Apolipoprotein B-100 of plasma low density lipoproteins undergoes proteolysis by contact activation factors when plasma is treated with dextran sulfate-500–$MgCl_2$

Robert E. Byrne* **and Angelo M. Scanu**[1,*,†]

Departments of Medicine* and Biochemistry and Molecular Biology,† The University of Chicago, Pritzker School of Medicine, Chicago, IL 60637

Abstract Human plasma low density lipoproteins (LDL) isolated by ultracentrifugation showed a single band corresponding to apolipoprotein B-100 (apoB-100) by SDS-gradient gel electrophoresis (GGE). In turn, apoB-100 of LDL precipitated from plasma by dextran sulfate-500 (DS)–$MgCl_2$ exhibited several bands indicative of a degradative process. The degradation was more extensive at 0°C than at either 23°C or 37°C, and appeared to be related to a protease activity that cleaved both the synthetic peptide, Z-Phe-Arg-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC) and apoB-100. Proteolysis was proportional to the DS added to the plasma, was prevented by the kallikrein inhibitor, D-Phe-L-Phe-L-Arg-$CHCl_2$, and was significantly decreased in plasma specimens of patients with either factor XII or prekallikrein deficiency. LDL pre-purified by ultracentrifugation and then precipitated by DS in the absence of plasma exhibited no proteolysis. However, proteolysis was observed when LDL interacted with kallikrein. The two main apolipoproteins of HDL_3, apoA-I and apoA-II, were not affected by this proteolytic process. We interpret the results to indicate that the negatively charged surface provided by DS accelerates in plasma the autoactivation of factor XII and the activation of prekallikrein, resulting in an increase of the effective concentration of kallikrein and possibly other proteases and proteolysis of LDL-apoB-100. The higher degree of the DS-induced proteolysis of apoB-100 at 0°C than at 23°C is likely the consequence of enhanced autoactivation of factor XII and a decreased efficiency of plasma inhibitors, such as C1-inhibitor. We speculate that the proteolysis of apoB-100 induced by DS is not limited to this polyanion, but may also be the property of other negatively charged agents, particularly at cold temperatures.—**Byrne, R. E., and A. M. Scanu.** Apolipoprotein B-100 of plasma low density lipoproteins undergoes proteolysis by contact activation factors when plasma is treated with dextran sulfate-500–$MgCl_2$. *J. Lipid Res.* 1989. **30:** 109–120.

Supplementary key words LDL-dextran sulfate interactions • proteolysis of apoB-100 • proteolytic enzymes

The isolation of plasma lipoproteins is commonly accomplished by ultracentrifugal techniques in salt solutions of varying densities (1). Lipoproteins may also be separated according to size by either gradient gel electrophoresis (GGE) (2), gel filtration chromatography (3) in native buffers, or immunoaffinity techniques that recognize specific apolipoprotein (apo) ligands (4–6). Lipoproteins can also be separated by exploiting differences in their mode of interaction with sulfated polysaccharides (e.g., 500-kDa dextran sulfate (DS), heparin, and sodium phosphotungstate) in combination with divalent cations (7, 8). Although found practical for use in determining the distribution of cholesterol between low density lipoproteins (LDL) and high density lipoproteins (HDL) (9), these techniques have also been used to remove LDL from plasma as one of the first steps in the isolation of lecithin:cholesterol acyltransferase (10) or lipid transfer protein(s) (11). Moreover, DS-cellulose beads have recently been used clinically as a specific sorbent for the selective removal of apoB-containing lipoproteins in the treatment of familial hypercholesterolemia (12).

In the present study we demonstrate that treatment of plasma with sulfated polysaccharides such as DS promotes the proteolysis of apoB-100 of LDL through the activation of the intrinsic pathway of the bloodclotting system. We also show that the extent of proteolysis is dependent on both

Abbreviations: GGE, gradient gel electrophoresis; DS, 500-kDa dextran sulfate; LDL, low density lipoproteins of d 1.019–1.045 g/ml; DS-precipitated LDL, LDL prepared by precipitation of plasma with DS followed by ultracentrifugation at d 1.019–1.045 g/ml; HDL_3, high density lipoprotein subclass 3, d 1.125–1.21 g/ml; apo, apolipoprotein; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Z-, benzyloxycarbonyl-; MeO-, methoxy-; Suc-, succinyl-; x-CH_2Cl, chloromethyl ketone derivative of peptide X; AMC, 7-amino-4-methylcoumarin; RFU, relative fluorescence unit; DFP, diisopropylfluorophosphate; PKD-plasma, prekallikrein-deficient plasma; FXIID-plasma, factor XII-deficient plasma; α-factor XII_a, the 80-kDa form of activated factor XII.

[1]To whom correspondence should be addressed at: Department of Medicine, Box 231, The University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637.

the concentration of the precipitating reagent and the temperature and may be prevented by specific proteolytic inhibitors. A preliminary account of these findings has appeared (13).

EXPERIMENTAL PROCEDURES

Materials

Chemicals. DS (sodium salt, mol wt 500,000), Sephacryl-S-200, and Sephadex G-25 were products of Pharmacia Fine Chemicals, Inc., Piscataway, NJ; electrophoresis grade acrylamide, bis-acrylamide, β-mercaptoethanol, and Coomassie brilliant blue R-250 were purchased from Bio-Rad, Richmond, CA; iodine monochloride was obtained from Eastman Co., Rochester, NY. All other chemicals used were of the highest available reagent grade.

Radiochemicals. [^{3}H]-Labeled DFP and carrier-free Na-^{125}I were purchased from Amersham Corp., Arlington Heights, IL.

Inhibitors and substrates. DFP and benzamidine were obtained from the Aldrich Chemical Co., Milwaukee, WI; D-Phe-L-Phe-L-Arg-CH_2Cl was from Calbiochem-Behring Co., San Diego, CA; benzyloxycarbonyl(Z)-Phe-Arg-7-amino-4-methylcoumarin(AMC) was from Peninsula Laboratories, Belmont, CA; methoxy-succinyl(MeO-Suc)-Ala-Ala-Pro-Val-AMC, Suc-Ala-Ala-Phe-AMC, Tos-Gly-Pro-Arg-AMC, and AMC were from Vega Biochemicals, Tucson, AZ.

Proteins. Purified human plasma kallikrein was purchased from Calbiochem-Behring; crystallized bovine serum albumin was from Miles Laboratory, Elkhart, IN; high molecular weight standards (40,000–250,000) and low molecular weight standards (10,000–100,000) were both purchased from Bio-Rad.

METHODS

Plasma. Human normolipidemic plasma was obtained from the blood of fasting healthy volunteers. The blood obtained by venipuncture was collected in polypropylene bottles containing 3 mM disodium ethylenediaminetetraacetic acid (EDTA), pH 7.0. Cells were removed from the plasma by centrifugation at 5,000 rpm in a Sorvall GSA rotor at 4°C for 30 min. The plasma was removed from the bulk of the cells and centrifugation was repeated as before. Subsequently, precautions were taken to avoid any contact of the separated plasma with glass surfaces. Prekallikrein-deficient (PKD) plasma and factor XII-deficient (FXIID) plasma were purchased from George King Bio-Medical, Inc., Overland Park, KS and were congenitally clotting factor-deficient; they were rapidly frozen in 1-ml plastic vials and maintained in this state until they were rapidly thawed to 37°C immediately before use. As assured by the supplier, the clotting factor activities in each deficient plasma were less than 1% of their respective clotting factor activities in normal pooled plasma, based on specific clotting assays and chromogenic substrate assays.

