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Abstract

Combination of data on molecular weight and on C- and N- terminal amino acids indicates that the human-serum lipoprotein of 1.093 g/ml hydrated density possesses two N-aspartic acid-C-threonine peptide chains, each of approximate molecular weight 95,000 to 100,000; the 1.149-g/ml lipoprotein a single N-aspartic acid-C-threonine chain of weight 95,000 to 100,000; the 1.029-g/ml two peptide chains, one of which is presumably N-glutamic acid-C-serine of weight 380,000, and the other probably the same; and the 1.006 to 1.002-g/ml at least an N-serine-C-alanine chain of maximum weight 120,000 and others about which the heterogeneity of the fraction precludes a definite statement. None of the N- and C- terminal amino acids, with the possible exception of the C- terminal of the 1.029-g/ml lipoprotein, seems phospholipid in origin. A thorough study is under way at present on the amino acids of the lipoprotein phospholipids. Preliminary results indicate that the C- terminal serine of the 1.029-g/ml lipoprotein is not phospholipid in origin.

C - AND N- TERMINAL AMINO ACIDS OF HUMAN SERUM LIPOPROTEINS

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Even if lipoproteins were not related to atherosclerosis, their ubiquitous biological occurrence, e.g., in mitochondria and microsomes, would demand their study. The elucidation of the nature of the binding of lipids to proteins awaits knowledge of at least the chemical constitution of the lipid and protein moieties. In extension of previous work on the protein components of serum lipoproteins,[1] we have determined their C- and N-terminal amino acids.

Several methods have been developed for the determination of N- terminal amino acid residues of the peptide chains of proteins. For N- terminal residues, the dinitrofluorobenzene (DNFB) method[2] is complemented by the phenylisothiocyanate (TPC) method;[3] the former is inapplicable for proline and glycine. For C-terminal amino acids, the hydrazinolysis method of Akabori and Ohno,[4, 5] followed by isolation of the C- terminal amino acids as dinitrophenyl (DNP) derivatives, has been satisfactory for a number of proteins.[6] In the study reported herein, these methods have been applied to some lipoprotein fractions of human serum.

Experimental

Lipoproteins were isolated from the serum of individual donors by centrifugation of the serum in media of varying density. For isolation of S_f 20–60 lipoproteins, serum from individual donors was centrifuged in 9-ml Lusteroid tubes in a Spinco preparative centrifuge for 26 hours at 20°C and at 80,000 g. The layer of material at the top of the centrifuge tube contained molecules of S_f rate greater than 20. Molecules of S_f rate greater than 60 were removed from this layer by centrifugation for 5 minutes at 110,000 g. The maximum ordinate of lipoproteins isolated in this manner usually corresponded to a lipoprotein of S_f rate 20–25. The S_f "60" lipoproteins were isolated in the customary manner.[7, 8]

After centrifugation of serum in 6-ml centrifuge tubes for 26 hours at 15°C at a solution density of d_4^{20} = 1.065 g/ml, the top 2.5 to 3 ml were removed and pooled. To the bottom 3 to 3.5 ml was added enough sodium chloride (or sodium chloride plus D_2O) to make the solution density d_4^{20} = 1.122 g/ml in the absence of protein. This solution was centrifuged in 6-ml centrifuge tubes for 36 hours at 16°C at 110,000 g. This centrifugation floated to the top 1/2 ml of the tube the lipoprotein of 1.093-g/ml hydrated density.* The schlieren diagram of this fraction, at a solution density of 1.122 g/ml, infrequently showed the presence of a component of flotation rate greater than that of the 1.093-g/ml lipoprotein. The concentration of this component was about 10% that of the 1.093-g/ml lipoprotein. This component is being investigated at present and will be the subject of a subsequent communication. C- and N- terminal amino acid data reported in this paper were obtained only on preparations free of this component.

To the bottom 4 to 4.5 ml of the 1.122-g/ml centrifugation was added enough of a D_2O–sodium nitrate or D_2O–sodium chloride solution to make the solution density 1.200 g/ml (at 20°) in the absence of protein. This solution was then centrifuged for 36 hours at 14°C at 110,000 g, in 6-ml centrifuge tubes. The 1.149-g/ml lipoprotein was contained in the top 1/2 ml of the centrifuge tube.

