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# IDENTIFICATION OF PROAPOA-I IN RAT LYMPH AND PLASMA: METABOLIC CONVERSION TO "MATURE" APOA-I

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**SUMMARY**: Rat apoA-I polymorphism has been analyzed in lymph and plasma. Two major proteins were present and their relative distribution was different in lymph and plasma lipoproteins. The basic protein (pI 5.60) was quantitatively most abundant among plasma lipoproteins and the acidic protein (pI 5.50) was predominant in lymph chylomicrons and lipoproteins. Microsequence amino acid analysis of the two proteins isolated by preparative isoelectrofocusing revealed that pI 5.50 apoA-I was proapoA-I with six additional amino acids ($H_2N$-Ser-Glu-Phe-Trp-Gln-Gln) at the N-terminal end of "mature" apoA-I (pI 5.60 apoA-I). When radioiodinated proapoA-I was injected in rats, a conversion to "mature" apoA-I was observed and the process reached 92% completion in six hours. These data demonstrate the origin of apoA-I polymorphism *in vivo*.

ApolipoproteinA-I (apoA-I) is the major protein constituent of high density lipoproteins (HDL) and chylomicrons, and is a polymorphic system in plasma composed of different isoproteins. ApoA-I is synthesized in the liver and the intestine as a preproprotein (1, 2). Cotranslational cleavage of the prepropeptide occurs intracellularly and gives rise to a proprotein, proapoA-I (3). In man apoA-I is secreted almost exclusively as proapoA-I and this protein has been identified in plasma and in the thoracic duct lymph particularly in the chylomicron fraction (4). Since proapoA-I has been found to be a minor circulating apoA-I form in man, it has been suggested that its conversion to "mature" isoproteins, which are abundant in plasma, is occurring after secretion (3-5).

In rat, apoA-I also is a major plasma apoprotein and is polymorphic. Rat apoA-I is synthesized as a prepro precursor protein (2, 3) but to date there

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is no evidence that proapoA-I is secreted or is present in plasma. In this report we have identified proapoA-I in the plasma of rats and in the intestinal lymph. We have identified the proprotein by amino acid microsequence analysis and provide the first evidence that proapoA-I isolated from rat intestinal lymph is actually converted in vivo within six hours to "mature" apoA-I. This proapoA-I to apoA-I catabolic pathway causes the observed plasma apoA-I polymorphism and explains the formation of plasma "mature" apoA-I. Furthermore, since plasma apoA-I polymorphism has been identified in several species, this proapoA-I to apoA-I catabolic pathway may be a common proteolytic process.

## MATERIALS AND METHODS

Male, Sprague Dawley, rats weighing 225-275 g, maintained on Purina rat chow and water ad libitum and light:dark (12 hr:12 hr) cycle-adapted were utilized throughout the study. Blood was drawn from the abdominal aorta of rats under ether anesthesia, collected in sterile tubes containing EDTA as anticoagulant and Trasylol as protease inhibitor and kept on ice. Lymph was collected from the main intestinal lymph duct via cannulation as previously described (6). The sterile collection flasks, which were kept on ice, contained EDTA, gentamicin and Trasylol in 0.9% NaCl. Lymph from overnight fasted rats was collected during a 14 hour continued fasting period after lymph cannulation. Lymph from fed animals was collected during an 18 hr period after cannulation. Both the fasted and fed rats were continuously infused with 0.45% NaCl via the gastric cannula at a rate of 1 ml/hr and, additionally, the fed animals received 1 ml bolus doses of corn oil intragastrically every 4 hrs during lymph drainage. All restrained animals had free access to water.

Chylomicrons were isolated from the lymph in a SW27 Beckman rotor at 27,000 rpm at 4°C for 90 mins. The fat cake was resuspended in saline (0.15M NaCl, d1.006 g/ml) and the chylomicrons washed twice by this same ultracentrifugation procedure. Chylomicrons, obtained from the final spin were dispersed in 0.08% ammonium bicarbonate and kept at 4°C until used. Lipoproteins from plasma and the lymph depleted of chylomicrons, were isolated in a Beckman 40.3 rotor, adjusting sequentially the solution density to 1.063 g/ml (d<1.063 g/ml lipoproteins) and then to 1.210 g/ml (HDL) (7). Before apolipoprotein analysis and/or radioactive counting, d<1.063 g/ml lipoproteins and HDL were extensively dialyzed against 0.08% ammonium bicarbonate and kept refrigerated until used.