Preparation of apolipoprotein B (apoB)-containing lipoproteins by treatment of plasma with 0.05% DS and 0.05 M $MgCl_2$ and isolation of the d 1.019–1.045 g/ml LDL fraction. The procedure utilized was a modification of that of Burstein, Scholnick, and Morfin (8). To 5 ml of plasma in a polypropylene tube was added 0.575 ml of 0.5% DS, pH 7.0, and 0.15 ml of 2.0 M $MgCl_2$ solution. After gentle mixing, the mixture was incubated for 20 min at either 0°C (melting ice) or 23°C. Next, the mixture was centrifuged at 6000 *g* for 10 in at 4°C. The supernatant, containing HDL and other plasma proteins, was removed and saved for the isolation of HDL_3 and other analyses of proteolytic activity (see below). The precipitate composed of apoB-containing lipoproteins and other proteins was dissolved in 1.67 M NaCl and ultracentrifuged at d 1.063 g/ml in a Ti 50.3 rotor at 40,000 rpm for 16 hr at 10°C. The floating lipoproteins were collected for further separation and the bottom 1.0-ml fraction was removed for assay of proteolytic activity.

The d 1.063 g/ml top fraction was diluted with 0.01% EDTA to a density of 1.019 g/ml and ultracentrifuged again as described above in order to remove VLDL and other lipoproteins. The infranatant was adjusted to d 1.045 g/ml and the LDL fraction, isolated by ultracentrifugal flotation at 40,000 rpm for 16 hr at 10°C, was dialyzed extensively against 0.15 M NaCl–0.01% Na_2 EDTA, pH 7.0. After sterilization by filtration (0.45 μm filter) it was stored under N_2 at 4°C. Overall, the stepwise procedure for ultracentrifugation in high salt provided a method for dissociating the proteolytic activity and other contaminants (including DS) from the lipoprotein under these conditions.

Preparation of LDL(d 1.019–1.045 g/ml). Plasma was adjusted to a density of 1.063 g/ml and the lipoproteins were isolated by ultracentrifugation in a Ti 50.3 rotor at 40,000 rpm for 16 hr at 10°C. The d 1.063 g/ml top fraction was diluted with 0.01% EDTA to a density of 1.019 g/ml and ultracentrifugal flotation was carried out sequentially as described above for the preparation of DS-precipitated LDL (d 1.019–1.063 g/ml). The LDL was dialyzed extensively against 0.15 M NaCl–0.01% EDTA, pH 7.0 and stored at 4°C.

Preparation of LDL labeled with ^{125}I(^{125}I-labeled LDL). Iodination of LDL was carried out by the method of Bilheimer, Eisenberg, and Levy (14). In particular, 1.0 ml of LDL (1.1 mg) in 0.15 M NaCl was carefully mixed with 0.5 ml of 2 M glycine, pH 10, and 0.5 ml of 4 M NaCl. Next, 0.01 ml of carrier-free Na-^{125}I (1 mCi, sp act 16.5 mCi/μg of iodine; Amersham, Arlington Heights, IL) followed by the addition of 0.02 ml of ICl (0.01 ml of stock

ICl diluted with 15.5 ml of 1 N HCl) in order to initiate the reaction. The reaction was allowed to continue for 15 min. The ^{125}I-labeled LDL was separated from unreacted iodide by Sephadex G-25 chromatography followed by dialysis against 0.15 M NaCl–0.01% Na_2EDTA, pH 7.0, and stored at 4°C under N_2. The ^{125}I-labeled LDL had a specific readioactivity of 125–150 cpm/ng and the radioactivity was 97% precipitable with 10% trichloroacetic acid.

Preparation of HDL_3. HDL_3 (d 1.125–1.21 g/ml) was prepared by sequential ultracentrifugal flotation of whole plasma as previously described (15). When the 0.05% DS–0.05 M $MgCl_2$ plasma supernatant was used as starting material, the density was adjusted to 1.21 g/ml by addition of 0.098 g NaCl/ml and 0.2 g NaBr/ml and ultracentrifugal flotation was performed in a 50.3 Ti rotor at 40,000 rpm for 24 hr at 10°C. The whole HDL top fraction was adjusted to d 1.125 g/ml by dilution with 0.01% Na_2EDTA and ultracentrifugal flotation was repeated as before. After removing the lipoprotein top fraction, the infranatant was adjusted to d 1.21 g/ml and ultracentrifugal flotation was repeated. After flotation, the HDL_3 was dialyzed extensively against 0.15 M NaCl, 0.01% EDTA, pH 7.0, and stored at 4°C under N_2.

Assay for amidolytic activity. Amidolytic activity was measured using the fluorogenic substrate Z-Phe-Arg-AMC as described by Morita et al. (16). The substrate (0.005 ml), dissolved in dimethylsulfoxide, was added to a quartz cell to a final concentration of 25 μM in 1.445 ml of assay buffer (0.01 M Tris–HCl, 0.15 M NaCl, pH 7.4) and equilibrated at 37°C. Then, 0.05 ml of the test sample was added to initiate the reaction. The increase in relative fluorescence of the liberated AMC (λ_{ex} = 370 nm, λ_{em} = 460 nm) was measured in a Perkin-Elmer MPF 44B recording spectrofluorimeter. Relative fluorescence units were converted to concentration units using a 0.5 μM standard solution of AMC, where 1 RFU = 7.90 pmol of substrate cleaved in a standard assay volume of 1.5 ml. One activity unit was defined as the quantity of enzyme that produced 1 RFU/min under standard assay conditions. For calculations of specific activity, the amount of protein was estimated by the modified method of Lowry et al. (17) in the presence of SDS as described by Markwell et al. (18). The kallikrein preparation had a specific Z-Phe-Arg-AMC amidolytic activity of 63.6 units/μg of protein. Assays using other peptide-AMC substrates were conducted in similar fashion and the units of activity defined as with Z-Phe-Arg-AMC.

Inhibition of degradation of apoB-100 in DS-precipitated LDL by various proteolytic inhibitors. The effectiveness of various proteolytic enzyme inhibitors on preventing the degradation of DS-precipitated LDL from plasma was assessed by addition of a given inhibitor to the plasma followed by precipitation with 0.05% DS–0.05 M $MgCl_2$. After removal of the plasma supernatant, the precipitate was dissolved in 1.67 M NaCl containing the inhibitor at the same initial concentration as added originally in the plasma. Next, the DS-precipitated LDL was isolated by ultracentrifugation (d 1.019–1.045 g/ml) as described previously (see above) except that the d 1.063 g/ml salt solution, used in the initial separation of the DS-precipitated lipoproteins from the rest of the precipitated proteins, also contained the initial plasma concentration of inhibitor. Subsequent ultracentrifugation steps of the d 1.063 g/ml top fraction were conducted in the absence of inhibitor.

