STUDIES OF THE COMPOSITION AND STRUCTURE OF PLASMA LIPOPROTEINS. C- AND N-TERMINAL AMINO ACIDS OF C-I POLYPEPTIDE ("R-Val") OF HUMAN PLASMA APOLIPOPROTEIN C

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1. Introduction

Several years ago, results from our laboratory demonstrated [1] that partially delipidized very low density lipoproteins (VLDL, $d < 1.006$ g/ml) isolated from hypertriglyceridemic plasma contained 3 separable phospholipid–protein residues; the protein moieties of two of these were identical to apolipoproteins A and B (ApoA and ApoB). The protein moiety of the third residue was characterized by a high capacity for phospholipid binding, immunological properties and peptide patterns different from those of ApoA and ApoB, and by serine and threonine as N-terminal amino acids. This newly recognized protein-moiety, designated as apolipoprotein C (ApoC), was also found in the chyle VLDL [2] and in all the major density lipoprotein classes isolated from normal, fasting human plasma [3]. Recently, Brown et al. [4] confirmed the presence in VLDL of 3 phospholipid–protein residues, one of which gave a specific immunoprecipitin reaction only with antibodies to VLDL. In addition, these authors separated the soluble protein moiety of totally delipidized VLDL by gel filtration into 2 major fractions. One fraction consisted of ApoA polypeptides, whereas the second contained a mixture of polypeptides characterized by serine and threonine as N-terminal amino acids. Chromatography of this fraction on a DEAE-cellulose column resulted in the isolation of 3 polypeptides which were designated on the basis of their C-terminal amino acids as apoLP-Val ("R-Val"), apoLP-Glu ("R-Glu"), and apoLP-Ala ("R-Ala") [5, 6]. To investigate the possible relationship between ApoC and polypeptides "R-Val", "R-Glu" and "R-Ala", we have isolated ApoC from VLDL by immunoprecipitation [7] and established [3, 8] that it consists of 3 immunologically distinct polypeptides which correspond in almost all chemical properties to those isolated by Brown et al. Since all 3 polypeptides seem to be required for the formation of a high density lipoprotein C (LP-C) [9] and an abnormal low density lipoprotein (LP-X) in obstructive jaundice [10], we have suggested that ApoC consists of three non-identical polypeptides designated as C-I ("R-Val"), C-II ("R-Glu") and C-III ("R-Ala").

In the course of our studies on the characterization of ApoC-polypeptides, we could not confirm valine as the C-terminal amino acid of C-I ("R-Val"). This report presents evidence that C-I polypeptide is characterized by serine as the C-terminal and threonine as the N-terminal amino acid.

The significance of this finding is discussed with respect to its possible influence on and significance for a new nomenclature of plasma lipoproteins.

2. Experimental procedure

2.1. *Isolation of human plasma VLDL*

VLDL were isolated from pooled human plasma by preparative ultracentrifugation at 105,000 *g* for 22 hr in the Ti60 rotor at plasma density. The top layer was removed by tube slicing, diluted with 0.15 M NaCl, and recentrifuged 2 times under the original isolation conditions. The VLDL, free of albumin as determined by double diffusion, was then dialyzed against distilled water and lyophilized.

2.2. *Isolation of C-I polypeptide*

Delipidization of the lyophilized VLDL was accomplished by 6 successive extractions with ethanol–diethyl ether (3:1, v/v) at 4° including one overnight extraction. This was followed by 3 extractions with diethyl ether. Totally delipidized VLDL (apoVLDL) were partially soluble in 0.1 M $(NH_4)_2CO_3$ at pH 9.0 and the soluble portion was lyophilized and fractionated by a modified procedure of Brown et al. [4]. The soluble apoVLDL was chromatographed on Sephadex G-100 column (2.5 × 100 cm) equilibrated with 0.1 M $(NH_4)_2CO_3$. Of the 2 major fractions collected, the first one consisted of ApoA and the second of ApoC polypeptides. The lyophilized ApoC fraction was applied to a DEAE-cellulose column (30 × 1.5 cm) prepared as described previously [11]. Elution with a linear gradient from 0.005 to 0.10 M NaCl containing 8 M urea at pH 7.2 yielded 4 major peaks. The first peak which contained C-I polypeptide was dialyzed against distilled water, lyophilized and utilized for subsequent investigation.

2.3. *Analytical and immunochemical methods*

The polypeptide was examined by double diffusion and immunoelectrophoresis in 1% agar according to previously described procedures [10]. Rabbit antisera to human LP-A, LP-B and LP-C were prepared as previously described [12]. Rabbit antibodies to human IgG, IgA, IgM, whole serum, and albumin were purchased from Behringwerke AG, Marburg an der Lahn, Germany.

