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Heterogeneity in Protein Subunits of Human Serum High-Density Lipoproteins*

B. Shore and V. Shore

ABSTRACT: The protein moieties of high-density lipoproteins of human serum contain comparable quantities of two polypeptide subunits of different amino acid sequence. These peptides in urea solutions were separated by polyacrylamide gel electrophoresis and by chromatography on DEAE-cellulose. The carboxyl-terminal sequences -Thr-Gln and probably -Lys-Tyr-Lys-Asn-Leu-Thr were elucidated by the actions of carboxypeptidases A and B on the lipid-free protein moieties of two fractions (1.083–1.124 and 1.126–1.195 g/cc) of lipoproteins and on the peptides fractionated on DEAE-cellulose. Glutamic acid γ-hydrazide, indicative of C-terminal glutamine, in addition to threonine, was found among the products of hydrazinolysis of the protein.

The protein contains approximately 2 moles of C-terminal glutamine plus threonine per 30,000 g of protein.

The protein moiety of the entire human serum high-density lipoprotein fraction (1.065–1.195 g/cc) is commonly thought to consist of one protein, the α protein, which occurs as identical subunits whose number varies with the density and molecular weight of the parent lipoprotein (Shore, 1957; Scanu, 1966; Levy and Fredrickson, 1965; Gustafson *et al.*, 1966). There are, however, some preliminary reports which do suggest heterogeneity in the proteins of high-density lipoproteins (Shore and Shore, 1966; Alaupovic *et al.*, 1967).

In the present study, the presence in high-density lipoproteins of two nonidentical peptides in comparable amounts is indicated (1) by the action of carboxypeptidases A and B on the protein moiety, (2) by the finding of glutamic acid γ-hydrazide, indicative of C-terminal glutamine, and threonine as products of hydrazinolysis of the protein, (3) by polyacrylamide gel disc electrophoresis of the protein in 8 M urea solution at pH 8.8, and (4) by separation of the peptide with C-terminal glutamine from that with C-terminal threonine by DEAE-cellulose column chromatography.

Materials and Methods

Lipoprotein fractions of densities 1.083–1.124 and 1.126–1.195 g/cc (HDL_2 and HDL_3, respectively) were isolated from human serum of individual donors. HDL_3 was prepared as described previously (Shore and Shore, 1967); HDL_2, the lipoproteins which floated between solvent densities 1.083 and 1.124 g/cc, were separated from less dense lipoproteins by two centrifugations at a solvent density of 1.083 g/cc (20°) for 36 hr at 39,000 rpm in a 40.3 rotor at 13–14° in a Spinco Model L centrifuge. The HDL_2 was then concentrated by two flotations at a solvent density of 1.124 g/cc for 48 hr at 39,000 rpm at 13–14°. The protein moieties of these lipoprotein fractions were obtained in lipid-free, water-soluble form with essentially complete recovery as described previously (Shore and Shore, 1967).

Carboxypeptidase A (COA-DFP 6131), a three-times-crystallized, diisopropylfluorophosphate-treated enzyme in water suspension, was obtained from Worthington Biochemical Corp. (Freehold, N. J.). Aliquots of the enzyme suspension were dissolved in 2 M LiCl at pH 8 before use. Carboxypeptidase B (COB-DFP 7GA) was obtained as a frozen solution from the same source. The presence of a small amount of carboxypeptidase A in the carboxypeptidase B preparation or *vice versa* was not excluded. The proteins in water solution (2 mg/ml) were digested with carboxypeptidase A, or

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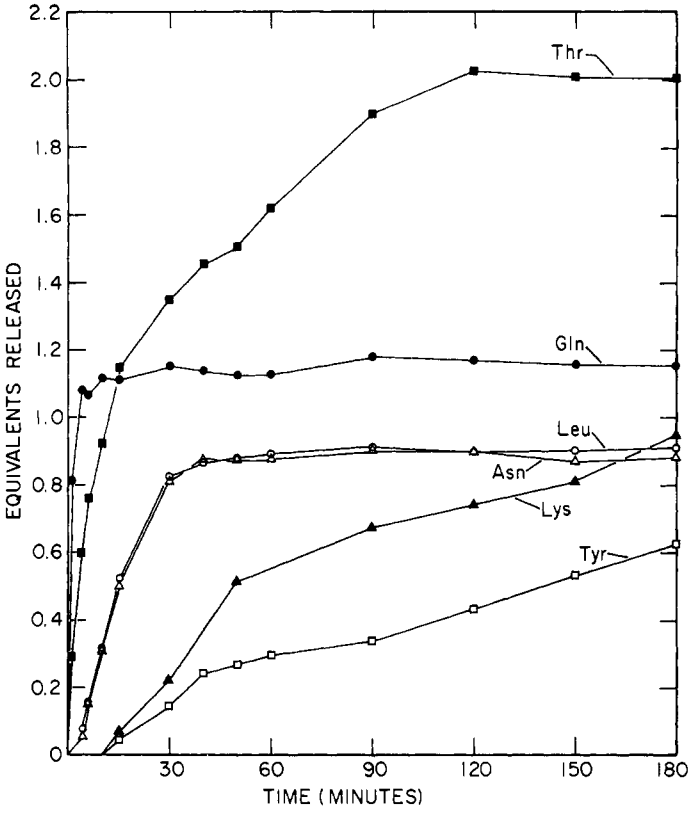