Autoradiograms of [^{3}H]-DFP-labeled proteases. A quantity of 0.3 ml (475 units of Z-Phe-Arg-AMC amidolytic activity) of the Sephacryl-S-200 column pooled fraction (28–31) in 0.15 M NaCl, 0.01 M Tris-HCl, 0.02% NaN_3, pH 8.0, was incubated with 60 μCi of ^{3}H-labeled DFP (4.0 Ci/mmol, Amersham Corp., Arlington Heights, IL) for 18 hr at 23°C. After incubation, the mixture was dialyzed extensively against 10% acetic acid at 4°C to remove unbound radionuclide followed by lyophilization. SDS-(4–12%)-GGE of the sample was performed, applying 12,000 cpm of labeled material to each lane. After staining with Coomassie blue R-250, the gel was prepared for autoradiography (19) and exposed to Kodak XAR-5 X-ray film at – 70°C for 1 week before development.

Electrophoretic procedures. For the separation of apoB-100 and apoB fragments, SDS-(3–6%)-GGE was performed using a modified Laemmli discontinuous buffer system (20). Routinely, the separating gel, in 0.45 M Tris-HCl (pH 8.8), consisted of a linear 3–6% polyacrylamide gradient in 0.2% SDS superimposed on a 0–20% sucrose gradient. The stacking gel, in 0.067 M Tris-HCl (pH 6.8), contained 2.75% polyacrylamide. The running buffer was 0.05 M Tris–0.38 M glycine-HCl, 0.2% SDS, pH 8.3. Gels were stained in 0.25% Coomassie blue R-250 in 25% isopropanol–10% acetic acid and destained in the same solvent followed by final destaining in 10% acetic acid until the background was clear. After staining, gels were scanned using an LKB Ultrascan XL laser densitometer according to the manufacturer's instructions. The apparent molecular weights of apoB peptides were estimated by extrapolation from a standard plot of the logarithm of the molecular weights versus the electrophoretic mobility of standard proteins. The following were used as molecular weight marker proteins: myosin (200,000); β-galactosidase (116,250); phosphorylase b (97,000); bovine serum albumin (66,200); and ovalbumin (42,500). For Western blotting, apolipoproteins and peptide fragments were transferred from gels to nitrocellulose paper (0.1 M μm pore size, Schleicher and Schuell, Inc., Keene, NH) using the Bio-Rad Trans-Blot® cell according to the method of Towbin, Staehelin, and Gordon (21). Transfer was done at either 30 V (HDL_3 apolipoproteins)

or 50 V (apoB-containing lipoproteins) overnight with cooling. Peptides were identified with a double antibody technique with rabbit anti-human LDL as the primary antibody, goat anti-rabbit IgG coupled to horseradish peroxidase (Miles Laboratories) as secondary antibody, and visualization by the peroxidase reaction using H_2O_2 and 4-chloro-1-naphthol as substrates (22). In the analogous procedures for detection of apoA-I and apoA-II, the corresponding rabbit anti-apoA-I and rabbit anti-apoA-II antibodies were used as the primary antibodies.

Nondenaturing GGE of LDL and DS-precipitated LDL was performed using 2.5–10% linear gradient gels in 0.09 M Tris, 0.08 M borate, 0.003 M Na_2EDTA, pH 8.3. After electrophoresis for 2700 V-hr, gels were stained and destained as described above for the SDS-polyacrylamide gels.

Agarose gel electrophoretic analyses of isolated lipoproteins were performed according to Noble (23) using precast Agarose Universal Electrophoresis Film (Corning, Palo Alto, CA) in a Corning cassette electrophoresis cell filled with 0.05 M barbital buffer containing 0.035% EDTA, pH 8.6, and run according to the manufacturer's instructions. The films were stained with Fat Red 7B for lipids.

RESULTS

In the initial phase of the study, we observed by SDS-GGE that proteolysis of apoB-100 occurred when LDL was precipitated from plasma with 0.4% sodium phosphotungstate–0.05 M $MgCl_2$, and with a variety of sulfated polysaccharide–metal ion combinations, namely DS with either $MgCl_2$ or $MnCl_2$, heparin–$MnCl_2$, and fucoidin–$MgCl_2$. The extent of apoB proteolysis varied depending on the precipitation conditions used. On the other hand, a single band was observed in LDL preparations isolated from plasma samples by ultracentrifugation (see Methods). In order to determine the mechanism by which polyanions induced apoB-100 proteolysis, we elected to use the DS–$MgCl_2$ model.

Effect of temperature

Five ml of plasma was incubated with 0.05% DS in the presence of 0.05 M $MgCl_2$, either in an ice bath (0°C) or at room temperature (23°C). The precipitated fractions were redissolved in 0.5 ml of 1.67 M NaCl–0.01% EDTA and ultracentrifuged in a 50.3 Ti rotor (40,000 rpm, 16 hr, 10°C). The d 1.063 g/ml top fraction was subjected to additional ultracentrifugation steps in order to isolate the d 1.019–1.045 g/ml LDL fraction. For comparison, LDL was also prepared by ultracentrifugation of plasma only. By SDS-GGE, LDL exhibited essentially only a single Coomassie blue stained band (band a) corresponding to apoB-100 (see **Fig. 1**, top pattern). By gel scanning, 86% of the stained material migrated as apoB-100, with bands

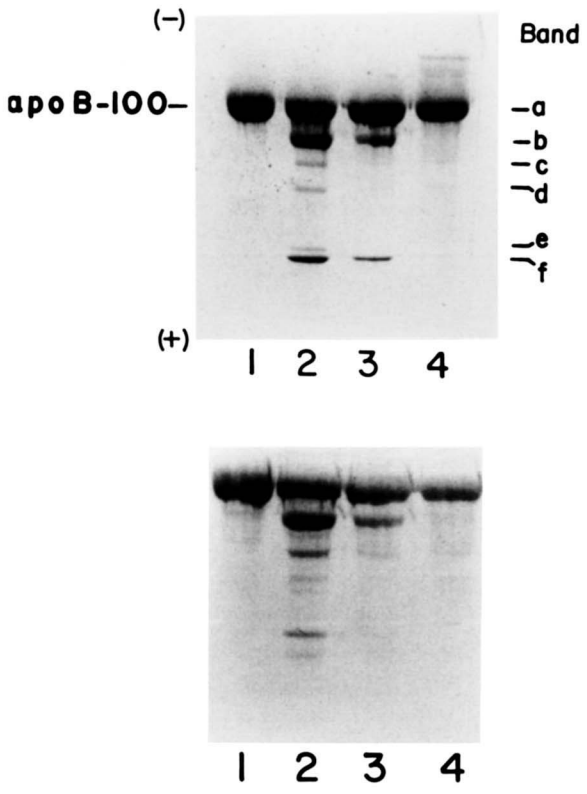

Fig. 1. The effect of temperature on the extent of proteolysis of apoB-100 in DS-precipitated LDL. Twenty five μg of LDL protein was loaded on wells of an SDS-(3–6%)polyacrylamide gradient gel. Top: Coomassie-blue stained patterns. Lane 1, LDL (d 1.019–1.045 g/ml); lane 2, DS-precipitated LDL (precipitation of plasma at 0°C for 20 min); lane 3, DS-precipitated LDL (precipitation of plasma at 23°C for 20 min); lane 4, LDL (d 1.019–1.045 g/ml) subjected to DS-precipitation at 0°C for 20 min. Bottom: Reactivity of LDL and DS-precipitated LDL with antibodies directed against apoB. Apolipoproteins and fragments were transferred from the gels to nitrocellulose paper and blots were developed using horseradish peroxidase-labeled second antibody, H_20_2, and 4-chloro-1-naphthol as substrate. There is a correspondence between stained bands in Fig. 1 (top), lanes 1–4 and immunoblots in Fig. 1 (bottom) lanes 1–4.

