Proteolytic digestion in the elucidation of the structure of low density lipoprotein

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Abstract The apoprotein (apoB) of low density lipoprotein (LDL) is reported to be a large polypeptide, and it is proposed that there are two similar-sized subunit proteins in LDL (Smith, Dawson, and Tanford. 1972. *J. Biol. Chem.* **247:** 3376–3381.). When apoB is isolated under conditions that minimize artifactual proteolysis, only a single, large molecular weight protein appears on polyacrylamide gel electrophoresis in SDS. To investigate the organization of apoB as it exists within native LDL, limited proteolysis with trypsin has been used as a structural probe. Tryptic digestion for 1 hr at pH 7.6 with enzyme-to-protein ratios of 1:100 and 1:5 results in the liberation of approximately 10% and 30% of apoB as smaller, water-soluble peptides. These peptides may be separated from the partially digested but still intact tryptic core (T-core) of the lipoprotein by chromatography on Sephadex G-75. Repeatedly, the 1:5 T-core of native LDL is found to contain a family of polypeptides of 14,000–100,000 molecular weight. Although they have lost significant quantities of apoprotein, these T-cores sustain an appearance of homogeneity, as studied by analytical ultracentrifugation. Their measured molecular weights do not differ appreciably from those of the native LDL, and the carbohydrate content of the 1:5 tryptic T-core of LDL is similar to that of the native LDL. In normolipemic individuals, LDL generally exists in a monodisperse state, but, in different individuals, monodisperse LDL may range in molecular weight from 2.4 to 3.9 $\times$ 10^6. Limited tryptic digestions were used to probe the organization of apoB in these different molecular weight LDL. As assayed by SDS–acrylamide gel electrophoresis of the larger polypeptides and fingerprinting of the smaller released peptides, those regions of LDL exposed to trypsin digestion are identical in monodisperse LDL of 2.5 and 3.4 $\times$ 10^6 molecular weight. Thus, the different quantities of lipid bound in these various LDL must interact with apoB so that the same regions of the apoprotein are exposed to the action of trypsin in these different molecular weight lipoproteins.

Supplementary key words apolipoprotein B · trypsin proteolysis

Apolipoprotein B, the apoprotein of LDL, continues to be an elusive substance whose structure is yet to be well described. For years there has been controversy with respect to the number and molecular weights of the polypeptides that comprise the protein moiety of LDL (2–8). In 1972 Smith, Dawson, and Tanford (1) reported that apoB is a protein of molecular weight approximating 250,000, and that LDL contains two such similar-sized subunits. If this value is correct, then apoB is a protein of some 1900 amino acid residues. The difficulty of studying this protein after lipid extraction is further compounded by its insolubility in virtually all aqueous solvents except in the presence of detergents or denaturants. Yet from genetic studies it seems likely that apoB may differ in primary structure from one person to the next and that these differences may reflect themselves in the physical as well as the biological properties of LDL (9).

In considering the structure of apoB, it is important to know how this protein is organized within native LDL. One may anticipate that certain regions of the apoprotein will be relatively exposed, while others will be less accessible to surface probes. The possibility of producing limited and reproducible cleavages of apoB should be feasible if the structure of native LDL is reasonably constant. If so, perhaps specific portions of the polypeptide sequence of apoB would be regularly cleaved upon proteolytic digestion under standardized conditions.

LDL is known to exist in either a monodisperse or a polydisperse state (10, 11), the former being more commonly observed. When one examines the physical properties of monodisperse LDL, it is seen that LDL differs in molecular size from one individual to the next. For a specific individual, however, the molecular weight of LDL appears to be invariant, suggesting that an ordered structure may prevail (9). Accordingly, it seemed of interest to explore the possibility

Abbreviations: LDL, low density lipoprotein; apoB, apolipoprotein B, the protein moiety of LDL; SDS, sodium dodecyl sulfate; T-core, the tryptic core of LDL.

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of utilizing limited enzymatic proteolysis of native LDL in order to determine whether one could obtain specific site cleavages of apoB and, if so, whether such a technique would yield further insights into the structure of this lipoprotein. Previous studies by Bernfeld and Kelley (12) and by Margolis and Langdon (13) had suggested that this approach might be fruitful. More recently, Ikai and Yagisawa have reported on the proteolysis of native LDL with subtilisin (14).[3]

METHODS

Isolation and purification of LDL

LDL was isolated from blood bank plasma and from the blood of individual nonlipemic and hyperlipemic fasting donors by ultracentrifugation following the method of Fisher, Hammond, and Warmke (10). The S_f4-20 LDL fractions from hyperlipemic individuals were prepared as described by Hammond and Fisher (11), except that sodium azide (0.02%), merthiolate (0.01%), and EDTA (0.01%) were added to the freshly drawn blood. For routine studies, LDL was isolated from blood bank plasma from a single donor using plasma that was less than one week old, and the ultracentrifugal isolation was performed in the presence of the indicated preservatives. Under these conditions, the variable proteolysis of apoB was a recurring finding and was monitored by performing acrylamide gel electrophoresis in SDS of all LDL preparations isolated.[4]

When attempting to suppress proteolysis completely, the drawn blood was added to tubes containing 20 mg of soybean trypsin inhibitor per 100 ml of blood in addition to the above-specified preservatives. To this solution, 150 mg of phenylmethylsulfonylfluoride in 0.5 ml of dimethylsulfoxide was immediately added per 100 ml blood, and the tubes were shaken gently. The blood was allowed to stand at 24°C or 4°C for 2 hr before beginning LDL preparation. In order to remove traces of trypsin inhibitor from purified LDL, the lipoprotein was dialyzed against a 0.05 M ammonium bicarbonate buffer, pH 7.6, containing 0.02% sodium azide, and purified by chromatography on a 1.8 × 105 cm Sepharose 4B column equilibrated with the same buffer. LDL, which eluted as a single symmetrical peak, was concentrated on an Amicon XM-100 membrane.

