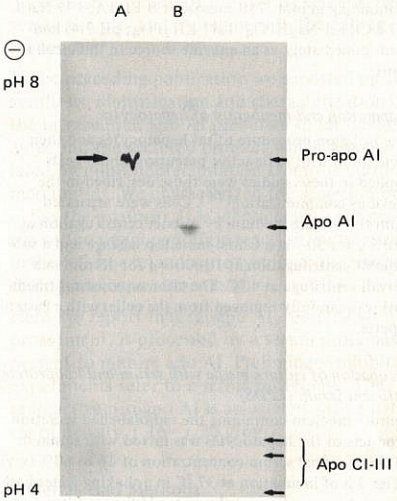


Fig. 1. Fluorogram of labelled pro-apo AI secreted by rat hepatocytes and separated by isoelectric focusing (Lane A). The proteins in Lane B were stained with Coomassie blue. The isoelectric point of pro-apo AI is distinctly more basic than mature apo AI.

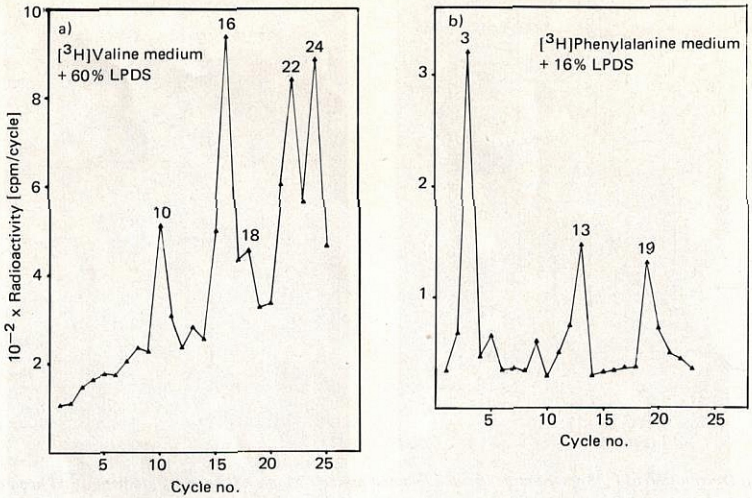


Fig. 2. N-Terminal amino-acid sequence analyses by automated Edman degradation of secreted pro-apo AI labelled with

a) [^{3}H]valine in Dulbecco's medium supplemented with 60% lipoprotein deficient serum (LPDS);

b) [^{3}H]phenylalanine in Dulbecco's medium containing 16% LPDS.

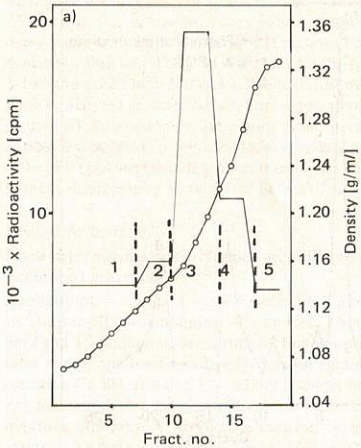
cess positive charges as compared to the mature apo AI. [^{35}S]Methionine and [^{3}H]valine were used as radioactive markers for the labelling of the apoproteins and their precursors. Automated Edman degradation positioned the hydantoin derivatives of [^{3}H]valine in positions 16, 22 and 24, which corresponds to the positions in pro-apo AI.

When the Dulbecco medium of the hepatocyte suspension was supplemented with [^{3}H]valine and 40% whole rat serum, a small fraction of mature apo AI was formed by proteolytic trimming of the hexapeptide at the N-terminus. This is demonstrated by the derivative of [^{3}H]valine in position 10. When the suspension medium was supplemented with lipoprotein-deficient serum effective processing to the mature apo AI occurred with the major radioactive hydantoin derivative of valine in Edman cycle 10 and 16. ^{3}H-Labelled phenylalanine which was found in positions 3 and 19 was then detected in cycles 3, 13 and 19, Fig. 2 (a, b). The activity in cycle 13 indicates the yield of the cleavage of pro-apo AI to mature apo AI by the serum proteinase.

Is proapolipoprotein AI secreted associated to a particle?