b–f accounting for the remaining 14% of the absorbing material (**Table 1**). In contrast, when LDL was precipitated by addition of DS and $MgCl_2$ to plasma at 0°C for 20 min and then purified by ultracentrifugation, at least six bands were seen in addition to apoB-100 (520 kDa): two major bands of 391 kDa and 137 kDa (bands b and f, respectively) and three minor components of 258 kDa (band c), 230 kDa (band d), and 156 kDa (band e). At 0°C, apoB-100 of DS-precipitated LDL decreased 58% compared to that of LDL (Table 1). Degradation of apoB-100 was also observed at 23°C (lane 3). Only bands a, b, and f were readily visible by Coomassie blue staining. By gel scanning, band a of DS-precipitated LDL (lane 3) decreased about 16% as compared to that of LDL (lane 1). No major differences in results were observed at either 0°C or 23°C in precipitated LDL removed from

TABLE 1. Densitometric scanning of SDS (3-6%)-GGE patterns for LDL and DS-precipitated LDL

Preparation	Band a	b	c	d	e	f
	% of stained material					
LDL[a]	85.6	5.7	3.4	3.3	0.9	1.1
DS-precipitated LDL from plasma (0°C)	35.6	27.4	6.7	4.7	3.4	22.3
DS-precipitated LDL from plasma (23°C)	73.4	11.6	2.6	3.3	1.1	8.0
LDL subjected to DS-precipitation	85.5	3.9	3.5	4.1	1.9	1.1

[a]The values given were averages of duplicate scans from three different electrophoretic runs for each sample.

the plasma supernatant either after 20 min or 18 hr of incubation (data not shown). Furthermore, incubation of DS-$MgCl_2$ with plasma at 37°C for either 20 min or 18 hr before separation of the precipitated lipoproteins resulted in a pattern of apoB proteolysis similar to that at 23°C (data not shown). Proteolysis of apoB-100 did not occur when prepurified LDL in the absence of plasma was subjected to precipitation with DS-$MgCl_2$ (lane 4). The high molecular weight material in lane 4 most likely represents aggregated apoB-100 not completely dissociated by SDS based on previous data from this laboratory (24).

Bands a and b were detected by the immunoblot technique using a polyclonal antibody to apoB (Fig. 1, bottom pattern). There were both qualitative and quantitative differences between the stained gel and the immunoblot (compare lanes 2 and 3, top and bottom).

Electrophoretic behavior of LDL and DS-precipitated LDL under nondenaturing conditions

As seen in **Fig. 2A**, in 1% agarose gels, LDL (lane 1) and DS-precipitated LDL (lane 2) exhibited the same net charge. Moreover, the two preparations exhibited identical LDL patterns by 2.5-10% GGE in the absence of denaturant. In one case (Fig. 2B), two bands with the same relative mobilities were observed in LDL (lane 1) and DS-precipitated LDL (lane 2).

Apolipoprotein degradation in lipoproteins other than LDL were also assessed following treatment of plasma incubated with 0.05% DS-0.05 M $MgCl_2$ for 20 min at 0°C. The apoB-containing lipoproteins of d < 1.019 g/ml underwent proteolysis after DS precipitation. The pattern was qualitatively similar to that observed with LDL-apoB-100. In these experiments it was also observed that Mg^{2+} ions were not necessary for the DS-induced proteolysis of apoB-100.

Studies on the amidolytic activity generated by incubation of plasma with DS-$MgCl_2$ at 0°C for 20 min

Having established that apoB-100 of DS-precipitated LDL underwent proteolysis during isolation from plasma we then wanted to establish the nature of the proteolytic agent that caused the degradation. To this end both supernatant and the precipitated material after resolubilization in 1.67 M NaCl were tested against synthetic substrates normally used for assessing trypsin-like activity (Z-Phe-Arg-AMC, Tos-Gly-Pro-Arg-AMC, Boc-Val-Leu-Lys-AMC, and Bz-Val-Gly-Arg-AMC), elastase-like activity (MeO-Suc-Ala-Ala-Pro-Val-AMC and Suc-Ala-Ala-Ala-AMC), and chymotrypsin-like activity (Suc-Ala-Pro-Phe-AMC). Both supernatant and lipoprotein fractions exhibited trypsin-like activity but neither elastase-like nor chymotrypsin-like activity. The substrate Z-Phe-Arg-AMC exhibited the highest rate of turnover and was utilized in all of the subsequent studies. Plasma incubated without DS, either in the presence or absence of 0.05 M $MgCl_2$, caused no cleavage of substrate. Overall, these data were taken to suggest that trypsin-like proteases such as those of the clotting system were being activated during the precipitation of the lipoproteins by DS-$MgCl_2$.

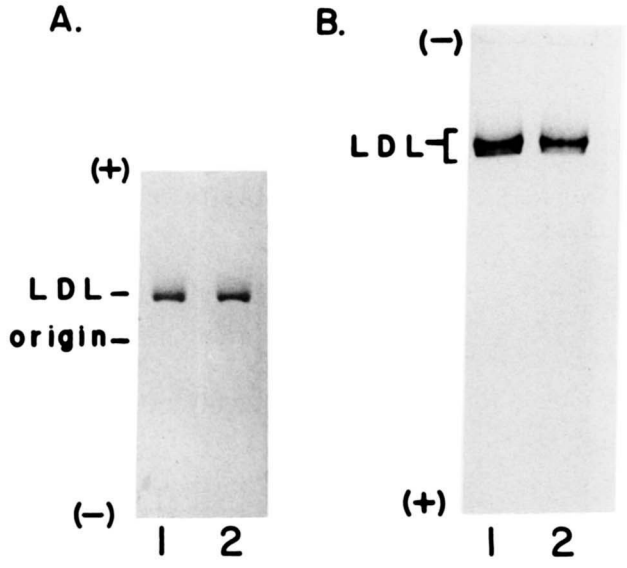

Fig. 2. Agarose gel electrophoresis (A) and GGE (B) of LDL and DS-precipitated LDL under nondenaturing conditions. A: Two μg of each LDL sample was applied to agarose films (1%) and subjected to electrophoresis in 0.065 M barbital, 0.035% EDTA, pH 8.6. Lipoproteins were fixed and stained with Fat Red 7B. Lane 1, LDL; lane 2, DS-precipitated LDL (precipitated from plasma at 0°C); B: Twenty μg of each LDL sample was applied to wells of a 2.5-10% gradient gel and electrophoresed in a buffer consisting of 0.09 M Tris, 0.08 M borate, 0.003 M Na_2EDTA, pH 8.3. The gels were then fixed and stained with Coomassie blue R 250. Lane 1, LDL; lane 2, DS-precipitated LDL (precipitated from plasma at 0°C).