N-terminal amino acids were determined with dansyl-chloride and carboxy-terminal amino acids by hydrazinolysis and carboxypeptidase A and B using procedures previously described [10]. Amino acid analyses were performed on a Beckman Model 120C analyzer by an accelerated procedure on spherical resins [12].

3. Results and discussion

3.1. *Characterization of the C-I polypeptide*

Double diffusion analysis of C-I revealed a single precipitin line with antibodies to LP-C and no reaction with antibodies to LP-A, LP-B, IgG, IgA, IgM, albumin or whole serum. On agar immunoelectrophoresis, the C-I polypeptide displayed a characteristic migration

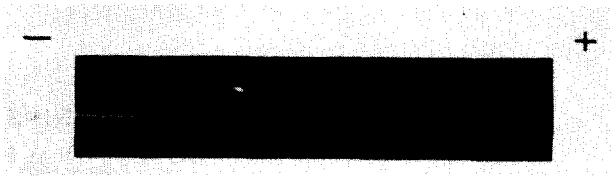

Fig. 1. Immunoelectrophoresis of C-I polypeptide on 1% agar. Trough contains antibodies to LP-C.

towards the cathode and gave a precipitin arc only with antibodies to LP-C (fig. 1). Amino acid analysis of C-I demonstrated the absence of tyrosine, histidine, cystine, and cysteine. From these results and the elution patterns on Sephadex G-100 and DEAE-cellulose columns, it was concluded that the C-I polypeptide corresponded to the "R-Val" polypeptide described by Brown et al. [4, 6].

3.2. *C-terminal amino acid*

Brown et al. [4] reported that digestion of C-I polypeptide ("R-Val") with carboxypeptidase A or B at 27° resulted in no release of amino acids. However, on the basis of a hydrazinolysis experiment, they concluded that this polypeptide contains valine as the C-terminal amino acid and suggested that it be called "R-Val" or apoLP-Val. Failure to confirm this result in routine characterization analyses of C-1 polypeptide

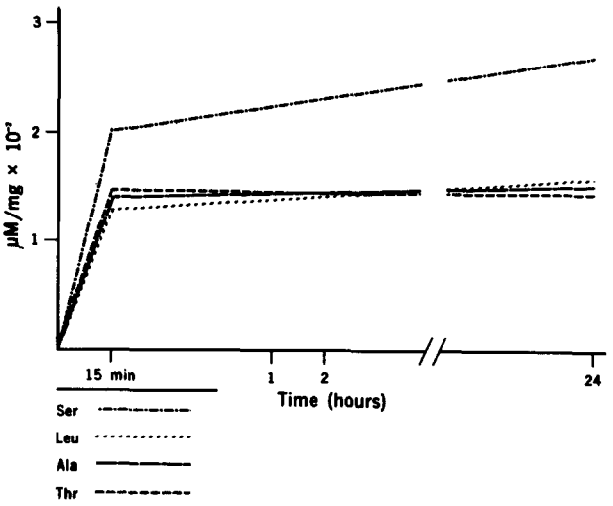

Fig. 2. Release of amino acids from C-I polypeptide with carboxypeptidases A and B at pH 5.8 and 37°. The ratio of enzymes to polypeptide was 1:80.

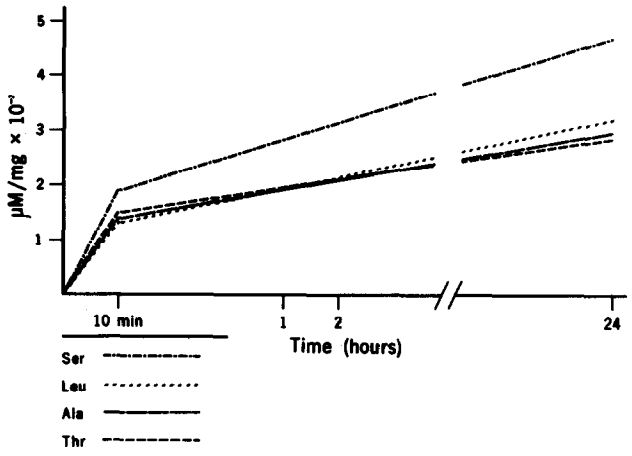

Fig. 3. Release of amino acids from C-I polypeptide with carboxypeptidases A and B at pH 8.5 and 37°. The ratio of enzymes to polypeptide was 1:80.