To answer this question rat hepatocytes were incubated in the presence of [^{35}S]methionine and the cell- and serum-free medium layered on top of a CsCl gradient. After equilibrium centrifugation the gradient was fractionated, each fraction dialysed and prepared for immunoprecipitation with anti-apo AI and gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis, Fig. 3.

According to the distribution of the ^{35}S-radioactivity, the labelled product(s) accumulate in Fract. 3, which has a density range of $d = 1.14$–1.22 g/*ml*. In the accompanying gel electrophoretic pattern, only Fract. 3 contains labelled proapolipoprotein AI with a molecular mass around 28–30 kDa as revealed by autoradiography.



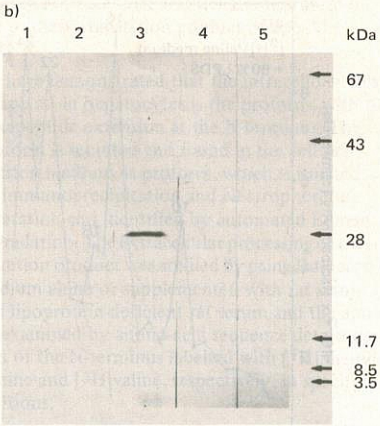


Fig. 3. a) Distribution of [^{35}S]methionine labelled secreted pro-apo AI in a CsCl-density gradient of rat hepatocyte medium.

Hepatocytes were labelled with [^{35}S]methionine in suspension culture. Serum- and methionine-free Dulbecco's medium was used. Fractions were collected and pooled (1–5), dialysed against 0.01M ammonium hydrogen carbonate and immunoprecipitated with rat apo AI-antiserum and protein A Sepharose CL-4B (see Materials and Methods). Fractions were separated by sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis and prepared for fluorography[21].

b) Autoradiogram of sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel with Fract. 1 to 5. Only Fract. 3 contained immunoprecipitated pro-apo AI.

Pro-apo AI was obviously associated with a particle secreted into the protein-free medium. For its selective isolation rat anti-apolipoprotein AI was covalently attached to Sepharose CL 4-B for immunoadsorption of the pro-apo AI-containing particle. The cell-free medium, containing all [^{35}S]methionine-labelled secreted proteins, was recycled over an anti-apo AI Sepharose immunoadsorbent, nonspecifically labelled protein thoroughly washed out with NaCl/P_i buffer and the apo AI-containing particle desorbed for electron microscopy and polyacrylamide gel electrophoresis-fluorography.

Fig. 4 shows the electron microscopy of

A) native, purified apo AI from rat serum,

B) the same adsorbed to and desorbed from the anti-apo AI affinity matrix,

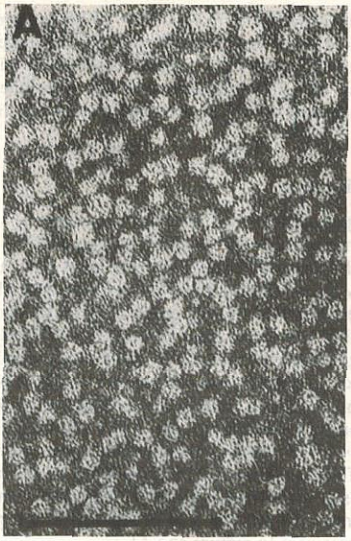
C) the particles isolated by the same affinity column from the medium of rat hepatocytes.

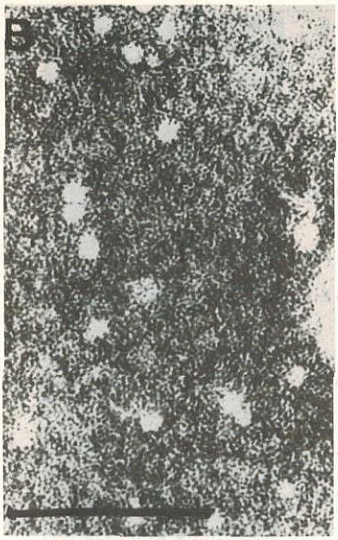
The sodium dodecyl sulfate polyacrylamide separation, together with the autoradiography, yielded the pattern of labelled bands shown in Fig. 5.