Effect of DS concentration on the extent of apoB proteolysis

In these studies we varied the DS concentration while keeping constant the concentration of $MgCl_2$ (0.05 M) which was able to stimulate the amidolytic activity approximately 2.6-fold. At the lowest concentration of DS tested (0.003%), the specific amidolytic activity of the plasma was 386 ± 54 pmol substrate cleaved/min per mg protein (average of triplicate determinations from two experiments). At 0.003% DS, LDL was not precipitated as assessed by an ^{125}I-labeled LDL tracer. As the concentration of DS increased, the extent of ^{125}I-labeled LDL that precipitated increased. At 0.05% DS, approximately 96% of the ^{125}I-labeled LDL was precipitated. The specific amidolytic activity in the supernatant was aproximately 26% less than that of the 0.003% DS-treated plasma, whereas the specific activity in the d 1.063 g/ml infranatant fraction was 960 ± 140 pmol substrate cleaved/min per mg protein, a value at least 3-fold higher than that of the plasma supernatant.

We also examined LDL from plasma incubated with 0.05 M $MgCl_2$ in the presence of either 0.05% DS or 0.003% DS by SDS polyacrylamide gel electrophoresis. As shown in **Fig. 3**, apoB proteolysis occurred even at the DS concentration of 0.003%, in the absence of lipoprotein precipitation (lane 2). However, the degree of proteolysis of apoB-100 of DS-precipitated LDL (lane 3) was more extensive than that of the unprecipitated plasma.

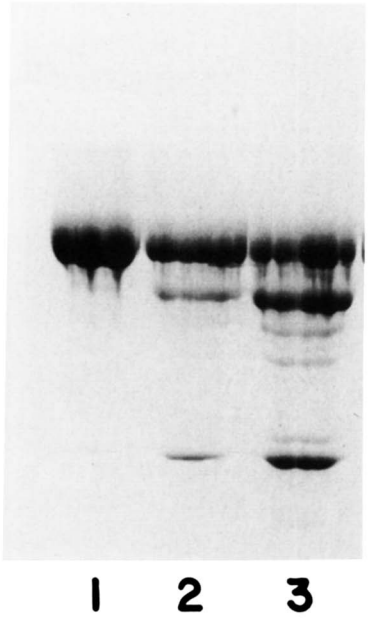

Fig. 3. SDS-GGE of LDL from plasma incubated at 0°C with sufficient (0.05%) and insufficient (0.003%) concentrations of DS to cause lipoprotein precipitation. Twenty five μg of LDL was loaded in wells of an SDS-(3–6%) polyacrylamide gradient gel. The gel was stained with Coomassie blue R-250. Lane 1, LDL; lane 2, LDL from plasma incubated with 0.003% DS–0.05 M $MgCl_2$ for 20 min followed by isolation of soluble LDL by ultracentrifugation (d 1.019–1.045 g/ml); lane 3, LDL from plasma incubated with 0.05% DS–0.05 M $MgCl_2$ for 20 min followed by isolation of precipitated LDL as in Fig. 1 (top, lane 2).

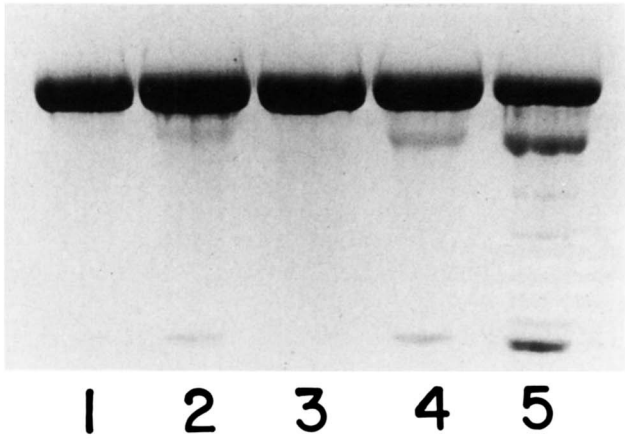

Fig. 4. Effect of proteolytic inhibitors on the degradation of DS-precipitated LDL prepared from plasma incubated with 0.05% DS–0.05 M $MgCl_2$ at 0°C. Proteolysis of apoB was assessed by SDS-(3–6%)-GGE. The gels were stained with Coomassie blue R-250. Lane 1, LDL; lane 2, DS-precipitated LDL from plasma incubated at 0°C for 20 min in the presence of 5 mM DFP; lane 3, DS-precipitated LDL from plasma incubated at 0°C for 20 min in the presence of 50 μM D-Phe-L-Phe-L-Arg-CH_2Cl; lane 4, DS-precipitated LDL from plasma incubated at 0°C for 20 min in the presence of 10 mM benzamidine; lane 5, DS-precipitated LDL from plasma incubated at 0°C for 20 min in the absence of inhibitors.

Effect of proteolytic inhibitors on the degradation of apoB-100 in DS-precipitated LDL

A variety of serine protease inhibitors was tested in an attempt to prevent apoB-100 degradation. In general, the given inhibitor was introduced in the system before precipitating the plasma with 0.05% DS–0.05 M $MgCl_2$ and was added again in the same concentrations to the precipitate that was dissolved in 1.67 M NaCl solution and ultracentrifuged at d 1.063 g/ml. As shown by SDS-GGE (**Fig. 4**), degradation of apoB-100 in DS-precipitated LDL was significantly less in the presence of 5 mM DFP (compare lanes 2 and 5). By gel scanning, approximately 6% in contrast to 53% of apoB-100 was cleaved in the presence and absence of 5 mM DFP, respectively (data not shown). Benzamidine (10 mM) was somewhat less effective than DFP (compare lanes 2 and 4). In contrast, 50 μM D-Phe-L-Phe-L-Arg-CH_2Cl (a kallikrein inhibitor) prevented cleavage of apoB-100 (lane 3).

The inhibitors of DS-induced apoB-100 cleavage also affected the degree of Z-Phe-Arg-AMC amidolytic activity. In the presence of either 5 mM DFP or 50 μM D-Phe-L-Phe-L-Arg-CH_2Cl, no amidolytic activity was detected in the supernatant after precipitation of the plasma with 0.05% DS–0.05 M $MgCl_2$. On the other hand, the amidolytic activity of the supernatant from plasma

precipitated with DS–$MgCl_2$ in the presence of 10 mM benzamidine was 67% of that observed in the absence of inhibitor. These results may be taken to indicate that the effect of benzamidine is reversible based on the observation that the sample was diluted 30-fold during the assay; whereas when assayed in the presence of 10 mM benzamidine, the activity was approximately 1% of that observed in the absence of inhibitor. When 5 mM DFP was added to a cuvette containing 60 μM substrate and 15 units of Z-Phe-Arg-AMC activity and the cuvette was immediately placed in the sample cell of the fluorimeter, the amidolytic activity decreased continuously and was completely absent in about 5 min. In contrast, when the system contained 50 μM D-Phe-L-Phe-L-Arg-CH_2Cl instead of 5 mM DFP, the amidolytic activity was completely inhibited within 10 sec. We interpret these results to indicate that both DFP and D-Phe-L-Phe-L-Arg-CH_2Cl completely inhibited the amidolytic activity, but the latter was a more efficient inhibitor than the former.

