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Synthesis and Processing of Human Serum Apolipoprotein AII in vitro and in Hep G2 Cells

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Dedicated to Prof. Dr. Karl Decker on the occasion of his 60th birthday

Summary: The synthesis and structure of the primary translation product of apo AII in a human liver poly(A+) mRNA primed cell-free system and its cotranslational modification was studied parallel to studies in vivo with Hep G2 cells, a human hepatoma cell line. The primary translation product is a preproprotein containing 100 amino acid residues, which is cleaved by the signal peptidase of endoplasmic reticulum to pro-apo AII with the loss of the N-terminal pre-sequence consisting of 18 amino acid residues. Hep G2 cells contain about equal amounts of the proform of apolipoprotein AII and of mature apo AII. Approximately in the same ratio pro- and mature apo AII are secreted into the medium. Determination of the partial amino-acid sequence by automated Edman degradation of the labelled prepro- and proforms of apo AII led to the segmentation of the N-terminus of the primary translation product, consisting of 23 amino acid residues, into the pre-sequence (18 residues) and the pro-sequence (5 residues) with terminal Arg-Arg-residues at the cleavage site to apo AII. We must therefore correct our previously postulated 17 and 6 residues containing segmentation.

So far no information has been obtained in which compartment and at what stage of posttranslational events the dimerization occurs by formation of the single disulfide bond at position Cys^6 in the mature apo AII structure, leading to the symmetrical molecule.

Synthese und Prozessierung des menschlichen Serum-Apolipoproteins AII in vitro und in Hep-G2-Zellen

Zusammenfassung: Die Synthese und Struktur des primären Translationsproduktes der Apolipoprotein-AII-mRNA aus menschlicher Leber wurden in einem zellfreien System zusammen mit der posttranslationalen Modifikation und parallel in vivo in Hep-G2-Zellen, einer menschlichen Hepatomzellinie, untersucht. Das primäre Translationsprodukt ist ein 100 Aminosäurereste enthaltendes Präproprotein, das durch eine Signalpeptidase des endoplasmatischen Reticulums unter Verlust der 18 Reste langen N-terminalen Präsequenz zum Pro-apo AII führt. Hep-G2-Zel-

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Abbreviations:
A_{260} unit, amount of material in 1 m*l* of solution which has an absorbance of 1 at 260 nm when measured in a 1-cm pathlength cell;
apo AI and apo AII, apolipoproteins AI and AII of human and rat serum high-density lipoproteins;
Aprotinin, Trasylol (Bayer AG);
HDL, serum high-density lipoprotein (d = 1.09 – 1.21 g/m*l*);
Hep G2, human hepatocarcinoma cell strain G2, kindly provided by Dr. B. B. Knowles, The Wistar-Institute, Philadelphia, U.S.A.;
MEM, minimal essential medium;
NaCl/P_i buffer, 0.14M NaCl containing 1.48mM KH_2PO_4, 7.4mM Na_2HPO_4 and 0.9mM $CaCl_2$, pH 7.4;
$NaDodSO_4$, sodium dodecylsulfate;
NP40, Nonidet P40, ethylphenylpoly(ethyleneglycol);
$PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

len enthalten das Pro-apo AII und das reife Apo AII in etwa gleichen Anteilen, in denen beide auch in das Medium sezerniert werden. Die partielle Bestimmung der Aminosäuresequenz durch automatischen Edman-Abbau der radioaktiv markierten Präpro- und Proform des Apo AII führte zu der obigen Segmentierung des N-Terminus des primären Translationsproduktes von Apo AII in die 18 Reste enthaltende Prä- und die aus 5 Resten bestehende Prosequenz mit Arg-Arg-Resten an der Spaltstelle zum reifen Apo AII. Damit korrigieren wir unsere früher postulierte Segmentierung (17 + 6 Aminosäurereste). Es bleibt zu klären, in welchem Kompartiment und auf welcher Stufe der Posttranslation die Dimerisation unter Bildung der einzigen Disulfidbrücke am Cys[6] der reifen Apo-AII-Struktur zum symmetrischen Apo AII erfolgt.

Key words: Apolipoprotein AII structure, synthesis in vivo, cotranslational processing, prepro- and pro-sequences, secretion by Hep G2 cells.