Electrophoresis

Acrylamide electrophoresis was performed as described by Kobylka et al. (17) on 10-cm 5% gels containing 0.1% sodium dodecyl sulfate. In those experiments where unproteolyzed or only minimally proteolyzed LDL was examined, 3% gels were used to enhance the migration of apoB into the gel. LDL samples for electrophoresis were solubilized by adding 0.1-ml aliquots to 0.2 ml of 3% SDS in a 0.2 M phosphate buffer, pH 7.8, containing 20% glycerol and 0.02% sodium azide. The samples were made 1% in mercaptoethanol, heated at 100°C for 3–5 min, cooled at 24°C, and 40-μg aliquots were subjected to electrophoresis. The gels were stained with 0.035% Coomassie Blue in 25% isopropanol and 10% acetic acid (18) and were destained in 7% acetic acid. Gels were calibrated with a mixture of marker proteins.

Aminoethylation

LDL and apoB were studied to determine free and bonded sulfhydryls. Aminoethylation was performed on apoB isolated from LDL solubilized in 6 M guanidine-HCl, 0.2 M NH_4HCO_3, 0.02% NaN_3, from which over 98% of the lipid was extracted at 4°C with diethyl ether–ethanol 3:1 followed by diethyl ether. The protein was then dialyzed repeatedly with exchanges of the same guanidine solution (19). Modification was performed according to Cole (20) using a 0.1 M phosphate buffer, pH 8.1, in the presence of either 6 M guanidine or 3% SDS. Before beginning the reaction with ethylenimine, the samples were incubated at 24°C for 5 hr in the presence or absence of 0.1% mercaptoethanol followed by dialysis against the buffer under a nitrogen atmosphere. Determination of *S*-aminoethylcysteine was by amino acid analysis after hydrolysis of the protein in 6 M HCl in vacuo at 110°C for 20 hr.

Amino acid analysis

Amino acid analysis was performed on a Beckman Model 120C amino acid analyzer after hydrolysis of the protein in 6 N hydrochloric acid for 20 hr at 110°C in vacuo. No corrections were made for the destruction of amino acids during hydrolysis. Cysteine was measured as cysteic acid by the method of Hirs (21), and tryptophan content was determined using the *p*-toluenesulfonic acid method of Liu and Chang (22).

[3] An abstract reporting the findings of this investigation has been published (15).

[4] It has proven convenient to store isolated LDL, at a protein concentration below 10 mg/ml, at −40°C in the presence of 20% sucrose for periods up to 6 mos. Upon thawing, LDL shows only a minimal precipitate, which is removed by filtration. LDL stored this way is similar to freshly prepared LDL with respect to those properties studied in this investigation.

Carbohydrate determination

The carbohydrate composition and content of apoB and of the polypeptides of trypsin-digested LDL were determined on the ethanol–diethylether–extracted protein by the method of Zanetta, Breckenridge, and Vincendon (23). The method permits determination of hexoses, hexosamines, and sialic acid. The identity of the hexoses was confirmed by gas–liquid chromatography of their alditol acetates (24).

Proteolytic digestion

Purified LDL was dialyzed against a 0.05 M ammonium bicarbonate buffer, pH 7.6, containing 0.02% sodium azide, at 4°C for 24 hr. Protein was determined by the method of Lowry et al. (25), and LDL protein concentration ranged from 2 to 8 mg/ml. Proteolysis with DCC-trypsin, α-chymotrypsin, or Pronase obtained from Sigma Chemical Co., St. Louis, MO, was performed at 24°C at pH 7.6 for 15 min or 60 min. Trypsin digestion was stopped by the addition of a twofold weight excess of soybean trypsin inhibitor.[5] For those samples that were to be electrophoresed, the digestion was stopped by the addition of 0.1-ml aliquots of the digest mixture to 0.2 ml of hot 3% SDS in a 0.1 M phosphate buffer, pH 7.8, containing 20% glycerol and 0.02% NaN_3. Mercaptoethanol was added to a concentration of 1%, and the samples were treated as described above.

Chromatographic separation of the tryptic digestion products

Following completion of the tryptic digestion and the addition of trypsin inhibitor, the solution was applied to a 2.5 × 40 cm Sephadex G-75 medium grade column, equilibrated with 0.05 M NH_4HCO_3, pH 7.6, and eluted with the same buffer. The eluate was monitored by measurement of absorbance at 220 nm and 280 nm.

Analytical ultracentrifugation

Intact LDL and trypsin-proteolyzed samples were ultracentrifuged in a Beckman-Spinco Model E analytical ultracentrifuge. Centrifugation was performed using double sector cells at 25°C and 42,040 rpm. Sedimentation coefficients were measured in KBr containing solutions of d 1.20 g/ml, and the buoyant density of the lipoproteins was determined by also measuring the sedimentation coefficient in solvents of differing densities and constructing S vs. density plots. Molecular weights were calculated as previously described (11).

Peptide mapping

The peptides recovered by gel filtration were repeatedly lyophilized to remove all traces of ammonium bicarbonate. The residue was taken up in 1.0 ml of water, extracted three times with ether, and the aqueous phase was relyophilized. The peptides were then dissolved in 10 μl of water and spotted on Whatman No. 3MM paper. Descending paper chromatography was performed in butanol–acetic acid–water for 16 hr, followed by high voltage electrophoresis in pyridine–acetic acid–water, pH 3.7, for 60 min at 2,500 V, as described by Katz, Dreyer, and Anfinsen (26). After thorough drying, the chromatograms were developed with a cadmium–ninhydrin reagent (27).