Proteolysis of apoB-100 in DS-precipitated LDL obtained from plasma deficient in either prekallikrein or factor XII

Since D-Phe-L-Phe-L-Arg-CH_2Cl was effective in preventing the degradation of apoB, we wished to determine whether enzymes of the contact phase of coagulation were responsible for the apoB-100 cleavage. For this purpose, plasma samples from subjects deficient in either prekallikrein or factor XII were subjected to DS–$MgCl_2$ precipitation. By specific clotting and chromogenic substrate assays, both the PKD-plasma and FXIID-plasma contained less than 1% of the normal levels of prekallikrein and factor XII, respectively. Since both the PKD- and FXIID-plasmas were frozen at − 80°C and thawed rapidly in a 37°C water bath prior to their use in DS-precipitation experiments, control plasma was subjected to the same manipulations. The Z-Phe-Arg-AMC activity generated after a 20 min incubation of the control plasma with 0.05% DS–0.05 M $MgCl_2$ at 0°C was 98% of that attained in plasma stored at 4°C without a freeze–thaw cycle. In contrast, no amidolytic activity was generated in either FXIID-plasma or PKD-plasma after DS–$MgCl_2$ precipitation. By SDS-GGE (**Fig. 5**) the patterns of degradation of apoB-100 were much less extensive in DS-precipitated LDL from PKD-plasma (lane 1) and FXIID-plasma (lane 3) than control plasma (lane 6). The higher molecular weight components in the LDL from PKD-plasma and FXIID-plasma were observed in both the LDL (lanes 2 and 4) and DS-precipitated LDL (lanes 1 and 3) samples. These bands may be due to the presence of aggregated material or to the apoB–apo[a] complex of lipoprotein[a] (24) but this was not verified. Nevertheless,

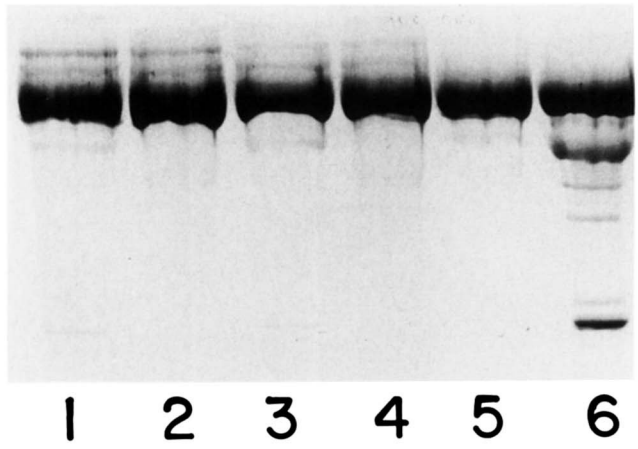

Fig. 5. Polyacrylamide gradient gels in the presence of SDS for DS-precipitated LDL prepared from normal plasma, PKD-plasma, and FXIID-plasma incubated with DS at 0°C. Degradation of apoB-100 was analyzed by SDS-(3–6%)-GGE. The gels were stained with Coomassie blue R-250. Plasma specimens were incubated with 0.05% DS–0.05 M $MgCl_2$ at 0°C for 20 min. Lane 1, DS-precipitated LDL from PKD-plasma; lane 2, LDL from PKD-plasma; lane 3, DS-precipitated LDL from FXIID-plasma; lane 4, LDL from FXIID-plasma; lane 5, LDL from normal plasma; lane 6, DS-precipitated LDL from normal plasma.

they were not observed in the normal plasma which underwent the same manipulations (lanes 5 and 6).

Proteolysis of apoB-100-LDL by either purified kallikrein or by a partially purified amidolytic activity in DS-treated plasma

The amidolytic activity present in DS-treated plasma was contained in the $d > 1.063$ g/ml fraction. Once dialyzed exhaustively against 0.15 M NaCl–0.01 M Tris, pH 8.0, and incubated with LDL at 37°C for 6 hr, it caused an extensive hydrolysis of apoB-100. By SDS-GGE criteria, positions and staining intensity of these fragments were identical to those seen when LDL was incubated with purified kallikrein under the same conditions (data not shown).

When separated by Sephacryl-S-200 column chromatography, the $d > 1.063$ g/ml bottom fraction exhibited the 280 nm-absorbing profile shown in **Fig. 6**. At least three major and two minor protein components (closed circles) were noted. In turn, the amidolytic activity eluted as one major peak within fractions 27–33 (open circles), at the trailing edge of the first major protein peak. By SDS GGE, most of the protein mass corresponded to mobilities of proteins > 97 kDa. Fractions 28–31 were pooled and concentrated by membrane filtration and incubated with LDL at 37°C for 2 hr at pH 8.0 in the presence or absence of the inhibitor, D-Phe-L-Phe-L-Arg-CH_2Cl. The resulting pattern of degradation of apoB-100 was similar to that obtained with purified kallikrein (**Fig. 7**, lanes 1 and 3). In both cases the degrada-

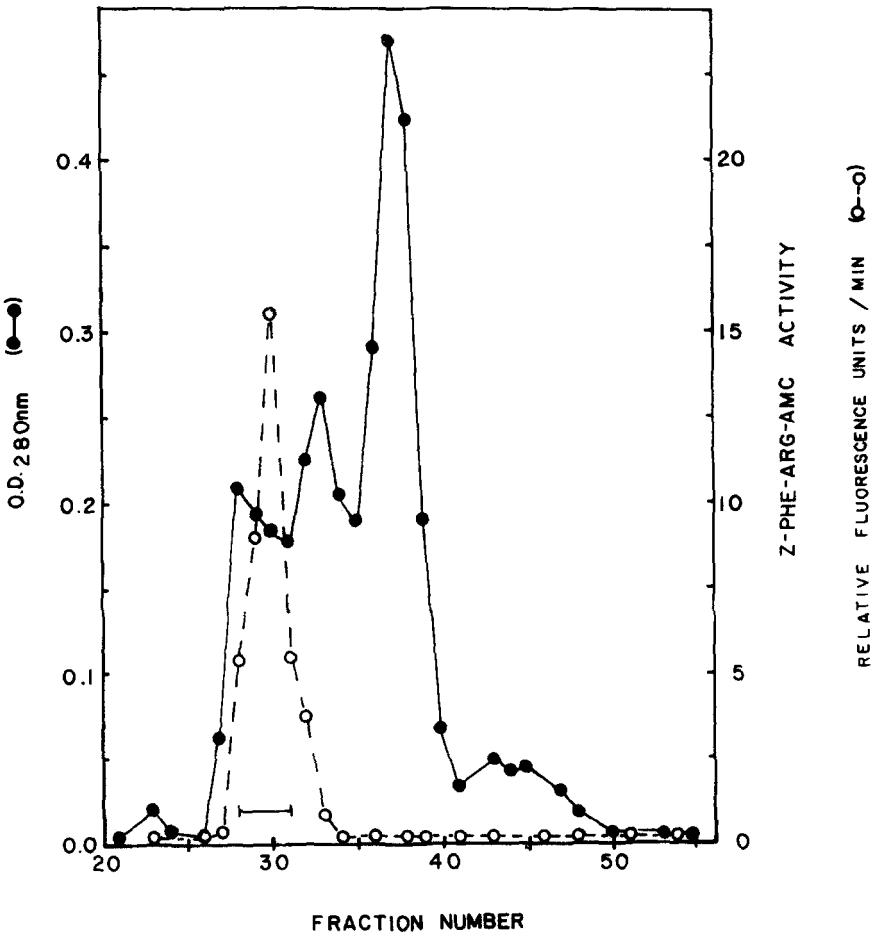

Fig. 6. Sephacryl-S-200 column chromatography of the Z-Phe-Arg-AMC amidolytic activity present in the d 1.063 g/ml bottom fraction from DS-precipitated LDL refloated at d 1.063 g/ml. One ml of the d 1.063 g/ml bottom fraction containing 15.5 mg protein and 1320 RFU of Z-Phe-Arg-AMC amidolytic activity was applied to a 1.5 × 90 cm column of Sephacryl-S-200 (4°C) equilibrated in 0.15 M NaCl, 0.01 M Tris, 0.02% NaN_3, pH 7.4. The column was developed with the same buffer at a flow rate of 13.8 ml/hr and 3.5-ml fractions were collected. The absorbance of the eluates was monitored at 280 nm (●—●) and 0.2-ml aliquots were assayed for amidolytic activity (O— —O) as described under Experimental Procedures.

tion was prevented by D-Phe-L-Phe-L-Arg-CH_2Cl (lanes 2 and 4).