FIGURE 1: The kinetics of amino acid release from the protein moiety of high-density lipoprotein during digestion with carboxypeptidase A at 25°. The molecular weight of the protein was taken as 30.5×10^3 (Shore and Shore, 1967).

carboxypeptidase B, or a mixture of A and B, either at 25 or 37°. The ratio of substrate to each enzyme was approximately 100:1 (w/w). The pH was maintained between 7.8 and 8.2 by addition of 0.1 M LiOH. Aliquots of the digestion mixture were withdrawn at the indicated times, added to 0.25 volume of either 1.5 N lithium citrate or 1.0 N sodium citrate buffer at pH 2.2, frozen in a Dry-Ice–ethanol bath, and kept at −15° until amino acid analysis was performed. The "blank" levels of amino acids in the preparations were determined by addition of an aliquot of the protein solution to pH 2.2 buffer just before freezing. The contribution from autodigestion was determined by incubating separately the carboxypeptidases in the absence of protein substrate. The amino acids released as a function of digestion time were determined quantitatively with a Beckman–Spinco Model 120B amino acid analyzer equipped with a high-sensitivity cuvet, an expanded-range recorder circuit, and a digital integrator system. The chromatographic procedure using UR-30 resin with lithium citrate buffers (as described in Bulletin A-TB-044 available from Beckman Instruments, Palo Alto, Calif.) was used for analysis of acidic and neutral amino acids; the basic amino acids were analyzed on a short column as described in Beckman Bulletin A-TB-033.

Carboxyl-terminal amino acids were also determined by hydrazinolysis as described by Braun and Schroeder (1967). After heating for 72 hr at 80° and subsequent removal of excess hydrazine, one-half of the hydrazinolysate (after removal of hydrazides on an Amberlite CG-50 column) was examined with the amino acid analyzer. The remainder was allowed to react with dinitrofluorobenzene and chromatographed as described by Kawanishi *et al.* (1964).

The protein in 8 M urea, before and after reduction with $NaBH_4$, was subjected to polyacrylamide gel disc electrophoresis (Davis, 1964; Poulik, 1966) in a Canalco Model 12 apparatus (Canal Industrial Corp., Bethesda, Md.). Electrophoresis was carried out for 2 hr at a current of 2.5 mA/tube. The acrylamide monomer concentrations were 7.5, 10, or 12.5%. Riboflavin in the presence of light was used instead of ammonium persulfate as the chain-initiating agent. Tris buffer at pH 8.8 (Davis, 1964) and acetate buffer at pH 4.5 (Reisfeld *et al.*, 1962) were used.

For column chromatography of the protein in 8 M urea, DEAE-cellulose in a microgranular, fully swollen and wet form was obtained from Reeve Angel and Co. (Clifton, N. J.). It was washed with 0.1 M Tris-HCl buffer (pH 8.0) before equilibration with the starting solution, which was 0.005 M Tris-HCl buffer (pH 8.0) containing 8 M urea. A linear elution gradient was formed by pumping 0.125 M Tris-HCl containing 8 M urea at 12 ml/hr into a 500-ml reservoir of starting buffer. Urea solutions were prepared just before use. Before chromatography, the protein solution was dialyzed for 24 hr against the starting solution. The column effluent was monitored continuously for absorbancy at 280 mμ and for conductivity. The fractions were dialyzed against water, concentrated, and analyzed for protein concentration. Some of each fraction was then taken for polyacrylamide gel electrophoresis and digestion with carboxypeptidases A and B.

Protein concentrations were determined by the method of Lowry *et al.* (1951). Glutaminase and asparaginase were obtained from Worthington Biochemical Corp.