RESULTS

Properties of the apoprotein

Following the usual isolation of LDL by differential density ultracentrifugal flotation, between d 1.006 and 1.06 g/ml, LDL was frequently found to contain several protein-staining bands upon electrophoresis on acrylamide gels in the presence of 0.1% SDS. The variable occurrence of large molecular weight polypeptides in LDL samples isolated on repeated occasions is shown in **Fig. 1,** which presents a series of 3% acrylamide gels performed on LDL isolated from the same subject under identical conditions. Gels *A* and *B* show samples of LDL isolated on different days from blood that was allowed to clot at 24°C for 2 hr before beginning preparation of LDL. Gels *C* and *D* are samples in which the drawn blood was divided into two different tubes, with tube *D* containing protease inhibitors, and the LDL samples were then isolated simultaneously. Gels *E* and *F* are identical to gels *C* and *D*, with the exception that the blood was drawn on a different occasion. Gel *A* shows five polypeptides, while gel *B* shows only a single protein. Gels *C* and *E* also show multiple bands. By contrast, the protease-inhibited LDL preparations show only a single high molecular weight protein, as does the uninhibited preparation shown in gel *B*.

Based upon its migration in acrylamide gels, as compared to standard proteins, apoB has an apparent molecular weight greater than the value of 250,000 reported by Smith et al. (1) as determined by physical chemical measurements in 6 M guanidine. Recently

[5] The effectiveness of the inhibitor in stopping further proteolysis of LDL was demonstrated by comparing the proteolytic products by SDS–acrylamide gel electophoresis immediately after digestion and at varying time intervals thereafter.

that same laboratory has reported the dimerization of apoB in SDS as measured ultracentrifugally (28). The single apoB band observed on our electrophoretic gels indicates a large protein with a molecular weight not inconsistent with the values reported from the above laboratory.

The half-cysteine content of apoB has been reported from a number of laboratories and calculates to an average of 15 mol assuming a 250,000-*g* subunit [summarized by Lee (29)]. In this laboratory, the cysteic acid content of performic acid-oxidized apoB is 1 mol% or 18 mol per subunit. It has been reported by Margolis and Langdon (30) that in native LDL approximately 15%, or two, cysteine residues may be determined as free-SH groups. Aminoethylation of native LDL and of apoB in our hands reveals no measurable aminoethylcysteine; however, aminoethylation after reduction with mercaptoethanol yielded 0.9 mol% aminoethylcysteine. We thus confirm the observations of Margolis and Langdon that most, if not all, the cysteine residues appear to be disulfide bonded. Does this bonding occur between subunits or intramolecularly? In their physical measurements of the molecular weight of apoB, Smith et al. (1) reduced and alkylated the protein and hence did not address this question. Accordingly, LDL was subjected to acrylamide gel electrophoresis in SDS at either pH 7.2 or 5.0, in the presence or absence of mercaptoethanol. In either case, the protein migrated an equal distance in the gel, suggesting an absence of disulfide bonding between subunits. It thus appears that apoB is not composed of subunits that are held together by disulfide bonds.[6]

Limited trypsinization of native LDL

Native LDL was subjected to graded proteolysis using the enzymes trypsin, chymotrypsin, and Pronase, and acrylamide gel electrophoresis was used to monitor the extent of proteolysis. **Fig. 2** shows the patterns obtained by digestion at 24°C for 1 hr at pH 7.6 using enzyme-to-protein ratios from 1:5000 to 1:5.

In each instance, sequential cleavage of apoB is evidenced. With Pronase, the cleavage is seen to progress most rapidly, while with trypsin there is a suggestion that proteolysis may progress in stages. Since limited proteolysis could be most readily achieved with trypsin, this enzyme was chosen for further study. To demonstrate the reproducibility of cleavage, a series of separate digestions was carried out with LDL using trypsin-to-protein ratios of 1:5000 and 1:100. **Fig. 3** shows that the cleavages obtained under specified conditions are reproducible. Using a 1:5 trypsin-to-protein mixture and monitoring the progression of digestion over 18 hr, the peptide pattern obtained upon electrophoresis is constant between 0.5 and 6 hr.

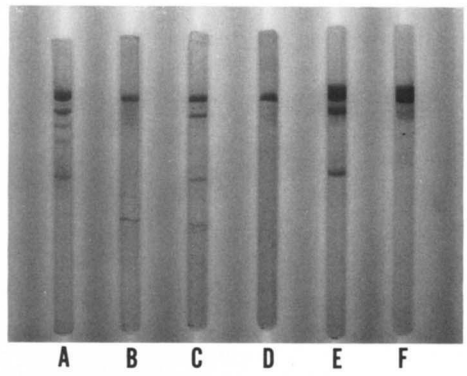

Fig. 1. Degrees of apparent proteolysis observed on different occasions during LDL isolation from the same subject. Blood was drawn on four different occasions over a 6-month span. The blood was allowed to clot in the presence or absence of protease inhibitors, these being added as described in Methods. LDL was isolated, in the presence of azide and merthiolate, from noninhibited and inhibited blood samples and subjected to electrophoresis on 3% acrylamide gels. Gels *A* and *B* represent two different LDL preparations and are the protein patterns of LDL from noninhibited blood. Gels *C* and *D* represent LDL protein isolated from noninhibited and inhibited blood samples, respectively, drawn on the same occasions. Gels *E* and *F* are the same as gels *C* and *D*, except the blood was drawn at a later date.