Apolipoproteins of the four main serum lipoprotein classes are synthesized in association with lipid synthesis and distribution in the organism for very divergent physiological functions. The regulation of these apolipoproteins, which are essential for the transport of the water-insoluble lipids, is not yet understood. However, recent studies in this and other laboratories[1–3] have been initiated in which cell-biological methods were applied to unravel the pertinent questions.

It has been demonstrated that cells from rat[1,2] and human liver[4] and intestine[3] synthesize the main apolipoprotein apo AI found in human serum HDL by programming cell-free synthesis in a reticulocyte lysate with the poly(A+) RNA of these cells. Similarly, apo AII, the other main protein component of HDL, obtained by cell-free translation of human liver poly(A+) RNA, was isolated by immunoprecipitation[1,5]. Na-DodSO$_4$-polyacrylamide gel electrophoresis and automated Edman degradation of the primary translation products revealed that rat[1,3] as well as human apo AI[4] and human apo AII[4,6] are extended by sequences of 24 and 23 amino-acid residues, respectively. These sequences are processed during the cotranslational translocation. A signal sequence of 18 amino-acid residues of apo AI is split off to form an intracellularly stable pro-apo AI N-terminally containing a hexapeptide ending in unusual Gln-Gln-residues. Pro-apo AI is secreted into the serum and slowly processed to mature apo AI by a serum proteinase. The secretion and final posttranslational processing has been mimicked in rat hepatocytes in suspension culture[7,8] and a hepatocarcinoma cell line, Hep G2[9–11]. Rat HDL contains only an ill-defined apo AII different from the human apolipoprotein[12]. We therefore analysed the primary translation product of human liver apo AII mRNA and its cotranslationally formed proform released after cleavage of the signal sequence. Due to the misplacement of [^{3}H]lysine in automated Edman degradation, we concluded in these experiments that a 17-residue signal sequence and a hexapeptide pro-sequence form the primary translation product containing 100 amino-acid residues. While this work was in progress Gordon et al.[6] confirmed the 23 residues containing prepro-sequence, but their analysis indicated that the signal sequence is 18 and the pro-sequence five residues long, ending in two arginine residues. Concomittant translations in vitro and studies on the synthesis and secretion in cell lines Hep G2 and Hep B3 described here are in full agreement. A primary translation product of 100 amino-acid residues is synthesized and processed into an 82-residue-pro-form and finally to the 77-residue-long mature apo AII. In this report we describe cell-free translations and cotranslational processing of liver poly(A+) RNA of apo AII, the immunoprecipitation of the apo AII precursors and their partial sequence analysis. The pro-form of apo AII resembles mature apo AII extended by a pentapeptide at the N-terminus. It was partially characterized by labelling experiments, subsequent isolation of the peptide and Edman degradation.

The secretion process of apo AII was analysed with Hep G2 and Hep B3 (not shown here) cells leading to identical conclusions: unlike pro-apo AI, which is entirely secreted in its pro-form, pro-apo AII and mature apo AII were isolated from the medium and the cells. The partial processing of the pro-form can be explained by the two basic residues (-Arg-Arg) terminating the prosequence, a structural feature common to other proproteins.

Materials and Methods

[^{35}S]Methionine (specific activity 600 Ci/mmol), [^{35}S]-cysteine (600 Ci/mmol), [4,5-$^3H_2$]leucine (190 Ci/mmol), [5-^{3}H]arginine (25 Ci/mmol) and [4,5-$^3H_2$]-lysine (100 Ci/mmol) were purchased from Amersham

Buchler (D-3300 Braunschweig). Anti-human apo AII-IgG was isolated from antisera raised in sheep immunized with purified S-carboxymethylated monomeric human serum apolipoprotein AII (apo AII/2).

Dog pancreatic endoplasmic reticulum membranes and reticulocyte lysates from anaemic rabbits were prepared following established procedures[5,13]. Human liver RNA was isolated according to Norgard et al.[14] and poly(A+) containing RNA by chromatography on oligo(dT)-cellulose[15].