ApoA-I isoform patterns in lymph and plasma lipoproteins were analyzed by two dimensional gel electrophoresis as previously described (4). Rat proapoA-I was isolated either from lymph chylomicrons or the d<1.21 g/ml lymph lipoprotein fraction. The "mature" apoA-I was isolated from plasma HDL. ProapoA-I and apoA-I were isolated by preparative isoelectrofocusing in polyacrylamide (7.5% acrylamide, pH gradient from 4 to 6) and eluted from the gel by isotachophoresis in 0.5% agarose containing 0.5M Tris-HCl, pH 6.8. Isolated apolipoproteins were extensively dialyzed against 0.08% ammonium bicarbonate and stored at -70°C until used. Identity and purity of the isolated proteins was checked by immunodiffusion against monospecific rat apoA-I antibodies, partial amino acid sequencing and by two dimensional gel electrophoresis (4) or SDS gel electrophoresis (8). The N-terminal sequence

of the isolated apoA-I forms was determined using 1-2 nmol of protein. Analysis was carried out in a Applied Biosystem (Model 470 A) gas phase sequenator.

For the turnover study in vivo, proapoA-I and apoA-I were radioiodinated respectively with $^{125}I$ and $^{131}I$ by the Chloramine T method. Briefly, 200 µg protein of each was dissolved in 100 µl of 0.6M phosphate buffer pH 7.2, followed by addition of 0.5 mCi radiolabeled iodide. Under constant mixing 20 µl of Chloramine T (33 µg/ml in phosphate buffer) was added and the iodination reaction stopped after five minutes by adding 5 µl of $Na_2S_2O_5$ (200 µg/ml in phosphate buffer). Radioiodinated protein and free iodine were separated by chromatography on a PD 10 (Pharmacia) column equilibrated with 0.15 M NaCl, 0.02M Tris-HCl, pH 7.4. The protein was extensively dialyzed overnight against the same buffer. Before injection, the radioiodinated protein solution was passed through a Millipore filter (0.22 µm). The specific activity of the radioiodinated proteins was between 6 to 10 cpm/ng. [$^{125}I$]ProapoA-I and [$^{131}I$]apoA-I ( 30,000 cpm each) were injected into the rats as a single bolus dose of 500 µl via a cannula inserted in one of the femoral veins (9). Three minutes after injection, an initial blood sample (300 µl) was obtained from each animal via the other femoral vein cannula. The animals were sacrified at intervals up to 12 hours after the injection of the radiolabelled proteins. Under ether anesthesia blood was drawn from the abdominal aorta and collected in sterile tubes containing EDTA and Trasylol. The d<1.063 g/ml lipoprotein density fraction and HDL were isolated by ultracentrifugation as detailed above and HDL dialyzed and delipidated for the analysis of the radioactivity distribution among proapoA-I and apoA-I by isoelectrofocusing (10).

## RESULTS

### Rat ApolipoproteinA-I Polymorphism in Lymph and Plasma Lipoproteins

Rat apoA-I exhibits a polymorphism in lymph and plasma consisting of two major proteins with pIs, respectively, of 5.50 and 5.60. The apoA-I patterns are shown in Figure 1, panels A-C, as analyzed by two dimensional gel electrophoresis. The more acidic of these proteins is the major apoA-I protein in lymph chylomicrons (Panel A) and the more basic is predominant in plasma (Panel C). The two proteins are quantitatively equal (Panel B) in the d<1.210 g/ml lymph supernatant depleted of chylomicrons by preliminary ultracentrifugation. The plasma distribution pattern (Panel C) remains identical for d<1.21 g/ml fraction, for d<1.063 g/ml fraction and for HDL, the d1.063-1.21 g/ml fraction. Also the lymph patterns (Panel A and B) are identical for lymph collected from fasted or fed rats. pI 5.60 ApoA-I was isolated by preparative isoelectrofocusing from plasma HDL and pI 5.50 apoA-I was isolated from either lymph chylomicrons or d<1.210 g/ml lymph lipoproteins. The proteins revealed slightly different molecular weights by SDS polyacrylamide gel electrophoresis. Using appropriate molecular weight