For the purpose of studying the products of the tryptic cleavage of LDL, larger-scale digests were performed at 1:5000, 1:500, 1:100, and 1:5 enzyme-to-protein ratios. After addition of trypsin inhibitor, the proteolytic digests of LDL were applied to a Sephadex G-75 column in a 0.05 M NH_4HCO_3 buffer, and the eluted fractions were monitored at 280 and 220 nm. The 1:5000 digest yielded only a lipoprotein product that eluted in the void volume, as did the 1:500 digest, except that here a small 220 nm absorption was recorded where the trypsin–trypsin inhibitor complex elutes.

Chromatography of the 1:100 digest yielded the elution pattern shown in **Fig. 4**. The void volume peak constituted partially digested LDL (1:100 T-core). The second small peak was the trypsin–trypsin inhibitor complex, and the third broad peak consti-

[6] It has previously been reported from this laboratory that the intrinsic viscosity of apoB solubilized in 6 M guanidine has a value larger than would be expected for a protein of 250,000 molecular weight and this observation is consistent with the finding by Steele and Reynolds that apoB is partially aggregated in this solvent (19). When apoB is reduced and carboxymethylated, the intrinsic viscosity is repeatedly found to increase about 8%. This observation supports the conclusion that disulfide bonding does not occur between apoB subunits but rather is intramolecular, presumably crosslinking short segments of the polypeptide chain (W. R. Fisher, unpublished results).

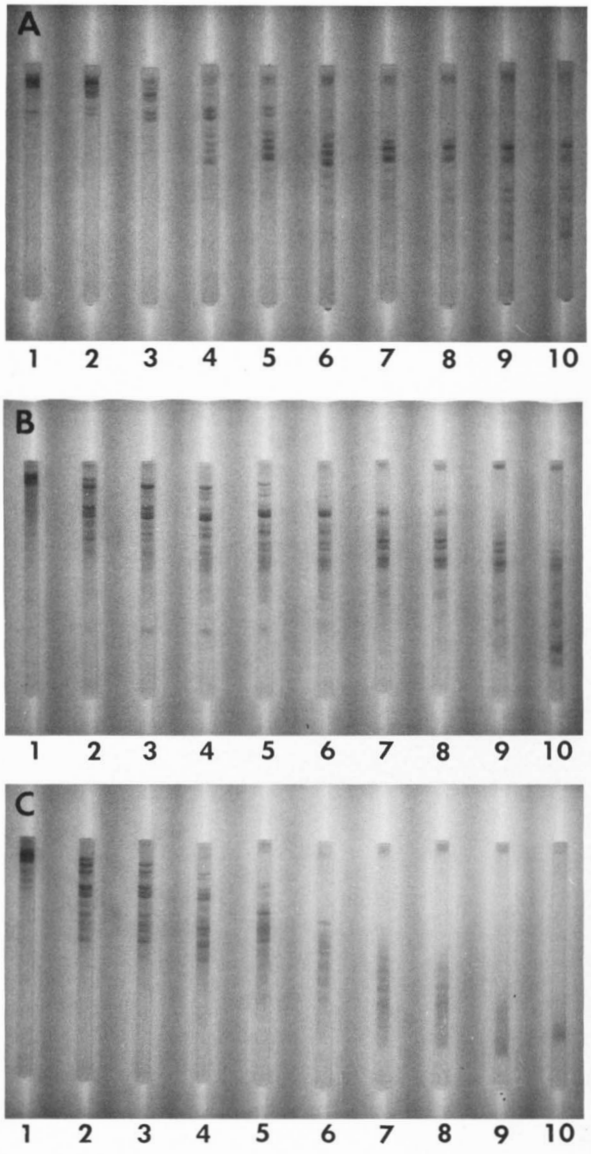

Fig. 2. Effects of trypsin, chymotrypsin, and Pronase digestion on LDL. Purified LDL from blood bank plasma was dialyzed against 0.05 M ammonium bicarbonate, 0.02% sodium azide, pH 7.6. Aliquots of 0.1 ml of LDL were digested with an enzyme-to-protein ratio between 1:5000 and 1:5 for 1 hr at 24°C. Digestion was stopped by the addition of 0.2 ml of hot 3% SDS in 0.1 M phosphate, pH 7.8, containing 20% glycerol and 0.02% sodium azide. The samples were made 1% in mercaptoethanol, heated to 100°C for 3–5 min, cooled to 24°C, and subjected to electrophoresis on 5% acrylamide gels. *A*, trypsin dependence; *B*, chymotrypsin dependence; *C*, Pronase dependence. Enzyme-to-protein weight ratios are: gel 1, 0; gel 2, 1:5000; gel 3, 1:2500; gel 4, 1:1000; gel 5, 1:500; gel 6, 1:250; gel 7, 1:100; gel 8, 1:50; gel 9, 1:25; gel 10, 1:5.

tuted small water-soluble peptides. SDS–acrylamide gel electrophoresis of the T-core fraction revealed the same pattern of large peptides seen in the total digest (inset, Fig. 4).