[^{3}H]DFP-labeling of serine protease(s) in the active fraction from Sephacryl-S-200 chromatography

The complete inhibition by DFP of the Z-Phe-Arg-MCA amidolytic activity of the active fractions from Sephacryl-S-200 column chromatography (fractions 28–31) allowed us to estimate its apparent molecular weights by using ^{3}H-labeled DFP as a label. **Fig. 8** shows the SDS-(6–14%)-GGE patterns of the [^{3}H]DFP-labeled components visualized by staining with Coomassie blue and by fluorography. Under nonreducing conditions, most of the protein mass migrated as components (lane 1) that exceeded the molecular weight of the phosphorylase b marker (97.4 kDa, lane 2). Numerous bands were stained when electrophoresis was carried out in the presence of a reducing agent (lane 3). The corresponding autoradiogram of the nonreduced material (lane 4) indicates that ^{3}H-labeled DFP labeled several components including major bands of 90 kDa and 86 kDa as well as minor bands of approximately 110 kDa and 77 kDa. Faint bands of molecular mass > 100 kDa as well as 30 kDa were also noted (lane 4). Upon reduction with β-mercaptoethanol, essentially all of the radiolabeled material was distributed in four components of 31 kDa, 31.5 kDa, 35 kDa (the major band), and 37 kDa (lane 5). These results indicate that most of the DFP-reactive material contained in the Sephacryl-S-200 fraction represented multiple polypeptide chains linked by disulfide bonds and that the active site serine of these proteases was approximately 31-37 kDa. Mandle and Kaplan (25) have characterized two species of single-chain prekal-

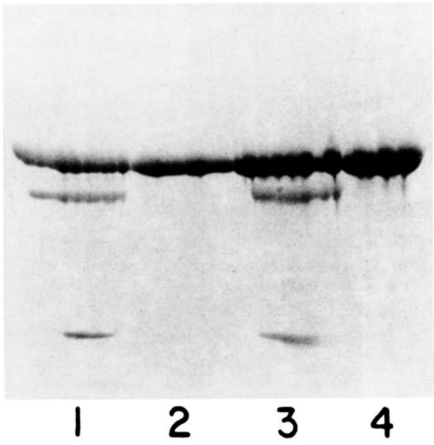

Fig. 7. SDS-GGE of LDL incubated for 2 hr at 37°C with either purified kallikrein or the active proteolytic fraction (28–31) of Sephacryl-S-200 in the absence or presence of D-Phe-L-Phe-L-Arg-CH_2Cl. Three hundred μg of LDL was incubated in 0.15 M NaCl, 0.01 M Tris, 0.02% NaN_3, pH 8.0, in a total volume of 0.1 ml, with 25 units of Z-Phe-Arg-AMC amidolytic activity of either purified kallikrein or the proteolytic activity of Sephacryl-S-200 pooled fraction (28–31). Lane 1, LDL + kallikrein; lane 2, LDL + kallikrein + 50 μM D-Phe-L-Phe-L-Arg-CH_2Cl; lane 3, LDL + Sephacryl-S-200 fraction(28–31); lane 4, LDL + Sephacryl-S-200 fraction (28–31) + 50 μM D-Phe-L-Phe-L-Arg-CH_2Cl.

likrein (85 kDa and 88 kDa) that retain their respective molecular weights during activation to the corresponding kallikrein. These kallikrein variants have two disulfide-linked polypeptide chains consisting of a 52-kDa heavy chain and either a 33-kDa or a 36-kDa light chain containing the active site serine (26). Moreover, the other two contact phase blood-clotting enzymes, namely factor α-XII_a (80 kDa) and factor XI_a (160 kDa), have disulfide-linked polypeptide chains that exhibit DFP-reactive light chains of approximately 28 kDa and 33 kDa, respectively, following SDS-PAGE in the presence of a reducing agent (26, 27). Overall, the patterns of the [^{3}H]DFP-labeled components shown in Fig. 8 indicate that factor α-XII_a, factor XI_a, kallikrein, and possibly other enzymes may be members of the active Sephacryl-S-200 fractions.

DISCUSSION

The results of our current studies have shown that apoB-100 of LDL particles that are precipitated from plasma by DS–$MgCl_2$ undergoes proteolysis with generation of fragments in the sizes of apoB-74 and apoB-26 as assessed by SDS-GGE. Our studies have also shown that apoB-100 proteolysis is more marked in plasma precipitated at 0°C than at 23°C although in neither case do changes in overall net charge and size of LDL occur (Fig. 2) and that cleavage of apoB-100 does not occur when LDL previously separated from plasma by ultracentrifugation is subjected to DS-precipitation. Thus a component or components of the plasma are necessary for apoB-100 proteolysis to occur.

The size of the major apoB-100 fragments in DS-precipitated LDL was similar to those designated as apoB-74 and apoB-26 in freshly isolated serum LDL (28) and to those generated in vitro following the digestion of LDL by kallikrein (29–31). The apoB-100 cleavage sites by kallikrein and thrombin are reported to be identical (32) as determined recently by sequence analyses of isolated peptides and by comparison with the amino acid sequence of apoB-100 deduced from nucleotide sequence data (31–33). From these data, cleavage sites have been identified at the carboxyl ends of Lys-1,297 and Lys-3,249 (32). Altogether, these data indicate that apoB-26 and apoB-74 consist of residues 1–1,297 and residues 1,298–4,536, respectively; apoB-44 and apoB-30 consist of residues 1,298–3,249 and 3,250–4,536, respectively, and apoB-70 consists of residues 1–3,249 (34). Although the fragments in DS-precipitated LDL require characterization to verify their identity with kallikrein-derived fragments, we anticipate that bands a,b,c,d,e, and f in Fig. 1 refer to apoB-100, -74, -70, -44, -30, and -26 respectively.