Cell-free translation of human liver poly(A+) mRNA and the cotranslational translocation in the presence of fortified reticulocyte lysate and dog pancreatic membranes followed by immunoprecipitation were carried out essentially as described before[1,4]. In short: The cocktail for preparative translation in vitro consisted of 500 µl rabbit reticulocyte lysate, 100 µl 1M potassium acetate, 25 µl 16mM magnesium acetate, 100 µl reticulocyte salts with the exception of the radioactive amino acid, and 1 mCi ^{35}S- or ^{3}H-labelled amino acid, brought to 1000 µl with bidistilled water. For translation 0.5 A_{260} units of human poly(A+) RNA was added and the volume adjusted to 1250 µl with water. 7.5 OD_{280} units of nuclease-treated dog pancreatic membranes[16] were added to the translation incubation mixture. Incubation time was 90 min at 37 °C. Immunoprecipitation: 312 µl $NaDodSO_4$ (25%) was added and the mixture boiled for 5 min. After addition of 4 ml Triton X-100 (19%) followed by 150 µl sheep anti-human apo AII/2 the mixture was incubated at 4 °C overnight on a rotating wheel. 1 ml *Staphylococcus aureus* (Cowan strain)[17] (10% suspension in $NaCl/P_i$ buffer) was added for adsorption of the antigen-antibody complex under constant rocking at 4 °C for 1–4 h. The *Staphylococcus* cell suspension was freed of unbound radioactive proteins by repeated suspension (10x) in aprotinin buffer and centrifugation. The cell suspension was reductively carboxymethylated, centrifuged and the supernatant prepared for $NaDodSO_4$ polyacrylamide gel electrophoresis.

All buffers used for the isolation and characterization of the labelled reaction products contained the proteinase inhibitors aprotinin (20 IU/ml) and $PhCH_2SO_2F$ (1mM). $NaDodSO_4$ polyacrylamide gradient (15–20%) electrophoresis of 1.5 mm slab gels was performed as described before and the gel either dried for fluorography[18] or, after staining with Coomassie blue, the apo AII/2 band was excised from the gel, electro-eluted in 50mM triethanolamine and 0.025% $NaDodSO_4$, and lyophilized. The dye and $NaDodSO_4$ were removed by acetone washing. After the addition of carrier myoglobin the labelled synthesis product was sequenced and the distribution of radioactivity in the phenylthiohydantoin derivatives of every cycle determined.

A Beckman sequencer Model 890 C was used for automated Edman degradation of the labelled product together with the carrier myoglobin.

Tissue culture

Eagle's MEM[19] supplemented with Earle's salts[20] and 10% fetal calf serum was used.

For each experiment Hep G2 cells were grown in 4 flasks (75 cm^2 surface each) to near confluency in the presence of MEM. The cells were rinsed with serum-free MEM, omitting the amino acid used in the labelling experiment, and then incubated with MEM and 1 mCi labelled amino acid for 6 h at 37 °C. The medium was collected by centrifugation at 10000 × *g* for 2 min. The supernatant was adjusted to 5% $NaDodSO_4$. For immunoprecipitation the five-fold excess of Triton X-100 over $NaDodSO_4$ in aprotinin-containing buffer (0.19M NaCl, 6mM EDTA, 50mM Tris/HCl, pH 7.4, 20 µl aprotinin/ml) was added.

The cell layer was washed three times with $NaCl/P_i$ buffer, and 2 ml NP40-buffer (10mM Tris-HCl pH 7.5, 1% NP40, 0.15M NaCl, 2mM EDTA, 1mM $PhCH_2SO_2F$) was added to each culture flask. For cell detachment and lysis they were shock-frozen and thawed three times. The lysed material was collected, the flask rinsed with NP40-buffer and the combined samples treated with $NaDodSO_4$ and Triton X-100 for immunoprecipitation as described for the medium.

Immunoprecipitation

150 µl sheep anti-human apo AII/2 was added to the detergent-denaturated medium and cell lysates and the mixture further processed as described for cell-free translation.

Results

Cell-free synthesis and processing of the primary translation product of human liver apo AII mRNA

Rabbit reticulocyte lysate was programmed with human liver poly(A+) RNA in the presence of [^{35}S]cysteine, [^{3}H]lysine, [^{3}H]alanine and [^{3}H]-arginine. For the first step of the processing nuclease-treated dog pancreatic endoplasmic reticulum membranes were added. Proteins with antigenic determinants of mature apo AII were isolated from the mixture of labelled proteins by precipitation with mono-specific sheep anti-apo AII and binding of the complex to Sepharose-protein A or *Staphylococcus aureus*.