Results

The action of carboxypeptidase A on the protein moieties of high-density lipoproteins from human serum indicates glutamine to be a C-terminal residue. Although it is conceivable that carboxypeptidase could release a non-C-terminal amino acid more rapidly than the C-terminal residue from a sequence such as -B-B-A, this is not probable in the release of glutamine from these proteins. After prolonged incubation approximately 1 mole of glutamine per 30×10^3 g of protein was released. Previous sedimentation equilibrium experiments on the protein in dilute NaCl solutions containing sodium dodecyl sulfate indicate $30–31 \times 10^3$ to be the protein subunit molecular weight (Shore and Shore, 1967). Glutamine was the amino acid most rapidly released from the protein moieties of both the 1.083–1.123- and the 1.126–1.195-g per cc lipoprotein fractions from each of five individuals (three males and two females). Threonine, which has been found to be C terminal by hydrazinolysis (Shore, 1957; Scanu and Granda, 1966), was released rapidly also, and eventually to the extent of approximately 2 moles per 30×10^3

TABLE I: Digestion of High-Density Lipoprotein Protein with Carboxypeptidases A and B.[a]

Protein Source[c]	Equiv of Amino Acid Released[b]						
	Lys	Thr	Asn	Gln	Leu	Tyr	Ala
HDL_2 (1)	1.27	2.00	0.81	1.21	0.82	0.81	0.42
HDL_3 (1)	1.25	2.24	0.74	1.38	0.77	0.65	0.03
HDL_2 (2)	1.14	1.97	0.88	1.08	0.92	0.79	0.40
HDL_3 (2)	1.33	2.07	0.72	1.30	0.77	0.64	0.09
HDL_2 (3)	1.11	1.86	0.86	1.05	0.88	0.67	0.19
HDL_3 (3)	1.21	2.07	0.91	1.16	0.93	0.77	0.02
HDL_2 (4)	*d*	2.04	0.92	0.86	0.98	0.80	0.16
HDL_3 (4)	1.33	1.98	0.86	0.93	0.94	0.85	0.09
HDL_3 (5)	1.21	2.12	0.83	1.16	0.89	0.74	0.11

[a] Incubation period, 3 hr at 37°. [b] The molecular weight of the protein was taken as 30.5×10^3 (Shore and Shore, 1967). [c] Samples 1, 4, and 5 were from male donors; samples 2 and 3 were from female donors. [d] Not determined.

g of protein. In addition, leucine, asparagine, lysine, and tyrosine were released by carboxypeptidase A from all the protein moieties; one example is shown in Figure 1. Carboxypeptidase B released, although more slowly, the same amino acids from these proteins (Figure 2). The identities of glutamine and asparagine were confirmed by conversion into glutamic acid and aspartic acid, respectively, by glutaminase and asparaginase.

The data of Figures 1 and 2 might seem to suggest that the lysine released is not in the same peptide chain as the leucine and asparagine residues, since carboxypeptidases A and B release lysine more slowly and more rapidly, respectively, than these amino acids. It is also possible, however, that these amino acids (Lys, Leu, and Asn) are derived from the same polypeptide chain, since possibly the B enzyme not only releases lysine more rapidly than does the A enzyme but that it also acts on two different lysine residues in the peptide chain. The first lysine residue follows leucine and asparagine (Figure 1) and precedes tyrosine; the second, released less readily than the first by carboxypeptidase B, follows tyrosine. During the digestion of the protein by carboxypeptidase B, the molar ratio of lysine to tyrosine is greater than two after short intervals and less than two after longer intervals.

The equivalents of glutamine and its molar ratio to other amino acids released by carboxypeptidase were similar but not constant among the protein samples analyzed (Table I). Variations were found among fractions of the same density from different persons and between HDL_2 and HDL_3 fractions from the same person.

The carboxypeptidase experiments suggest that at least two peptide chains of different amino acid sequence are present in the protein moieties of high-density lipoproteins. However, leucine, lysine, and tyrosine have not been found to be C terminal by hydrazinolysis of these proteins; C-terminal glutamine and asparagine, which are converted into glutamic acid γ-hydrazide and aspartic acid β-hydrazide, respectively, would have been lost with the aldehyde–hydrazide fraction in previously used procedures. In this reinvestigation of the products of hydrazinolysis, glutamic acid γ-hydrazide was identified by chromatography of the di-DNP compound. The uncorrected recovery was 1 mole per 40×10^3 g of protein or less. The uncorrected yield of threonine was 1 mole per 50×10^3 g of protein or less. These data, together with the carboxypeptidase results, indicate that glutamine and threonine are C-terminal amino acids in these proteins.