Although the affinity column to which the pro-apo AI containing particle was bound, had been rinsed excessively with NaCl/P_i buffer to remove all nonspecifically bound proteins, the spherical particles with an average diameter of 12 nm, visualized in the electron microscope, Fig. 5, contained a combination of albumin, apo AIV, apo E and apo AI, of which apo E ist most heavily labelled.

Inhibition of the extracellular final processing of pro-apo AI by serum proteinase

The proform of apo AI cannot yet be made available in the amounts required for a detailed characterization of the complex in which pro-





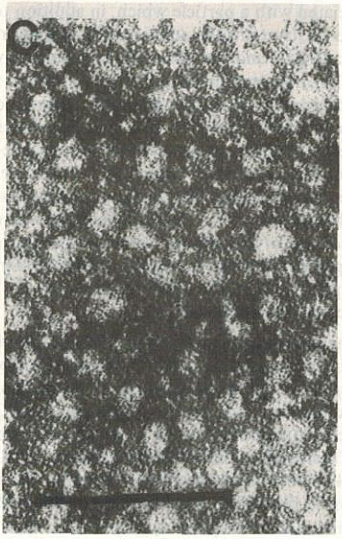


Fig. 4. Electron micrographs of
A) native rat serum HDL particles;
B) native rat serum HDL adsorbed to the rat anti-apo AI-Sepharose CL-4B matrix and desorbed with 0.1M glycine buffer pH 2.8;
C) particles secreted by rat hepatocytes in suspension culture, which were isolated by adsorption to rat anti-apo AI-Sepharose CL-4B and desorbed as above after *thorough washing with NaCl/P_i buffer* (50 volumes).
The bar represents 0.1 μm.

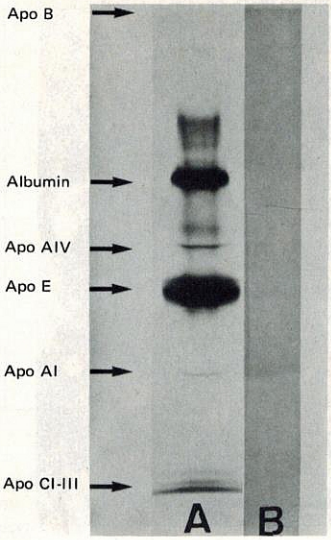


Fig. 5. A: Fluorography of proteins associated with the secreted particles (Fig. 4C) purified by immunoadsorption.

They were separated by sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis. B: Same gel after Coomassie blue staining.
Labelled particles (110000 cpm in 80 μl) were added to 300 μg of rat HDL-apoproteins and the particles delipidated and precipitated with ethanol/ether 1:1 (v/v). The precipitate was dissolved in sample buffer and separated as described above.

apo AI must be present for the specific proteolytic cleavage at the Gln-Asp bond and the study of the nature of the serum proteinase (metallo-, cysteine, serine proteinase). It is interesting however to note the following inhibitory effects: When the incubation medium (Dulbecco's medium) was supplemented with whole serum (40 vol %) the prosegment was cleaved only in a minute yield as indicated from Fig. 6 in which valine in position 16 of the proform did not shift to position 10 in a considerable yield.

Freezing of the lipoprotein deficient serum, added in the same amount under which effective cleavage occurred with unfrozen LPDS, led to the inactivation of the serum proteinase.

The experiment, in which the inhibitor phenylmethanesulfonyl fluoride was applied, suggests that the specific proteinase is a serine proteinase, Fig. 6c. Other inhibitors such as *p*-chloromercurisulfonate and EDTA were ineffective, Fig. 6d.

Discussion

Apolipoprotein AI undergoes a number of steps until it reaches its final destination which is the HDL-particle. These steps have been elucidated by a combination of in vitro experiments which demonstrated the cell-free translation of apo AI mRNA into preproapolipoprotein AI, the cleavage of the 18 amino-acid residues long signal peptide of dog microsomal membranes to the proform of apo AI, which is characterized by the N-terminal extension of apo AI by a hexapeptide [6,7,10]. Finally, we demonstrated the secretion of this pro-apo AI into the medium of hepatocytes in suspension culture[15]. This paper demonstrates the processing of pro-apo AI by a serum proteinase to mature apo AI.