The degree of homogeneity of all the isolated fractions was estimated by analytical ultracentrifugation. Only one symmetrical schlieren peak

* In subsequent expressions of the density of lipoprotein, "hydrated density" is omitted for simplicity's sake.

was evident in all the lipoprotein fractions but the S_f 20–60. Since that group of lipoproteins is already present in low cencentration in the serum of normal persons, no attempt was made at purification. Some centrifugal characteristics of the lipoproteins are included in Table I. The symbols S_f^0 and $s_{20,w}^0$ have their customary significance; sedimentation coefficients at infinite dilution are calculated by use of the relation

$$S_f^0,\ s^0 = S_f, s\ (1 - kc)^{-1},$$

where c is the concentration of lipoprotein. Measurement of the sedimentation constant and the concentration of each lipoprotein fraction at different concentrations determined k for the particular lipoprotein. The density of zero sedimentation, assumed to be the hydrated density of the lipoprotein, was determined by linear interpolation of the values of $\eta_{rel}s$ for at least three densities; η_{rel} is the viscosity of the solvent relative to that of water at the same temperature, and s is the measured flotation or sedimentation coefficient. All the flotation and sedimentation coefficients of Table I, with the exception of that for S_f 20–60, were weight average values.[9] These agreed within 3% with those determined by measurement of the maximum ordinate of the schlieren peak, the lipoproteins being centrifuged at 52,640 rpm.

N-terminal amino acid analysis by means of the dinitrofluorobenzene technique was carried out in the following manner. The isolated lipoproteins were first dialyzed against copper-free, redistilled water.[10] (It is of interest that the 1.093- and 1.149-g/ml lipoproteins are more stable on dialysis against distilled water than the 1.029- and 1.006–1.002-g/ml lipoproteins). DNP proteins were then prepared by shaking a mixture of one volume of aqueous protein solution containing 2% sodium bicarbonate and two volumes 2.5 DNFB (2,4 dinitrofluorobenzene, Eastman) in ethanol solution for 80 minutes at room temperature. The insoluble DNP-protein, after being washed with water, alcohol, and ether, was placed in the dark in a desiccator over phosphorus pentoxide.

Ten to 20 mg DNP-protein was then hydrolyzed with constant-boiling, redistilled hydrochloric acid in a sealed, evacuated Pyrex tube at 105°C for 12 to 16 hours. The ether-soluble DNP-amino acids were transferred

TABLE I

C- AND N- TERMINAL AMINO ACIDS OF HUMAN SERUM LIPOPROTEINS[e]

Sedimentation characteristics	Hydrated density (g/ml)	Molecular weight	Protein, %	Weight of peptide chain[a], (g) / 1.0 mole N-terminal amino acid: DNFB[b]	Weight of peptide chain[a], (g) / 1.0 mole N-terminal amino acid: PTC[c]	Weight of peptide chain[a], (g) / 1.0 mole C-terminal amino acid: Hydrazinolysis
$s^o_{20,w}$ 5.0_1	1.149	165,000 (Stokes sphere)	52	90,000–100,000 aspartic acid	90,000–100,000 aspartic acid	85,000–100,000 threonine
		195,000 (sedimentation)[d]		Trace amounts of glutamic acid, serine, and threonine		Trace amounts of serine, alanine, and glycine
$s^o_{20,w}$ 5.5_0	1.093	365,000 (Stokes sphere)	46	90,000–100,000 aspartic acid	90,000–100,000 aspartic acid	90,000–100,000 threonine
		435,000 (sedimentation)[d]		Trace amounts of glutamic acid, serine, and threonine		Trace amounts of serine, alanine, and glycine
S^o_f 7.9_2	1.029	3,200,000 (light scattering)[g]	24	390,000–400,000 glutamic acid	380,000–400,000 glutamic acid	400,000–450,000 serine
				Trace amounts of aspartic acid, serine, and threonine		670,000–800,000 alanine
						600,000–1,000,000 glycine
S^o_f 20–60	1.006–1.002	50–250 million (Stokes sphere)	9	110,000–125,000 serine		115,000–130,000 alanine
				700,000–800,000 glutamic acid		250,000–300,000 serine
				670,000–800,000 threonine		

[a] Calculated from the terminal amino acid analysis. [b] Dinitrofluorobenzene method. [c] Phenylisothiocyanate method. [d] Obtained by the method of Klainer and Kegeles (J. Phys Chem. 59, 852 (1955)). [e] The terminal amino acid data in this table represent the range of variation of determinations on at least four different lipoprotein isolations.