The radioactive proteins migrated in a molecular mass range of 8–9 kDa in $NaDodSO_4$-polyacrylamide gel electrophoresis, which corresponds to the apo AII monomer. They were recovered from the gel by electro-elution as described before[1], diluted with myoglobin and sequenced by automated Edman degradation in the number of cycles indicated in the figures. The radioactivity recovered in the thiazolinones of each cycle was determined. Fig. 1 visualizes the distribution of [^{35}S]cysteine (a), [^{3}H]alanine (b), [^{3}H]lysine (c) and [^{3}H]arginine (d) in the N-terminal sequence of the apo AII monomer which had been synthesized in vitro and processed. ^{3}H-Labelled amino acids were always incubated together with [^{35}S]methionine in order to obtain the position of the ^{3}H-labelled amino-acid residue relative to

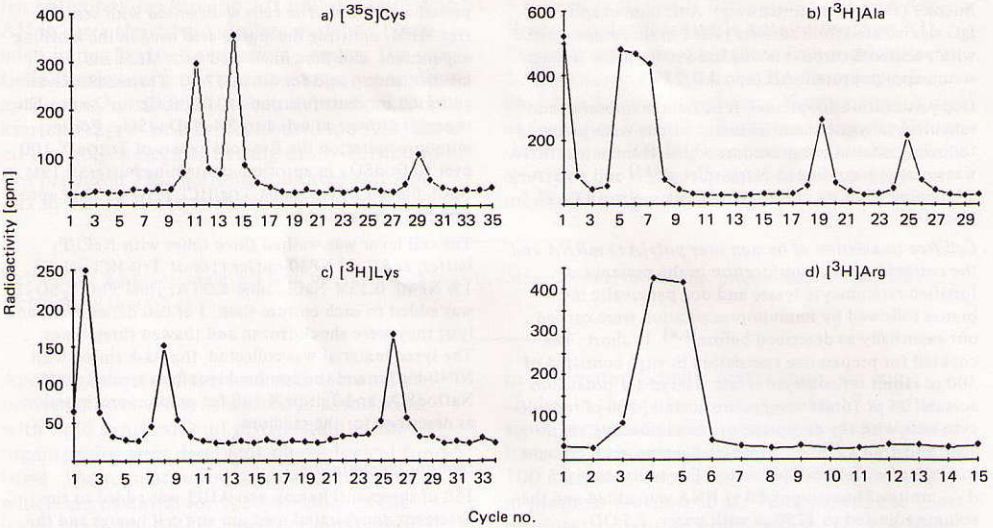

Fig. 1. N-terminal sequence analyses of processed apo AII-mRNA primary translation product.

Human liver poly(A+) RNA was translated in a rabbit reticulocyte cell-free translation system containing the labelled and 19 unlabelled amino acids in the presence of dog pancreatic membranes.
Radioactivity pattern of the thiazolinones after Edman degradation of immunoprecipitated apo AII, which was purified by $NaDodSO_4$-polyacrylamide gel electrophoresis and electro-elution as described under Materials and Methods. Amounts of radiolabelled apo AII subjected to automated Edman degradation: a) [^{35}S]cysteine 7500 dpm; b) [^{3}H]-alanine 10500 dpm; c) [^{3}H]lysine 8400 dpm; d) [^{3}H]arginine 9500 dpm.

methionine, which we found only in position 1 of the prepro-sequence of apo AII in our previous study[4]. [^{35}S]Cysteine residues were located in positions 11, 14 and 29 of the polypeptides isolated from the cotranslational processing experiment, indicating that about 40–50% of the prepro-apo AII had been translocated with the splitting off of the signal sequence containing 18 amino-acid residues, as deduced from the radioactivity in cycle 11 on the basis of a 98% repetitive yield of the phenylthiohydantoin derivatives of the amino acids of myoglobin as internal standard in each automated Edman degradation. [^{3}H]-Alanine was found in positions 1, 5, 6, 7, 19 and 25, of which 1 and 7 are derived from pro-apo AII, the other from the prepro-sequence.

The radioactivity of [^{3}H]lysine as labelled precursor appeared in cycles 2, 8 and 26. The ratio of lysine-2/lysine-8 was 1.7 taking a 98% repetitive yield into account. Since the first lysine residue within the mature apo AII sequence occurs at position 3, lysine at position 8 is derived from the pro-apo AII sequence in which the N-terminus must be extended by a pentapeptide. This pentapeptide ends with two adjacent arginine residues as demonstrated with [^{3}H]-arginine as precursor. The total radioactivity was detected in cycles 4 and 5.

