STABILITY OF THE APOPROTEIN OF HUMAN SERUM LOW DENSITY LIPOPROTEIN: ABSENCE OF ENDOGENOUS ENDOPEPTIDASE ACTIVITY

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SUMMARY

Protease activity, inhibitable by DFP, has been reported in purified preparations of low density lipoprotein from human serum. Such activity could introduce serious error in studies of structure of its subunit proteins. In this study low density lipoprotein was prepared and stored in an effective antimicrobial medium. The protein moiety was examined for evidence of proteolysis by electrophoresis with sodium dodecyl sulfate, gel permeation chromatography, solubility in tetramethylurea, and measurement of amino and carboxyl termini. No evidence of proteolytic cleavage of the apoprotein was found indicating that low density lipoprotein lacks endogenous protease activity.

INTRODUCTION

Estimates of the molecular weight of subunits of the apoprotein of LDL[1] from human serum vary from 8,000 to 250,000 (1). Recently Krishnaiah and Weigandt have described endopeptidase activity associated with LDL that is inhibited by DFP and phenylmethylsulfonyl fluoride (2). In their study the protein moiety of fresh preparations of LDL yielded a broad band in gel electrophoresis with SDS[2] with a mobility corresponding to a molecular weight of 250,000 to 270,000, whereas additional bands of apparent molecular weights as low as 70,000 were observed after storage of LDL for 5 days or more at 4° despite the presence of azide in the medium. The smaller proteins appeared at an accelerated rate when the lipoprotein was incubated at 37°. An increase in amino groups reactive with fluorescamine was also observed on storage

[1] low density lipoprotein
[2] sodium decyl sulfate

and was attributed to liberation of free alpha amino groups by proteolytic cleavage.

Whether endogenous endopeptidase activity is present in LDL is critical to study of its subunit structure. In this report we adduce evidence for the absence of endopeptidase activity in LDL incubated in an effective antimicrobial medium.

MATERIALS AND METHODS

Blood samples were drawn from fasting healthy male donors. EDTA was added immediately to one-half of each specimen to a concentration of 6 mM for preparation of plasma. Serum was obtained from the remainder after the blood had clotted at 23°. Each preparation of serum and plasma was divided and DFP was added to one-half at a concentration of 1 mM. The following antimicrobial agents were added to serum and plasma and were maintained in the solutions at all subsequent stages of the preparation of LDL: sodium azide .05%, thimerosal (merthiolate) 0.005% and chloramphenicol 50 µg/ml. All solutions also contained 1 mM EDTA.

LDL ($1.024<d<1.050$) was prepared by repetitive ultracentrifugation (3), and dialyzed at 4° for 48 hours against 0.15 M NaCl. No viable bacteria or fungi could be recovered on culture of LDL prepared in the presence of the antimicrobial agents even after incubation for 196 hours at 37°. On electrophoresis in agarose gel (4) the LDL gave a single, narrow band of beta mobility. Electron microscopy of preparations negatively stained with 2% potassium phosphotungstate (5) showed a homogeneous population of spherical particles (mean diameter of 220 Å ± 15 Å S.D.). On immunodiffusion (6), a single precipitin line formed with anti-human serum and with antiserum to human LDL and no reaction occurred with several dilutions of anti-albumin. Apoprotein soluble in 1,1,3,3 tetramethylurea (4.2 M) comprised 1-5% of the total protein in the different preparations and was essentially completely accounted for by the R-serine, R-glutamic and R-alanine apoproteins observed on electrophoresis in 7.5% polyacrylamide gel (7).

LDL, prepared from serum and plasma with and without DFP, were stored at 4° for up to 49 days and were incubated at 37° for up to 196 hours at a concentration of 2-3 mg/ml. After incubation the lipids were removed by extraction at 23° with ethanol-ether 3:1 (v/v) for 20 hours and again for 2 hours. The protein was then incubated in 0.1 M sodium phosphate, pH 8.5, containing 1% SDS (w/v) and 1% 2-mercaptoethanol (v/v) at 37° for 16 hours. An optically clear solution resulted. 150-400 μg of protein from each sample was subjected to electrophoresis in 4% polyacrylamide gels with SDS by the technique of Weber and Osborn (8). Gels were stained with a 0.2% (w/v) solution of Coomassie Blue in ethanol:H_2O:acetic acid; 9:9:2 for 2 hours and then were destained in 10% acetic acid for 2 days.

As a further comparison of the content of proteins of molecular weights below 150,000, LDL from serum and plasma (both ± DFP) was delipidated and subjected to gel permeation chromatography on Sephadex G-100 (9) following storage for 21 days at 4°. The protein was incubated for 1 hour with 1% 2-mercaptoethanol before application. The void volume and the included volume fractions were each concentrated by ultrafiltration in an Amicon ultrafiltration cell using the UM 2 membrane. Content of protein was then determined (10).

Since fragments derived from apolipoprotein B by proteolytic cleavage might not share its insolubility in 4.2 M tetramethylurea, an increase in the soluble protein fraction might result from proteolysis. The soluble fraction of the protein moiety of serum LDL prepared without DFP and incubated at 23° for 72 hours immediately after isolation was compared with that of the same preparations which had been held at 4° (7).

The appearance of new free amino groups and carboxyl terminal amino acids was also measured to determine whether proteolytic cleavage had occurred in the LDL during storage. To evaluate any increase in the number of free amino groups the reactivity of LDL with fluorescamine (11) was measured, without succinylation, on samples that had been removed and frozen at intervals during storage (4° for up to 16 days or 23° for up to 8 days). Analysis of carboxyl-

terminal amino acids was performed with carboxypeptidases A and B by the method of Ambler (12).