prompted us to reinvestigate this problem by submitting a preparation of C-I polypeptide to a systematic, quantitative study by both enzymatic digestion with carboxypeptidases A and B and by hydrazinolysis. Since Brown et al. [4] observed no release of amino acids by carboxypeptidase A or B at pH 8.5, the digestion of C-I was first performed at pH 5.8 and 37° [13]. The results of this time study (fig. 2) showed that the initial release of serine was 1.5–2 times greater than those of threonine, alanine and leucine. However, an almost identical result was obtained by digestion of C-I polypeptide with a mixture of carboxypeptidases A and B at pH 8.5 and 37° (fig. 3). The alkaline pH had a slight influence on the overall rate of reaction rather than on the sequence of liberated amino acids. The amount of serine increased linearly with time and there was no leveling-off in the amount of this amino acid after 24 hr. Due to a small quantity of available polypeptide preparation, longer digestion times were not investigated. To verify the results of enzymatic cleavage, the C-1 polypeptide was submitted to hydrazinolysis at 3 different reaction times (10, 50 and 90 hr). In each instance, serine was identified by the amino acid analyzer as the only major acid (table 1); the optimal recovery of serine (52.3% of the theoretical value based on the molecular weight of 7000 [6] for C-I polypeptide) was achieved after 90 hr of hydrazinolysis. We concluded from these

Table 1
The release of amino acids from C-I polypeptide by hydrazinolysis.

Amino acids released	μmoles/10^2 mg of polypeptide		
	10 hr	50 hr	90 hr
Serine	3.5	5.0	7.5
Glycine	1.2	0.6	1.9
Alanine	0.70	0.4	1.1

studies that serine is the C-terminal amino acid of C-I polypeptide.

Although it is difficult to explain the discrepancy between results presented in this study and those in the literature [4, 6], the reported failure to observe a release of amino acids by enzymatic digestion could have been caused by either the conformational state of the C-I polypeptide, its poor solubility, or methodological differences. One should not overlook the enormous methodological difficulties in securing a quantity of C-I polypeptide sufficient for systematic time studies, and the possible lack of an adequate amount of this polypeptide in the original study [4] may have been an additional reason for failure to recognize serine as the C-terminal amino acid. Although one would expect the presence of C-I polypeptide in the S_f 20–100 lipoproteins, Shore and Shore [14] reported that hydrazinolysis of the corresponding protein moiety failed to reveal valine as one of the C-terminal amino acids.

3.3. *N-terminal amino acid*

In agreement with the previously published reports [4, 6], results of a qualitative N-terminal amino acid analysis of C-I polypeptide by dansylation method confirmed threonine as the only N-terminus.

Detection of serine as the C-terminal amino acid of C-I polypeptide may necessitate a reassessment of the C-terminal amino acid of apolipoprotein B (ApoB), the major protein moiety of very low- as well as low-density lipoproteins. Several years ago, Shore reported [15] that hydrazinolysis of low density lipoproteins with S_f 7.9 and 7.1 yielded serine (2.2–2.5 μmoles/10^3 mg protein), alanine (1.3–1.5 μmoles/10^3 mg protein), glycine (1.0–1.3 μmoles/10^3 mg protein) and threonine. This result has been interpreted as

evidence that serine is the C-terminus of ApoB which has been referred to as "R-Ser" [16, 17]. However, serine has not yet been confirmed as the C-terminal amino acid of ApoB. Moreover, Shore and Shore indicated in a recent report [14] that hydrazinolysis of a low-density lipoprotein with S_f 4–8 performed with Amberlite CG50 resin as catalyst for 40 or 60 hr at 80° did not yield serine. Since recent immunochemical studies have revealed the presence of LP-C in the LDL subfractions [12], the small amount of detectable serine by hydrazinolysis of LDL preparations may stem from and indicate the presence of C-I polypeptide rather than apolipoprotein B.

The finding of serine as the C-terminal amino acid of C-I polypeptide is significant not only because it raises the question of the C-terminus of ApoB, but also because it renders untenable the prevailing practice of identifying the apolipoproteins or polypeptides by their terminal amino acids. It has been shown recently that glutamine is the C-terminal amino acid of both ApoA polypeptides [11]. The possible existence of serine as the C-terminus of both ApoB and C-I polypeptide represents an additional argument supporting the recently proposed nomenclature [8, 18] which is based on letters and numerals as codes for apolipoproteins and their constitutive polypeptides. According to this proposal, the polypeptides of ApoA are called A-I (C-glutamine-N-aspartic acid) and A-II (C-glutamine-N-blocked), and the polypeptides of ApoC are designated as C-I (C-serine-N-threonine), C-II (C-glutamic acid-N-threonine) and C-III (C-alanine-N-serine). The polymorphic forms of C-III are identified by arabic numerals such as C-III-0 (absence of sialic acid), C-III-1 (one mole of sialic acid), C-III-2 (two moles of sialic acid), etc. If necessary, the same coding system can be applied to the polymorphic forms of ApoA polypeptides. In absence of unequivocal evidence for the presence of non-identical polypeptides, the protein moiety of major low-density lipoprotein is simply called ApoB.

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