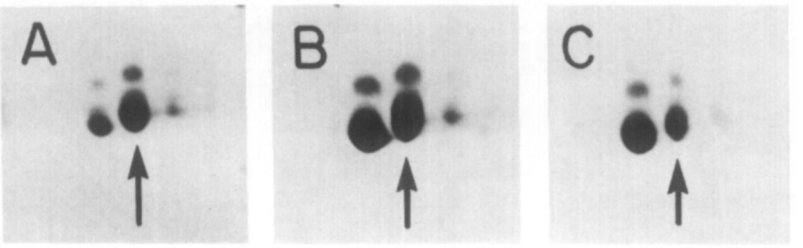


Figure 1 **Two Dimensional Gel Electrophoresis of Rat ApolipoproteinA-I**
The portion of the electrophoretogram showing apoA-I is presented. The arrow indicates the position of proapoA-I. The apoA-I pattern in lymph chylomicrons (Panel A); in chylomicron depleted - lymph lipoproteins (d<1.21 g/ml) (Panel B) and in the plasma lipoproteins (d<1.21 g/ml) (Panel C) are illustrated.

standards, pI 5.60 apoA-I and pI 5.50 apoA-I had apparent molecular weights of 27,000 and 28,000 daltons, respectively (Fig. 2). Purity of the proteins was based on their electrophoretic and immunological behavior. Automatic N-terminal sequence analysis showed that pI 5.50 apoA-I contained an additional sequence of six amino acids at the amino terminal end of pI 5.60 apoA-I. The sequence of the additional hexapeptide of the proprotein was $H_2N$-Ser-Glu-Phe-Trp-Gln-Gln. The sequence of the proprotein was continued to residue 3 confirming the length of the propeptide and the reported sequence of the N-terminal of the plasma mature protein. The partial amino acid sequence of the rat propeptide has been reported recently by Gordon et al (3) utilizing the radiolabelled cell free translation product of rat intestinal apoA-I. Their four identified amino acid residues are identical to those reported here. pI 5.60 ApoA-I was analyzed through twenty residues and the results match the amino acid sequence already published by Gordon et al (3) confirming that pI 5.60 apoA-I corresponds to "mature" apoA-I which is the predominant apoA-I protein in plasma (3).

## In Vivo Conversion of ProapoA-I to "Mature" ApoA-I

To gain insight into the mechanism of processing in plasma of proapoA-I to "mature" apoA-I, the two proteins were radioiodinated respectively with $^{125}I$ and $^{131}I$ and injected simultaneously into rats. They were then sacrificed at intervals to 12 hours and plasma subjected to ultracentri-

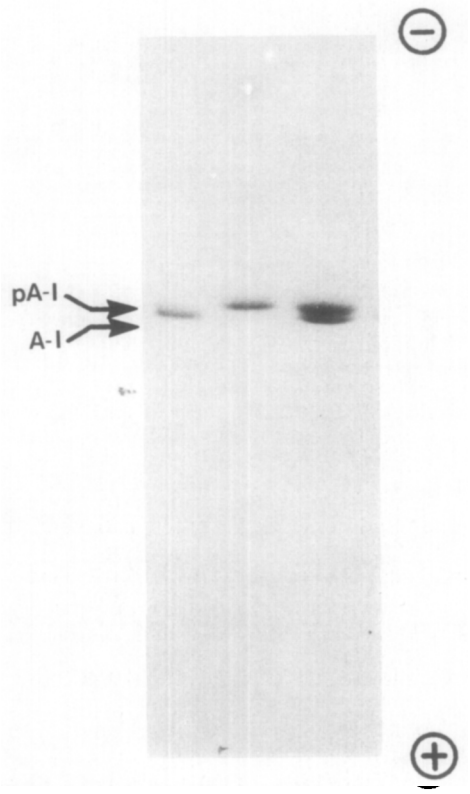


**Figure 2** **SDS Polyacrylamide Gel Electrophoresis of Purified Rat ApoA-I and ProapoA-I**
Five µg of each protein were loaded: apoA-I (left lane), proapoA-I (central lane) and a mixture of the two apolipoproteins (right lane).

fugation for isolation of HDL. Delipidated apo-HDL were analyzed by isoelectrofocusing and the radioactivity associated with proapoA-I and apoA-I bands counted. The results demonstrate that proapoA-I is converted in plasma to apoA-I. The time course of this conversion process is shown in Figure 3. ProapoA-I conversion to apoA-I reached 27% in two hours and the process reached 92% completion in six hours.