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to Whatman No. 1 paper for two-dimensional chromatography according to Levy,[11] or to Whatman No. 4 paper (previously sprayed with pH 5 phthalate buffer) for one-dimensional chromatography according to Blackburn and Lowther.[12] The water-soluble DNP amino acids were concentrated in vacuo and transferred to Whatman No. 4 paper (previously sprayed with pH 6 phthalate buffer) for one-dimensional chromatography according to Blackburn and Lowther. After elution in 1% bicarbonate, the amount in each amino acid spot was determined spectrophotometrically. Readings of the optical density were taken at 360 and 390 mμ and the amount of amino acid was calculated assuming the molar extinction coefficient at 360 mμ to be 17,000.

For the phenylthiocarbamyl method, 20 to 50 mg of protein in aqueous solution was mixed with 2 ml dioxane and stirred at 40°C with 0.1 ml PTC for 3 hours; a pH of about 9 was maintained by the addition of 0.01 N sodium hydroxide. The mixture was then extracted repeatedly with benzene. Dissolved benzene was removed with a stream of air and the aqueous phase evaporated to dryness in vacuo over phosphorus pentoxide and sodium hydroxide flakes. The residue was dissolved in 8 ml water, brought to 1 N in hydrochloric acid by the addition of 6 N acid, and heated in a boiling water bath for 1 hour. The phenylthiohydantoins (PTH's) were then extracted with ether. Traces of other absorbing material made direct reading in the spectrophotometer unreliable. For qualitative identification, the PTH's were chromatographed according to the method of Sjoquist.[13] Alternately, the PTH's were hydrolyzed with glass-distilled, constant-boiling hydrochloric acid in a sealed, evacuated Pyrex tube at 150°C for 16 hours. After it had been evaporated to dryness, the residue was dissolved in 1 ml water, and 0.1 ml was taken for two-dimensional chromatography as described by Levy and Chung.[14] To the remaining 0.9 ml was added 0.5 ml 6% sodium bicarbonate, 0.1 ml water, and 3 ml of 2.5% DNFB in ethanol. This mixture was shaken for 80 minutes at room temperature, 20 ml water added, and the excess DNFB removed with ether. The aqueous phase was acidified and extracted with ethyl acetate. The solvent from both phases was removed in vacuo, and from the residue of the organic phase dinitrophenol was removed by sublimation, in a cold finger under a vacuum of less than 1 mm, at 60°C. The DNP amino acids were chromatographed and read as described above.

The procedure used in hydrazinolysis followed very closely that described by Niu and Fraenkel-Conrat.[6] For C- terminal analysis by this method, the lipoproteins were delipidized directly with methanol-ether or after precipitation with cold 10% trichloroacetic acid in ether. (The appreciable solubility of the 1.093- and 1.149-g/ml lipoproteins in organic solvents often made precipitation necessary. Results were not affected by this procedure). Fifteen to 25 mg of delipidized protein that had been thoroughly dried in a vacuum oven over phosphorus pentoxide at 50°C was taken for analysis. To the protein was added 0.5 ml anhydrous hydrazine in a dry chamber. The tube containing the protein and hydrazine was sealed and heated for 10 hours at 100°C. The remainder of the procedure was not significantly different from that of Niu and Fraenkel-Conrat.[6]

Results

By the dinitrophenylation technique, the 1.093- and 1.149-g/ml lipoproteins were found to have, as N-terminal amino acid, $0.10\text{-}0.11 \times 10^{-6}$ mole aspartic acid per 10 mg protein. Thus, there is one mole aspartic acid per 90,000–100,000 grams protein. Smaller amounts (0.01×10^{-6} mole per 10 mg protein) of glutamic acid, serine, and threonine were found for both these lipoproteins. If these were actually N-terminal amino acids, a highly improbable molecular weight would result. The results with the DNP technique were qualitatively and quantitatively confirmed by the PTC method.