Chromatography on Sephadex G-75 of the 1:5 tryptic digest yielded an elution pattern as shown in **Fig. 5**. Again, the void volume peak is the partially digested LDL. The next two peaks comprise the trypsin–trypsin inhibitor complex and free, excess trypsin inhibitor. The smaller fourth and larger fifth peaks constitute families of water-soluble peptides as revealed by peptide mapping. **Fig. 6** shows SDS–acrylamide gels of a 1:5 tryptic digest sequence of LDL, including *A*) native LDL; *B*) the total digest; *C*) the T-core isolated from the G-75 column, which was separated from *D*) the trypsin and trypsin inhibitor, and gel *E*) shows the delipidated resolubilized T-core proteins. In this instance, delipidation was accomplished by three extractions of the T-core with ethanol–diethylether 1:3 followed by resolubilization in 3% SDS. This procedure for the recovery of the 1:5 T-core is highly reproducible and is now being performed routinely as the initial step in the isolation of the tryptic polypeptides of apoB.

Limited proteolysis of native LDL with increasing concentrations of trypsin thus apparently modifies the structure of LDL, and yet the modified lipoprotein may be recovered intact. On repeated occasions, the isolated 1:5 T-core has shown seven bands on gel electrophoresis, which range in molecular weight from

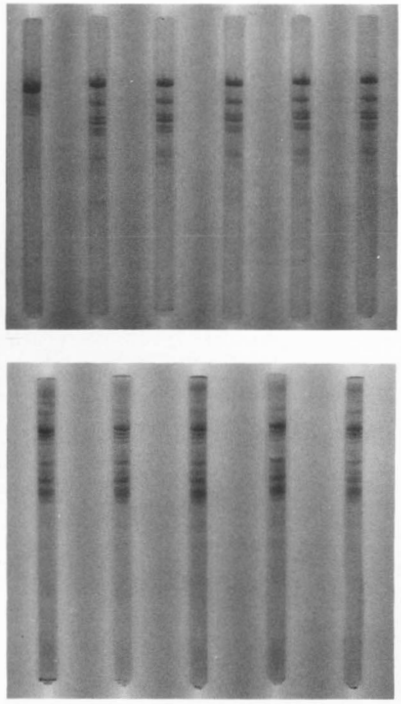

Fig. 3. Digestion of separate LDL samples at trypsin-to-protein ratios of (top) 1:5000 and (bottom) 1:100. Top: 1:5000 digest. Gel on left shows predigested LDL. Gels 2–6 are separate digests run on 3% acrylamide gels. Digestion time was 15 min, but otherwise conditions were as described in Methods. Bottom: 1:100 digest. Separate digests of LDL separated in 5% acrylamide gels. Digestion for 1 hr as described in Methods.

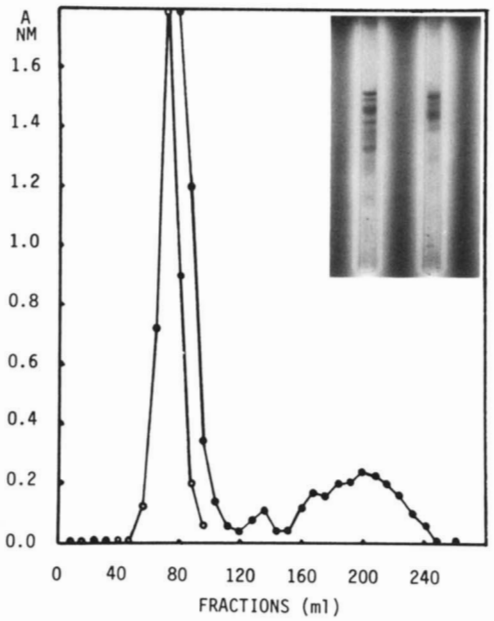

Fig. 4. Chromatography of 1:100 trypsin digest of LDL on Sephadex G-75, as described in Methods, monitored at 280 nm (● – – – ●) and 220 nm (○ – – – ○). The elution profile is described in the text. Inset: 5% acrylamide gel electrophoretic pattern of the total 1:100 digest and of the T-core eluted in the major, initial, void volume peak.

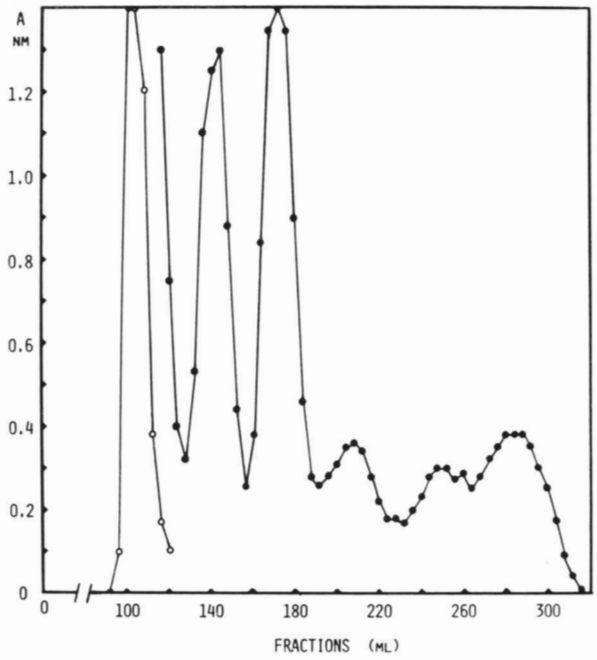

Fig. 5. Chromatography of 1:5 trypsin digest of LDL on Sephadex G-75 column similar to that shown in Fig. 4.

about 14,000 to 100,000 as estimated from their positions on the gels.

In order to estimate the extent of release of water-soluble peptides, the peptide fractions from the Sephadex G-75 columns were pooled, hydrolyzed with 6 N HCl, and the total mass of amino acids was measured on the amino acid analyzer. Such an estimate is only an approximation, since losses occur during chromatography; however, the data show that in the 1:100 digestion approximately 90% of the apoprotein is recovered in the T-core fraction. After 1:5 trypsin digestion, 61% of the apoprotein is recovered in the T-core, 6% in the large peptide fraction, and 20% in the small peptide fraction (Fig. 5).