## DISCUSSION

Rat apoA-I is synthesized as a precursor protein, preproapoA-I. PreproapoA-I undergoes co-translational proteolytic cleavage to proapoA-I intracellularly (2). In man proapoA-I is the major apoA-I form secreted by the liver and the intestine (3,4). The data presented here demonstrates that proapoA-I is also secreted in the rat and is present in lymph and plasma. Rat

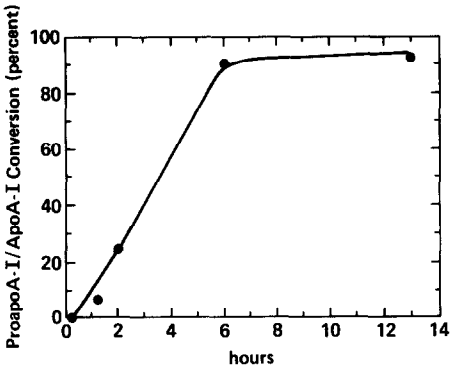


Figure 3 **Time Course of the ProapoA-I to ApoA-I Conversion Process In Vivo in Rat**

After injection of radioiodinated proapoA-I, blood samples were obtained serially from the injected animals as described in the Materials and Methods section. The analysis of the distribution of the counts between proapoA-I and apoA-I in HDL were determined by isoelectrofocusing.

perfused liver secretes almost exclusively proapoA-I (11). Thus the biosynthetic pathway of apoA-I in rat seems analogous to that seen in man.

However several major differences exist, in particular, the mass of proapoA-I in the rat is more than 25% of the total circulating apoA-I while in man proapoA-I is less than 2%. Moreover, the rate of conversion of proapoA-I to apoA-I in the rat requires 6 hours, a time comparable to the residence time of the "mature" apoA-I which is 7-8 hours. In contrast, in man the conversion process is rapid, being approximately three hours (unpublished observation, Ghiselli), relative to the plasma residence time of apoA-I of 3-4 days. Despite this significant difference between rat and man of the conversion rates relative to residence times, the conversion rates themselves (3 or 6 hours) are more similar suggesting that the responsible lipoprotein protease (apoA-I propeptidase) from the two species may be identical or similar.

The amino acid sequence of rat proapoA-I peptide is also different from that recently reported in human (1). In particular, Ser for Arg and Glu for His are substituted at residues -6 and -5 in rat proapoA-I peptide as compared to human. Rat and human peptides also have different net charges. The electrophoretic mobility of rat proapoA-I is one charge unit more acidic than rat apoA-I and human proapoA-I is two charge units more basic than human apoA-

I consistent with the differences in the hexapeptide sequences of the proproteins. An interesting homology is, however, evident from residues -4 to 1, i.e., Phe-Trp-Gln-Gln-Asp in both species. This suggests that the apoA-I propeptidase responsible for the cleavage of the propeptides has a high degree of specificity and that in evolution the mechanism of the proapoA-I cleavage has been maintained. This amino acid sequence and specificity of propeptide cleavage also may explain the comparable in vivo rates of proapoA-I to apoA-I conversion found in rats and humans.

The functional significance of a proapoA-I - apoA-I system is still obscure. ProapoA-I is the major A-I isoprotein in rat chylomicrons whether isolated from lymph collected during a fasting state or during active fat absorption. Human lymph chylomicrons are similarly enriched in proapoA-I. These data suggest that cleavage of the propeptide occurs only when chylomicrons reach the circulation. It is possible that proapoA-I plays a crucial role in the plasma catabolism of the triglyceride-rich intestinal lipoproteins.

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