Synthesis and secretion of apo AII by hepatocarcinoma cells (Hep G2)

The secretion process of apo AII and the subsequent elucidation of the structure of the secreted polypeptide were achieved by experiments with hepatocarcinoma cells (Hep G2), an established cell line of human origin known to possess the major secretory functions of the hepatocyte[21]. In each case two flasks (75 cm^2 surface) with a nearly confluent Hep G2 cell layer were incubated with [^{35}S]methionine, [^{35}S]cysteine and [^{3}H]arginine (0.5 mCi/flask). No serum was added to the medium and the amino acid of the respective labelling experiment was omitted from the medium (MEM plus Earle's salts)[19,20] during the incubation period of 6 h.

Proteinase inhibitors (EDTA, aprotinin, iodoacetamide and benzamidine) was added as well as 100 μl human serum inactivated for 30 min at 56 °C for apo AII dilution. The medium was collected and a rinse (2 m*l*) of the cell-layer pooled with the MEM, proteins denatured with

detergents ($NaDodSO_4$ and Triton X-100) and sheep anti-human apo AII IgG fraction or serum added for immunoprecipitation.

The cell-layer was detached and lysed with detergents and repeated freezing and thawing. The solubilized apo AII was isolated from the protein mixture by immunoprecipitation, complexed to Sepharose-protein A or equally effectively to denatured *Staphylococcus aureus* (Cowan strain)[17]. After reductive carboxymethylation in $NaDodSO_4$-solution the solubilized proteins were separated by polyacrylamide gel electrophoresis. The radioactive bands with molecular masses around 8–9 kDa were excised from the gel and the protein was obtained by electro-elution ready for Edman degradation.

Figure 2 diagramatically summarizes the distribution of the radioactivity in the cycles of the automated sequencing of anti-apo AII-precipitable labelled protein from the medium and cells.

The [^{35}S]methionine experiment (Fig. 2a) clearly demonstrates that the prepro form can neither be detected in the cell extract nor as secretion product in the medium. The presence of [^{35}S]-methionine in cycles 26 and 31 however indicates that the proform (cycle 31) and the mature apo AII sequence (cycle 26) are secreted in about equal amounts, which is the ratio also found inside the hepatocytes. A pentapeptide sequence as N-terminal extension is also obviously indicated by the [^{35}S]methionine in cycle 31.

In accordance with the cotranslational translocation experiments in vitro [^{35}S]cysteine residues were detected in cycles 6 and 11, Cys^{11} in the proform and Cys^{6} in the mature apo AII (Fig. 2b). [^{3}H]Arginine labelled the product only in residues 4 and 5 of the first 15 degradation cycles. This analysis reflects the degradation of the proform of apo AII (Fig. 2c), as the mature protein contains no Arg within that sequence area.

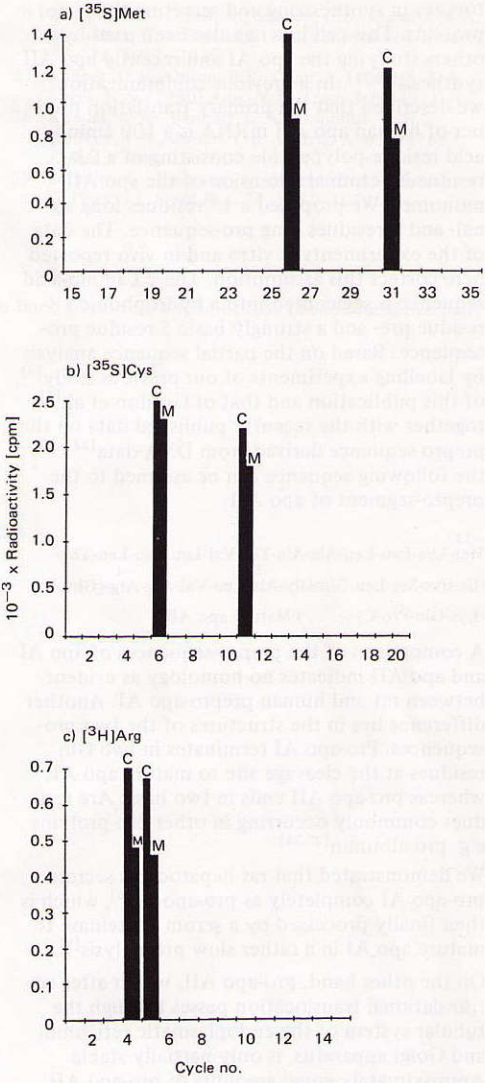

Fig. 2. N-terminal sequence analyses of apo AII isolated by immunoprecipitation from the medium (M) and lysed Hep G2 cells (C).