RESULTS AND DISCUSSION

The band pattern obtained by electrophoresis of LDL protein with SDS is shown in Figure 1. An intensely staining doublet is observed. The mobilities of its bands correspond to molecular weights of approximately 318,000 and 276,000 Below this complex are three clearly separated bands, less intensely stained, with mobilities corresponding to molecular weights of approximately 234,000, 184,000 and 151,000, respectively.

The patterns obtained on electrophoresis of LDL prepared from serum or plasma were identical in each of four preparations. Also, the LDL prepared with DFP gave patterns indistinguishable from those of the portions of the same

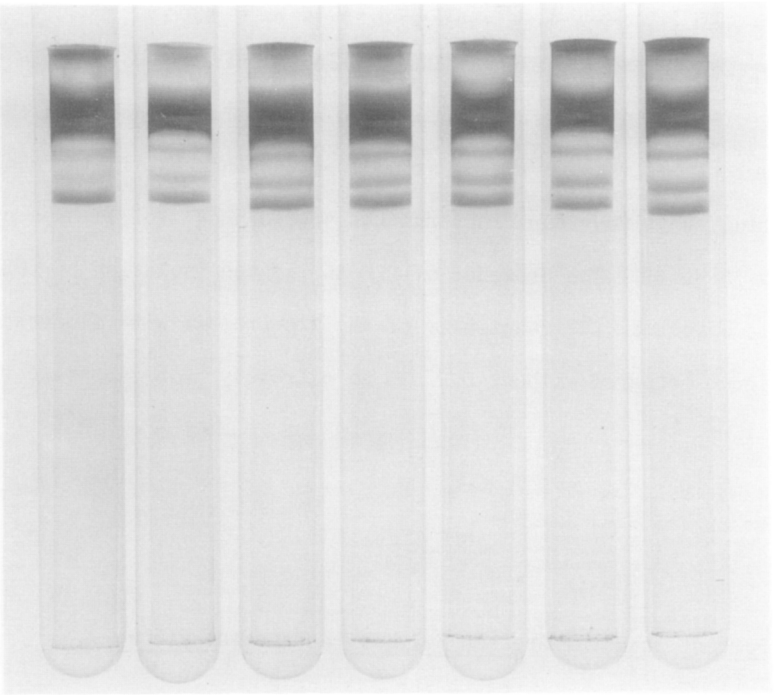

Figure 1: Electrophoresis of LDL protein in polyacrylamide gel with SDS. Origin at top, anode at bottom. LDL from serum, prepared without DFP, incubated with antimicrobial agents at 37° for (left to right): 0, 24, 48, 72, 96, 144, 196 hours. 150 µg of protein was applied to each gel.

preparations from which DFP had been omitted. The pattern in all preparations remained stable upon storage at 4° for 49 days and upon incubation at 37° for up to 196 hours (Fig. 1). The delipidated protein of LDL incubated for comparable periods with SDS in the presence of the antimicrobial agents gave a band pattern indistinguishable from that obtained after the incubation of whole LDL. In contrast with the findings of Krishnaiah and Weigandt (2) no new protein appeared with molecular weights in the range of 70,000 to 150,000 upon storage. However, such a distribution was observed after storage of some preparations of LDL for 30-60 days in the absence of the antimicrobial agents.

Gel chromatography of LDL protein gave a large symmetrical peak at the void volume and a very small protein peak in the included volume in each case. The protein in the included volume varied from 2.3 to 5.9 percent of the total in different preparations of LDL but the values for LDL from serum or plasma from each donor, with or without DFP, were in close agreement.

The portion of the protein of LDL from serum that was soluble in tetramethylurea did not increase upon incubation at 37° for 72 hours in the absence of DFP (Table I). Likewise there was no apparent increase in the number of fluorescamine-reactive groups in serum LDL stored at 4° for 16 days or at 23° for 8 days (Fig. 2). The presence of DFP during the isolation of LDL had no discernible effect on the reactivity of the lipoprotein with fluorescamine. LDL from serum prepared without DFP was an extremely poor substrate for carbo-

TABLE I

Percent of Total Protein Soluble in Tetramethylurea

	Serum LDL	
Preparation	4° Control	After Incubation at 37° (72 h)
1	1.29	1.24
2	1.87	1.60

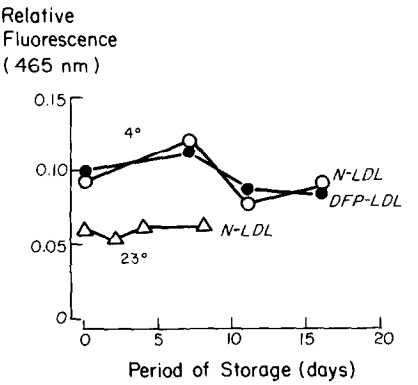

Figure 2: Fluorescamine reactivity of LDL during storage. N-LDL indicates LDL prepared from serum; DFP-LDL indicates LDL prepared from serum in the presence of 1 mM DFP. Ordinate: relative fluorescence. Abscissa: period of storage.

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xypeptidases. Even after incubation at 37° for 24 hours yields of less than 1 μmole of amino acid (principally serine) were obtained per gram of protein on two preparations of LDL.

These data indicate that there is no endogenous endopeptidase activity in LDL from human serum. We have cultured several species of bacteria from LDL prepared in the presence of azide alone, indicating that microbial proteases may be present in such preparations. Therefore it is imperative that a demonstrably effective antimicrobial medium be maintained in all stages of preparation of LDL when structural studies are to be undertaken.

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