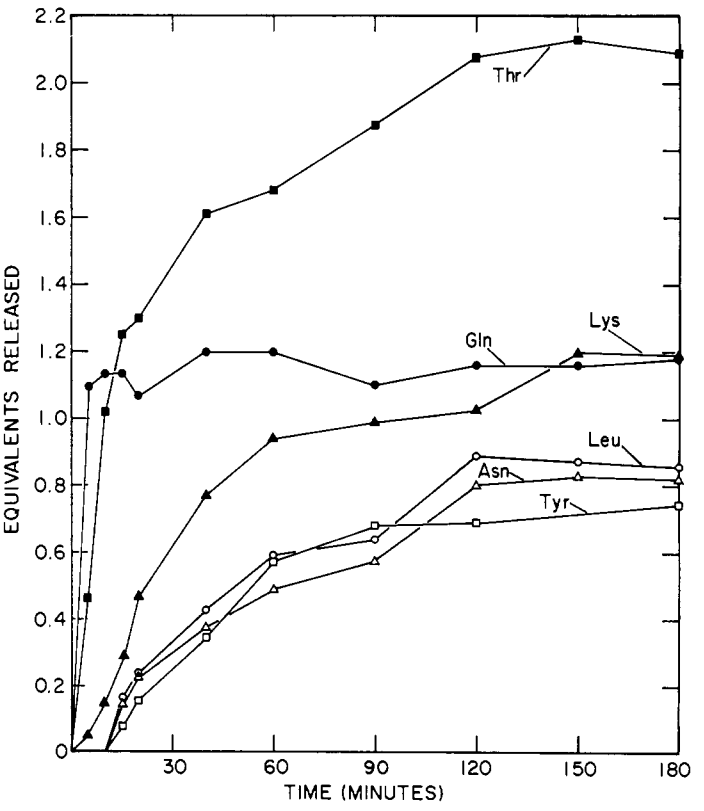

FIGURE 2: The release of amino acids (moles per 30.5×10^3 g of protein) from high-density lipoprotein protein during digestion with carboxypeptidase B at 37°.

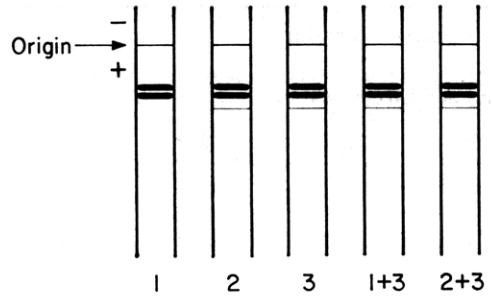

FIGURE 3: Polyacrylamide gel disc electrophoresis patterns of high-density lipoprotein proteins in 8 M urea at pH 8.8. 1, 2, and 3 correspond to the protein moieties of HDL_3 (3), HDL_3 (4), and HDL_2 (2), respectively, of Table I. The proteins were stained with Amido Schwarz.

The presence of two different polypeptides in high-density lipoproteins was supported by the finding of two major protein bands in polyacrylamide gel electrophoresis of the protein in 8 M urea at pH 8.8 (Figure 3). These data are consistent with the finding of two chains with nonidentical carboxyl-terminal amino acids. At pH 4.5 the protein migrated in one major band. Reduction of the protein disulfide group with $NaBH_4$ before gel electrophoresis did not affect the resolution of the protein during electrophoresis. The protein migrated in 12.5% gels, but 10% was optimum for resolution into bands.

The existence of two different polypeptides in serum high-density lipoproteins was further substantiated by the results of experiments on the fractions from DEAE-cellulose chromatography of the protein moiety of HDL_3 (Figure 4). The fractions were analyzed for protein content, for amino acids released by carboxypeptidases, and by polyacrylamide gel disc electrophoresis. Fractions 1 and 2 contained less than 5% of the total protein; fractions 3, 4, and 5 contained approximately 30, 40, and 25%, respectively, of the starting protein. Subsequent chromatographic separations of HDL_2 or HDL_3 proteins have always given fractions 3, 4, and 5, although in somewhat different proportions (fraction 5 is usually relatively smaller); fractions 1 and 2 are usually not found. The results of polyacrylamide gel electrophoresis, which are shown in Figure 5, suggest that fraction 3 contains only the faster moving component of the unfractionated protein (Figure 3), that fraction 4 is predominantly the slower moving component of the unfractionated protein with some of the polypeptide found in fraction 3, and that fraction 5 contains both of the peptides found in fractions 3 and 4. The results of experiments with carboxypeptidases (Table II) indicate that fraction 3 contains a polypeptide of molecular weight about 14,000 and with the carboxyl-terminal sequence R-Thr-Gln. No other amino acids were released by carboxypeptidases A and B from fraction 3. Fraction 4 contains the polypeptide with carboxyl-terminal threonine. These results also indicate that the lysine released from the unfractionated protein (Figures 1 and 2) is derived from the same polypeptide as are the asparagine, leucine, and tyrosine residues. Fraction 5 appears to contain in approximately equimolar amounts both polypeptides R-Gln and R-Thr. Perhaps these peptides are not completely dissociated by 8 M urea under the conditions of the chromatographic procedure.