Pro-apo AI is secreted from the hepatocyte associated with a particle which, in addition to pro-apo AI, contains the arginine-rich apolipoprotein E, apo AIV and also albumin. This particle was isolated by immunoadsorption to a rat apo AI-antibody-Sepharose CL-4B matrix. The lipid components of this particle of 12 nm diameter are presently being studied. The inhibitory effect of lipoproteins in whole serum on the cleavage of the prosequence awaits an experimental answer, although one could speculate that a premature discharge of pro-apo AI from the substrate partice might prohibit the exposure of the Gln-Asp bond of pro-apo AI for proteolytic attack by the serum pro-apo AI proteinase.

Whereas the enzymatic steps leading from the primary translation product of apo AI mRNA to mature apo AI are now clear, the assembly and detailed composition of the secreted "HDL-precursor particle" which bears few similarities to the particle, described by Hamilton et al.[22], have to be elucidated further. Finally, the transition to the mature HDL-particle of normal serum which harbours apo AI synthesized in liver and mucosa cells, is completely obscure but can be approached experimentally.

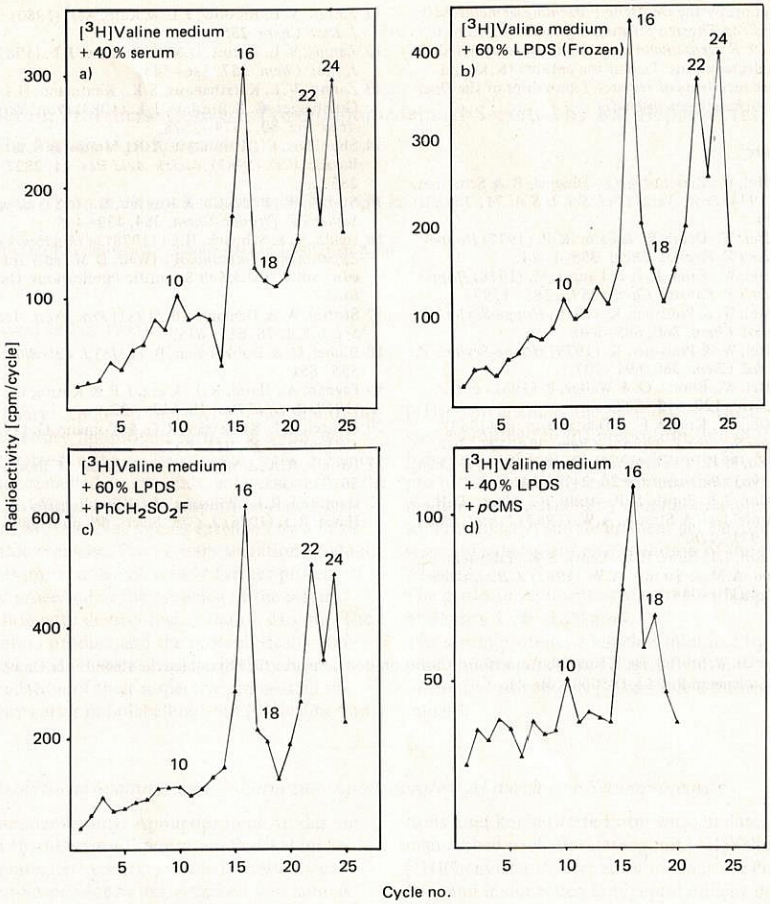


Fig. 6. N-Terminal sequence analyses by automated Edman degradation of pro-apo AI secreted by rat hepatocytes in suspension.

[^{3}H]Valine was used as radioactive precursor. Suspension medium: Dulbecco's medium supplemented with

a) 40% whole rat serum,

b) 60% frozen LPDS,

c) 60% LPDS and phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) (1mM).

d) 60% LPDS and *p*-chloromercuriphenylsulfonate (*p*CMS) (0.1mM).

The labelled products (pro-apo AI and processed pro-apo AI were isolated as described under Materials and Methods.

The support by the *Deutsche Forschungsgemeinschaft* and the *Fritz Thyssen Stiftung* is gratefully acknowledged. *Dr. R. Deutzmann* carried out the automated Edman degradations. Two of the authors (K.K. and C.B.) are recipients of research fellowships of the *Deutsche Forschungsgemeinschaft*.

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