Amino acid analyses of these digestion products as compared with that of whole LDL are reported in **Table 1**. The amino acid composition of LDL in these studies agrees with that of previous reports (1, 30–32). The composition of the 1:100 and 1:5 T-cores likewise does not differ from LDL, and the similarity in the liberated 1:100 peptides and 1:5 small peptides to native LDL suggests that the distribution of amino acids in apoB, as represented by these various fractions, is reasonably uniform. Thus, the amino acid composition appears very similar between those regions of apoB that are inaccessible to trypsin and those portions that are liberated by proteolysis.

The cysteine content of the delipidated urea-solubilized 1:5 T-core was approximately 1 mol% when determined as cysteic acid and as *S*-aminoethylcysteine after reduction with mercaptoethanol (Table 1). The unreduced T-core revealed no measurable *S*-aminoethylcysteine, thus indicating that the cysteine exists primarily in the disulfide-bonded state in the T-core as in the intact apoB. SDS acrylamide gel electrophoresis also revealed a shift in peptide pattern toward smaller peptides following S–S bond cleavage with mercaptoethanol, presumably resulting from dissociation of disulfide-bonded peptides.

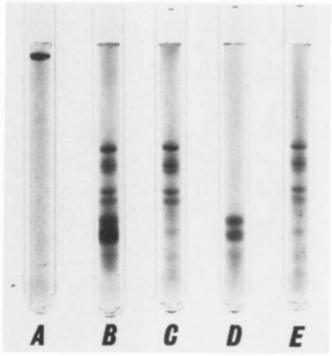

Fig. 6. Acrylamide gel electrophoresis on 5% gels in SDS of the 1:5 tryptic digestion products of LDL. *A*, native LDL prior to digestion; *B*, LDL at completion of digestion after addition of soybean trypsin inhibitor; *C*, 1:5 T-core isolated in void volume peak from Sephadex G-75 (see Fig. 5); *D*, second peak fraction from the same column, showing trypsin and trypsin inhibitor; *E*, delipidated T-core resolubilized in 3% SDS.

TABLE 1. Amino acid composition of LDL and products of tryptic digestion[a] of low density lipoprotein[b]

Amino Acid	ApoB[c]	1:100 Trypsin		1:5 Trypsin		
		Core	Peptides	Core	Large Peptides	Small Peptides
Lys	7	7	9	7	5	9
His	2	2	2	3	1	2
Arg	3	3	5	3	2	4
Asp	11	11	10	11	12	11
Thr	7	7	6	6	6	6
Ser	9	9	7	9	11	7
Glu	12	13	12	13	10	12
Pro	4	4	5	4	6	4
Gly	5	5	5	6	9	6
Ala	6	6	7	6	6	6
Cys[d]	1	—[f]	—[f]	1[g]	—[f]	—[f]
Val	5	5	8	5	6	7
Met	2	2	2	2	2	1
Ile	5	6	5	5	5	6
Leu	12	13	11	12	6	14
Tyr	3	3	2	3	2	3
Phe	5	5	4	5	2	4
Trp[e]	1	—[f]	—[f]	—[f]	—[f]	—[f]

[a] Products of tryptic digestion were prepared as described in the text.
[b] Values expressed as mol percent of amino acids.
[c] Average of four hydrolyses including LDL from pooled blood bank plasma and three different molecular weight LDL.
[d] Determined as cysteic acid.
[e] Determined after hydrolysis with 3 N *p*-toluenesulfonic acid.
[f] Not determined.
[g] Determined as the average of cysteic acid and of aminoethylcysteine after reduction of disulfide bonds.

Carbohydrate analysis of apoB after delipidation with diethylether–ethanol is in agreement with previous reports [summarized by Swaminathan and Aladjem (33)], and the values are recorded in **Table 2**. Also shown is the carbohydrate content of the 1:5 T-core apoprotein which is essentially the same as that of apoB when expressed as weight percent carbohydrate. Apparently those regions of apoB to which carbohydrate is attached are not located within native LDL so as to be preferentially cleaved and released by trypsinization. Margolis and Langdon have previously shown that trypsinization of LDL results in the loss of sialic acid in association with protein, and thus our results are in agreement (13).

TABLE 2. Carbohydrate content of apoB and 1:5 T-core apoprotein

	ApoB	T-Core Apoprotein
	% by wt	
Galactose	0.7 (0.3)[a]	0.8
Mannose	2.5 (0.8)	2.7
Glucosamine	2.6 (0.6)	1.6
Sialic acid	2.0 (0.15)	2.6
Total	7.8	7.7

[a] Standard deviations in parentheses.

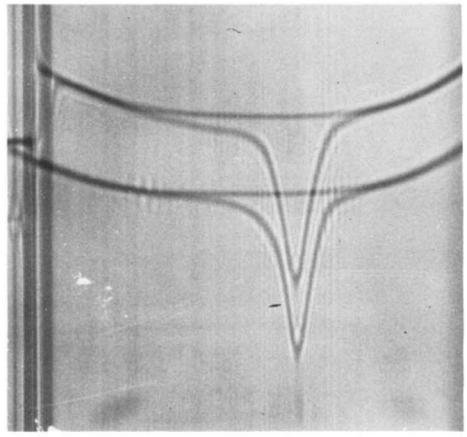

Fig. 7. Analytical ultracentrifugation of native and 1:5 trypsin-digested LDL. Upper pattern is the digested LDL, and the lower pattern is native LDL. Concentration 15 mg/ml. Centrifugation was at 42,040 rpm at 25°C, and the photograph was taken 19 min after the centrifuge reached speed. Solvent was KBr of 1.20 g/ml.