By hydrazinolysis, threonine was found to be the principal C- terminal amino acid for the 1.093- and 1.149-g/ml lipoproteins. Assuming 35% recovery,[6] 0.10 to 0.12×10^{-6} mole threonine per 10 mg protein was present, corresponding to 1 mole threonine per 85,000 to 100,000 g protein. Smaller amounts (0.01 to 0.03×10^{-6} mole amino acid/10 mg protein) of serine, alanine, and glycine were also found. These three amino acids varied in proportion and amount from sample to sample. If these were actually C-terminal amino acids, a highly improbable lipoprotein molecular weight would result.

Combination of the data for terminal amino acid and molecular weight—percent protein indicates that the 1.149-g/ml lipoprotein possesses one N-aspartic acid-C-threonine peptide chain of approximate molecular weight 95,000 to 100,000. The 1.093-g/ml lipoprotein contains 2 N-aspartic acid-C-threonine chains, each of approximate molecular weight 95,000 to 100,000. These data do not agree with those of other workers,[15] who found 0.76 mole aspartic acid per mole protein (at most 1 peptide chain) for a fraction of density 1.063 to 1.21. The nonidentity of data may be the result of differences in technique or in the degree of homogeneity of the investigated fractions.

For the 1.029-g/ml lipoprotein, 0.025×10^{-6} mole glutamic acid per 10 mg protein was found by the dinitrophenylation technique. This corresponds to 1.0 mole glutamic acid per 400,000 g protein. Traces ($< 0.01 \times 10^{-6}$ mole per 10 mg protein) of aspartic acid, serine, and threonine were found. Were these N-terminal amino acids, an improbable lipoprotein molecular weight would result. However, in one of six of the samples investigated (S_f^0 of 7.8, hydrated density of 1.029 g/ml) 1 mole threonine per 400,000 g protein was found by the DNP method. The PTC method indicated the presence of 1 mole glutamic acid per 380,000 g protein. Hydrazinolysis indicated the presence of 1.0 mole serine per 400,000 to 450,000 g protein. The C-terminal data also indicated the presence of 1.0 mole alanine per 670,000 to 800,000 g protein, 1.0 mole glycine per 600,000 to 1,000,000 g protein, and 1.0 mole threonine per 1,200,000 g protein. The consistency of the data, and the light scattering molecular weight and percent protein of the lipoprotein, indicate the C-terminal amino acid probably to be serine. Alanine, glycine, and threonine easily arise as artifacts (perhaps through unwanted protein hydrolysis) in the hydrazinolysis procedure.[6]

Our data indicate that the 1.029-g/ml lipoprotein contains two peptide chains, one of which is presumably N-glutamic acid-C-serine of molecular weight 380,000 and the other probably of the same weight and terminal amino acid composition. Other workers[15] found 0.49 mole glutamic acid per mole protein (at most one peptide chain even if the molecular weight of the fraction is as high as 3,200,000). Again the difference between data may be in technique or degree of heterogeneity of the investigated fractions.

For the S_f 20–60 lipoproteins, the DNP technique gave data corresponding to 1.0 mole serine per 110,000 to 125,000 g protein, 1.0 mole glutamic acid

per 700,000 g protein, and 1.0 moles threonine per 670,000 to 800,000 g protein. Trace amounts of DNP aspartic acid and DNP phenylalanine were also found. Phenylthiocarbamylation was not attempted on this fraction. Hydrazinolysis indicated the presence of 1.0 mole alanine per 115,000 to 130,000 g protein and 1.0 mole serine per 250,000 to 300,000 g protein. These data indicate the presence of at least an N-serine-C-alanine peptide chain of maximum weight 120,000. The heterogeneity of the fraction precludes a definite statement about the other peptide chains. This fraction was not investigated by Avigan and co-workers.[15]

The C- and N- terminal amino acid data, as well as some physical properties, are summarized in Table I. None of the N-terminal amino acids or C-terminal amino acids of the proteins of the 1.149;-1,093;-1.006-1.002 g/ml lipoproteins seem to be phospholipid in origin. It is unlikely that the C-terminal serin of the 1.029-g/ml lipoprotein is phospholipid in origin, but a thorough study is under way at present on the amino acid components of the lipoprotein phospholipids. Preliminary results indicate that the C-terminal serine of the 1.029-g/ml lipoprotein is not of phospholipid origin.

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