Cells grown to more confluency were incubated with the labelled amino acid as described under Materials and Methods. The immunoprecipitated labelled apoprotein was further purified by $NaDodSO_4$-polyacrylamide gel electrophoresis, electro-eluted and subjected to Edman degradation.

a) [^{35}S]methionine medium 10000 dpm, cell lysate 5400 dpm;
b) [^{35}S]cysteine medium 12000 dpm, cell lysate 13500 dpm;
c) [^{3}H]arginine medium 5800 dpm, cell lysate 6500 dpm.

Discussion

In this report we describe experiments in vitro and in tissue culture which contribute to a further understanding of the synthesis, processing and secretion of human apolipoprotein AII besides apo AI, the other major protein component of human HDL particles. Whereas the protein synthesis in vitro programmed with human liver poly(A+) RNA and the cotranslational translocation was studied with cell-biological procedures as described for apo AI, the secretion process could be analysed with a hepatoma cell line (Hep G2) potent like non-transformed hepa-

tocytes in synthesizing and secreting the major proteins. This cell line has also been used by others studying the apo AI and recently apo AII synthesis[9–11]. In a previous communication[4] we described that the primary translation product of human apo AII mRNA is a 100 amino-acid residue-polypeptide consisting of a 23-residue-N-terminal extension of the apo AII monomer. We proposed a 17 residues long signal- and 6 residues long pro-sequence. The data of the experiments in vitro and in vivo reported here correct this assumption. The 23 amino-acid sequence is segmented into a hydrophobic 18-residue pre- and a strongly basic 5-residue pro-sequence. Based on the partial sequence analysis by labelling experiments of our previous study[4], of this publication and that of Gordon et al.[6] together with the recently published data on the prepro-sequence derived from DNA-data[22–26] the following sequence can be assigned to the prepro-segment of apo AII:

$$\overset{-23}{\text{Met}}\text{-Lys-Leu-Leu-Ala-Ala-Thr-Val-Leu-Leu-Leu-Thr-}$$
$$\text{-Ile-Cys-Ser-Leu-Glu-Gly-}\overset{-5}{\text{Ala}}\text{-Leu-Val-Arg-}\overset{-1}{\text{Arg}}\text{-(Gln-Ala-}$$
$$\text{-Lys-Glu-Pro-Cys- . . .) Mature apo AII}$$

A comparison of the prepro-sequences of apo AI and apo AII indicates no homology as evident between rat and human prepro-apo AI. Another difference lies in the structures of the two pro-sequences. Pro-apo AI terminates in two Gln residues at the cleavage site to mature apo AI, whereas pro-apo AII ends in two basic Arg residues commonly occurring in other pro-proteins e.g. pro-albumin[27,28].

We demonstrated that rat hepatocytes secrete pro-apo AI completely as pro-apo AI[8], which is then finally processed by a serum proteinase to mature apo AI in a rather slow proteolysis[9].

On the other hand, pro-apo AII, which after co-translational translocation passes through the tubular system of the endoplasmatic reticulum and Golgi apparatus, is only partially stable. Approximately equal amounts of pro-apo AII and mature apo AII monomer were isolated from the lysed Hep G2 cells and found in a similar ratio in the medium as secretion products. These results are in agreement with those recently reported by Gordon et al.[6]. The secretion of pro-apo AI and pro-apo AII with two additional basic charges on the pro-sequence is responsible for the difference in their positions on isoelectric focusing. The slow proteolytic cleavage demonstrated for pro-apo AI leads to their permanent presence in serum. At least one of the isoproteins of apo AI[10,11] and apo AII[29], isoprotein AI-2 and presumably isoprotein AII-1 are these proforms.

So far no information regarding the function of the pro-sequences is available. Whether the final processing to the mature apo AI and AII occurs at the surface of the assembled HDL particle of the lipoprotein complex secreted from either the hepatocyte or the enterocyte remains to be investigated. Apo AII is present in HDL particles as a dimer linked by one S-S-bridge between the Cys^6 of two monomers. So far only the monomer and its pro- and prepro-forms have been isolated. Different techniques will have to be applied to settle the site and stage of the disulfide-bridge formation.

Future site-directed mutation studies might shed light on the physiological meaning of the N-terminal pro-sequences.

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