In order to assess the physical changes in LDL resulting from controlled partial tryptic digestion, three separate monodisperse LDL preparations were studied by analytical ultracentrifugation to determine the molecular weight of the native lipoprotein and of the isolated T-core. **Fig. 7** shows a schlieren pattern of native LDL and the 1:5 T-core obtained during a sedimentation run; it indicates the apparent homogeneity of the T-core and the similar flotation rates of the two lipoproteins. The schlieren peaks derived from the 1:500 and 1:100 T-cores behaved similarly. **Table 3** presents the physical data derived from these studies. The data indicate that digestion with trypsin, even in a 1:5 ratio, does not measurably alter the molecular weight of LDL despite the obvious proteolysis of the apoprotein. In this instance, a loss of approximately 30% of the apoprotein alone would result in approximately a 5% decrease in lipoprotein molecu-

TABLE 3. Physical parameters of LDL and isolated T-cores

	Buoyant Density[a]	$S^0(25^0; \rho\ 1.20)$	Mol Wt[b]
	g/ml	*S*	$\times 10^6$
1:500 trypsin to protein digest			
Native LDL	1.032	−38.9	2.7_5
T-core	1.037	−37.8	2.7_5
1:100 trypsin to protein digest			
Native LDL	1.034	−41.0	3.0_2
T-core	1.033	−40.4	2.9_3
1:5 trypsin to protein digest			
Native LDL	1.033	−42.6	3.1_8
T-core	1.027	−45.1	3.2_6

[a] Determined from sedimentation velocity measurements in solvents of different densities as previously reported (11).
[b] Calculated as previously described assuming a frictional ratio of 1:11 (11).

lar weight, a change too small to be measured in these ultracentrifugational studies.

Trypsin as a probe of the structure of LDL of different molecular weights

LDL isolated from individuals may exist in either a monodisperse or a polydisperse state (34). The molecular weight of native monodisperse LDL has been shown to range from 2.4 to 3.9 × 10^6 (9), and individuals with polydisperse LDL frequently have lipoproteins in the LDL class which range in molecular weight from 2.5 to 4.9 × 10^6 (16). In all instances, the lipoproteins contain the same weight of apoprotein per molecule, and molecular weight differences result from different quantities of bound lipid. The apoproteins of various molecular weight LDL have been examined by SDS–acrylamide gel electrophoresis, and the gels are shown in **Fig. 8**. Each apoprotein migrated as a single molecular species and entered the gels by an equal distance. Thus, each of these lipoproteins appears to contain a similar apoprotein subunit and, if one accepts a mol wt of 250,000 for apoB, then based on stoichiometric considerations there must be two apoB subunits per native LDL over the molecular weight range examined from 2.5 to 4.9 × 10^6, since monodisperse LDL contain the same weight of apoprotein per gram mol of LDL (10).

How then are these apoB subunits oriented within LDL macromolecules which differ widely in their lipid content and molecular size? As a start toward answering this question, it seemed appropriate to use limited trypsinization as a probe of differences in the structure of these lipoproteins. The question posed was whether those regions of apoB exposed to limited

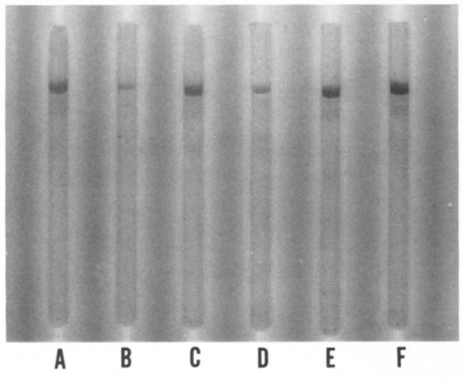

Fig. 8. Electrophoresis of various molecular weight LDL on 3% acrylamide gels. LDL was prepared from protease-inhibited blood drawn from nonlipemic and hyperlipemic individuals as described in Methods. Gels *A*–*C* are from monodisperse LDL and molecular weights are: gel *A*, 2.5 × 10^6; gel *B*, 2.9 × 10^6; gel *C*, 3.4 × 10^6. Gels *D*–*F* are polydisperse LDL samples, and the molecular weights are: gel *D*, 4.9 × 10^6, gel *E*, 3.2 × 10^6; gel *F*, 2.5 × 10^6.

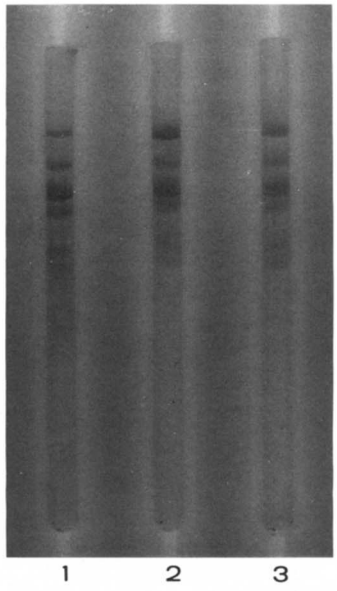

Fig. 9. Digestion of three different molecular weight monodisperse LDL with trypsin-to-protein ratios of 1:5000 for 15 min as described in Methods. Digests electrophoresed on 3% acrylamide gels. LDL molecular weights are *A*: 2.5 × 10^6, *B*: 2.9 × 10^6, *C*: 3.4 × 10^6. The samples were the same as in Fig. 8.

trypsinization were the same in these different molecular weight LDL.