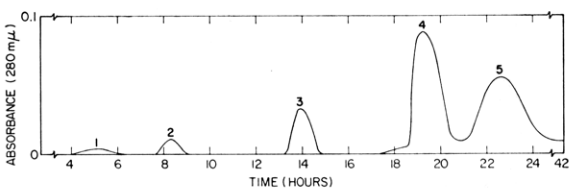

FIGURE 4: Chromatographic separation of high-density lipoprotein protein on DEAE-cellulose. The protein (4 mg in starting buffer, which was 0.005 M Tris-HCl (pH 8) containing 8 M urea) was applied to a 0.9 × 26 cm column and eluted with a linear gradient of Tris-HCl buffer (pH 8) in 8 M urea and a flow rate of 12 ml/hr. Elution was complete at 0.06 M Tris-HCl.

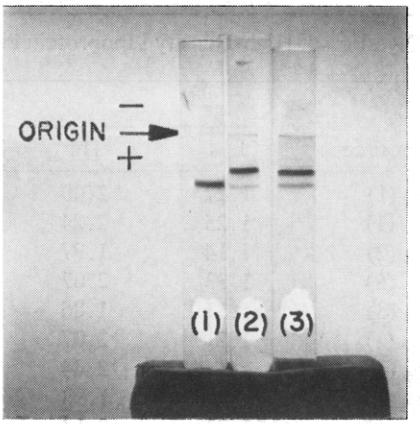

FIGURE 5: Polyacrylamide gel disc electrophoresis of polypeptide fractions from DEAE-cellulose chromatography of high-density lipoprotein protein. 1, 2, and 3 correspond to fractions 3 , 4, and 5, respectively, of Figure 4. The peptides were stained with Amido Schwarz.

The kinetics and stoichiometry of carboxypeptidase action on the unfractionated protein moieties of the serum high-density lipoproteins, the results of carboxypeptidase on the peptides obtained by DEAE-cellulose chromatography, the hydrazinolysis results, and the results of polyacrylamide gel disc electrophoresis are all consistent with the presence in these lipoproteins of two different peptides having carboxyl-terminal sequences R-Thr-Gln and, probably, R-Lys-Tyr-Lys-Asn-Leu-Thr.

Discussion

The subunit of mol wt 30–31 × 10^3 g which was observed in sedimentation equilibrium experiments of the lipid-free protein moiety in dilute NaCl solutions containing sodium dodecyl sulfate now appears to contain two peptide chains, since the sum of C-terminal threonine and glutamine is approximately 2 moles per 30 × 10^3 g of protein. At this point, it cannot be said that lipoprotein subunits which contain one or two of these peptides exist in the molecule, or that the subunit, if it exists, contains one or two kinds of peptides. The

TABLE II: Action of Carboxypeptidases on HDL_3 Polypeptide Fractions from DEAE-cellulose Chromatography.

Fraction[a]	Incubn (°C)	g of Protein/Mole of Amino Acid Released					
		Gln	Thr	Asn	Leu	Lys	Tyr
3	1 min (37)	14,200	31,200				
3	5 min (37)	14,100	14,000				
3	2 hr (37)	13,900	13,700				
4	2 hr (25)	63,000	15,000	20,000	19,500	12,900	22,000
5	2 hr (25)	34,800	15,900	30,400	30,800	18,400	32,100

[a] Corresponds to peaks shown in Figure 4.

molar ratio of these peptides appears not always to be an integral number and is not constant among different high-density lipoprotein preparations. However, the elution from DEAE-cellulose of a fraction containing both peptides, in addition to fractions containing the individual peptides, suggests that the two peptides do form mixed complexes. These peptides are probably very similar in size. The molecular weight of about 14×10^3 g estimated from the amount of C-terminal glutamine of the peptide isolated on DEAE-cellulose is approximately one-half that for the mixture of peptides.

The source of alanine found in some carboxypeptidase digests of high-density lipoprotein protein (Table I) is not yet explained. It probably is not derived from the polypeptides R-Gln and R-Thr since it was not found in these fractions from DEAE-cellulose chromatography. It is possible that the high-density lipoprotein proteins (usually HDL_2) which give rise to alanine contain a third, as yet unidentified, polypeptide moiety.

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