Accordingly, monodisperse LDL of mol wt 2.5, 2.9, and 3.4 × 10^6 were exposed to tryptic digestion at an enzyme-to-protein ratio of 1:5000, at pH 7.6 for 15 min at 24°C. **Fig. 9** shows the resulting patterns of apoB cleavage as revealed by electrophoresis in SDS on 3% acrylamide gels, and the gels are identical. In like manner, LDL of mol wt 2.5 and 3.4 × 10^6 were digested with trypsin at enzyme-to-protein ratios of 1:100 at pH 7.6 for 1 hr at 24°C, and once again the gels showed identical cleavage patterns. The enzymatic digests of the two lipoproteins were then chromatographed on a Sephadex G-75 column, yielding elution patterns similar to that shown in Fig. 4. Electrophoretic gels run on the two tryptic cores eluted in the large, initial peak gave identical patterns which appear the same as shown in the inset of Fig. 4. The smaller peptides eluted in the third, broad peak from this column were recovered following lyophilization, and **Fig. 10** shows that the peptide maps obtained are also identical.

Thus, the electrophoretic gels suggest that the same regions of apoB are exposed to the action of trypsin in different molecular weight, monodisperse LDL, and fingerprinting of the liberated, water-soluble peptides shows these to be similar in LDL of mol wt 2.5 and 3.4 × 10^6.

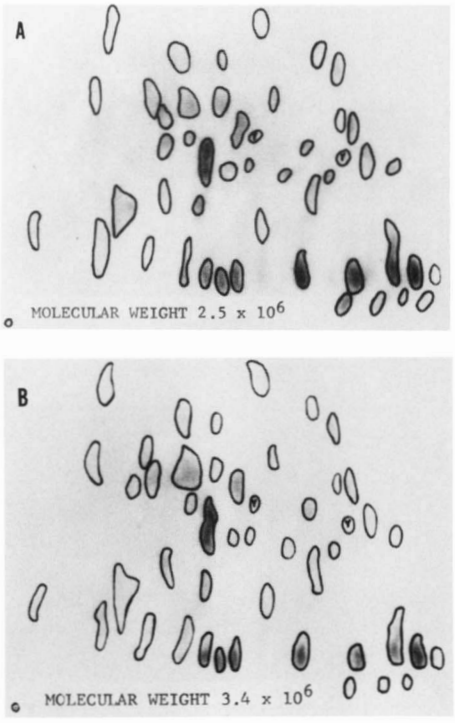

Fig. 10. Peptide maps from 1:100 tryptic digest of two different molecular weight LDL isolated from individual donors. The peptides at spots "Y" turned yellow during the color development. *A*, peptides from LDL of molecular weight 2.4×10^6; *B*, peptides from LDL of molecular weight 3.4×10^6. Peptide mapping was performed as described in Methods.

DISCUSSION

The studies of Smith, Dawson, and Tanford (1) provide evidence that apoB is a large protein and that the apoprotein of LDL contains two similar-sized apoB subunits. The observations reported here are in agreement with those findings and suggest that these subunits are not crosslinked by disulfide bonds.

Once again, the ease with which LDL may undergo proteolysis has been demonstrated; this finding has been a subject of interest in several other laboratories (35–38). In our hands, to isolate unproteolyzed apoB consistently, it is necessary to add bacterial and general protease inhibitors to freshly drawn blood and to maintain constant suppression of bacterial growth thereafter.

One may, however, systematically cleave apoB as it exists within native LDL by using a variety of proteases, and the specificity of cleavage is reproducible. Apparently, the inherent structure of the native lipoprotein results in the selective exposure of limited regions of apoB for proteolytic cleavage. Following trypsinization, there remains an intact lipoprotein which may be isolated. Hydrodynamic characterization of the T-cores shows that though they may lose up to 30% of their initial apoB content, this is lost without significantly disrupting the integrity of the lipoprotein. This finding, plus the reproducibility of the tryptic proteolysis patterns, provide further evidence that native LDL is a structured molecule with a considerable degree of inherent organizational stability.

The products resulting from selective proteolysis of LDL with trypsin have already proven to be of interest. Using this technique, evidence has been accumulated to indicate that the same regions of apoB are exposed to tryptic cleavage in monodisperse LDL differing in mol wt from 2.5 to 3.4×10^6.

On the basis of genetic studies in humans, it has been proposed that the extent of lipid binding by apoB, and hence the molecular weight of LDL, is an inherited property (9). If so, then one might anticipate structural differences in apoB influencing its interaction with lipid in such a manner as to permit the accommodation of the different amount of lipid bound in high and low molecular weight LDL. Using trypsin as a probe of LDL structure, the data suggest that those regions of apoB that are accessible to limited tryptic cleavage within monodisperse, native LDL appear identical regardless of LDL molecular weight. Tentatively, we would propose that those regions of apoB that may differ in structure must be inaccessible to limited trypsinization and thus would either be buried within the lipid domains or folded in inaccessible conformations within these different molecular weight LDL.

The LDL T-cores display many of the biological and structural properties of native LDL. Thus, the polypeptides remaining within this T-core retain the ability to interact with lipid. They also contain much of the carbohydrate of apoB that is not preferentially released during trypsinization. Of special interest is the finding that the 1:5 T-core has the ability to bind with specificity to the high-affinity LDL binding site on fibroblasts (36). It is also precipitated by anti-LDL serum, thus displaying many of the antigenic determinants of native LDL. Yet the 1:5 T-core contains a family of polypeptides, the largest of which is slightly larger than serum albumin. By isolating and studying these peptides, it should be possible to further our understanding of the biochemical properties of apoB.

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