When the DS supernatant and the DS-precipitated fractions from plasma were examined for protease activity with fluorogenic peptide amide substrates, both fractions exhibited activity with several X-Arg-AMC and X-Lys-AMC substrates, indicating trypsin-like activity. Neither fraction was reactive against substrates for chymotrypsin or neutrophil elastase, the latter enzyme having been previously shown in our laboratory to cause proteolysis of HDL_3 (35) and LDL (36). The substrate that exhibited the highest rate of amidolytic activity, Z-Phe-Arg-AMC,

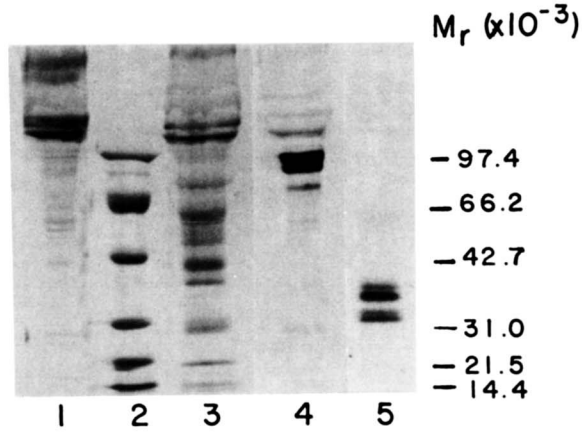

Fig. 8. SDS-(6–14%)-GGE of [^{3}H]DFP-labeled serine proteases contained in Sephacryl S-200 fractions 28–31. Sephacryl-S-200 fractions 28–31 were pooled, concentrated 10-fold by ultrafiltration, labeled with [^{3}H]DFP, and separated by electrophoresis as described under Experimental Procedures. Lanes 1–3 were stained with Coomassie blue; lanes 4 and 5 were visualized by fluorography. Lane 1, pooled fractions (28–31); lane 2, molecular weight standards; lane 3, pooled fractions(28–31) + 1% β-mercaptoethanol; lane 4, as in lane 1; lane 5, as in lane 3.

suggests that a kallikrein-like enzyme (16) was responsible for the degradation of apoB-100. It must be emphasized that plasma proteases other than kallikrein may also cleave Z-Phe-Arg-AMC.

In this work we show that at 0.003% DS, a concentration where a maximum amidolytic activity in plasma is attained, no precipitation of ^{125}I-labeled LDL occurs. Moreover, at 0.05% DS, a concentration at which precipitation of ^{125}I-labeled LDL reaches completion, the specific activity of the precipitated fraction is higher than in the plasma supernatant. This indicates that the activation of plasma by DS does not require LDL-DS precipitation. However, the results do suggest that the proteolytic activity was associated preferentially with the DS-precipitated lipoprotein complex as compared to that of the plasma alone. In addition, apoB-100 proteolysis was more extensive at 0.05% DS than at 0.003% DS (Fig. 3). We believe that this was likely due to the increase in the effective LDL and protease concentrations in the immediate vicinity of the DS-lipoprotein complex relative to the remainder of the plasma. In this aspect apoB-100 of the DS-precipitated lipoproteins of $d < 1.019$ g/ml was degraded while the apolipoproteins of HDL_3, namely apoA-I and apoA-II, which remained soluble in the plasma supernatant, were not. Further studies are required in order to evaluate the specificity of kallikrein-like activities for apoB-containing lipoproteins versus the apolipoproteins of HDL.

The kallikrein inhibitor D-Phe-L-Phe-L-Arg-CH_2Cl (37) was effective in preventing apoB-100 proteolysis in DS-precipitated LDL (Fig. 4). Although 5 mM DFP completely inhibited the active fraction from Sephacryl S-200 column chromatography, the inactivation was not immediate and this provides an explanation for the incomplete protection of DS-precipitated LDL from proteolysis (see Fig. 6 and text of Results). Moreover, the incomplete inhibition of DS-precipitated LDL proteolysis by benzamidine may be explained by the reversibility of this competitive inhibitor.

The absence of Z-Phe-Arg-AMC amidolytic activity following incubation of either PKD-plasma or FXIID-plasma with 0.05% DS–0.05 M $MgCl_2$ at 0°C for 20 min, and only minor fragmentation of apoB-100 in the DS-precipitated LDL from either plasma (Fig. 5), indicates that contact activation reactions played a major role in the DS-dependent proteolysis of apoB-100-containing lipoproteins in plasma. Why there was a discrepancy between the lack of amidolytic activity in the DS-treated clotting factor-deficient plasmas and the minor proteolysis of apoB-100 in the DS-precipitated LDL from these plasmas is unresolved. Perhaps the prekallikrein and factor XII in the respective deficient plasmas, albeit at low levels, underwent activation during incubation with DS–$MgCl_2$, and rapidly cleaved a minor fraction of the apoB-100 in plasma before the enzymes were inactivated by inhibitors. On the other hand, we cannot rule out the possibility that other enzyme activities, lacking Z-Phe-Arg-AMC amidolytic activity, may play a minor role in the proteolysis of DS-precipitated LDL.

The active fraction from Sephacryl-S-200 column chromatography of DS-treated normal plasma, when incubated with LDL at 37°C for 2 hr in buffer at pH 8.0, generated an apoB proteolysis pattern similar to purified kallikrein and was completely inhibited by D-Phe-L-Phe-L-Arg-Ch_2Cl (Fig. 7). Furthermore, radioactive labeling of the partially purified activity with [^{3}H]DFP followed by autoradiography of the SDS-GGE patterns under non-reducing and reducing conditions (Fig. 8) indicated that most of the radioactivity was incorporated into components with mobility consistent with the reported size range and heterogeneity of plasma kallikrein (25). Nevertheless, minor components of the size of α-factor XII_a (77 kDa) and other higher ($>$ 100 kDa) and lower ($<$ 66 kDa) molecular mass material were also present.

We do not currently have information regarding a comparison of the kinetics of LDL proteolysis by kallikrein, factor αXII_a, and factor XI_a. It is possible that all of these enzymes may cleave the same peptide bonds in apoB-100. By the same token, Kettner and Shaw (37) have shown that D-Phe-L-Phe-L-Arg-CH_2Cl effectively inhibits other plasma proteases in addition to kallikrein including plasmin, thrombin, and factor X_a. Thus, it is likely that the proteolysis of apoB-100 that occurs in DS-treated plasma is due to the action of kallikrein in concert with another protease(s) that is either directly inactivated by D-Phe-L-Phe-L-Arg-CH_2Cl or requires kallikrein or another enzyme activity, which is susceptible to this inhibitor, for expression of its activity.

The results of the present findings are important for several reasons. For the first time, we have demonstrated that techniques using precipitation of plasma with sulfated polysaccharides result in proteolytic modification of apoB-100 in LDL and perhaps in all apoB-containing lipoproteins. From these results we anticipate that factors that promote the activation of the contact system in plasma, such as exposure of plasma to negatively charged surfaces and divalent cations, may also promote degradation of apoB. We also predict that when due precautions are taken to eliminate contact activation of plasma by glass surfaces and inclusion of D-Phe-L-Phe-L-Arg-CH_2Cl as an inhibitor, LDL from normolipidemic plasma can be prepared containing undegraded apoB-100.

We have observed that proteolysis of apoB-100 of LDL from EDTA-plasma occurs when stored for 3 days at 4°C but not at 23°C. In contrast, LDL degradation does not take place when the same plasma is stored in an identical fashion at 4°C but in the presence of D-Phe-L-Phe-L-

Arg-CH_2Cl. (R. E. Byrne and A. M. Scanu, unpublished observations). It is probable, however, that in this case the previously documented phenomenon of cold-activation of plasma may also have played a role in the proteolysis of apoB-100 (38, 39). Czendlik, Lämmle, and Duckert (4) have proposed that cold activation may result from a decrease in the efficiency of C-1-inhibitor at low temperatures.

The significance of kallikrein modification of apoB-100 in LDL metabolism remains obscure since the cleavage specificity of this reaction appears to have little effect on LDL receptor binding and uptake (41, 42). This contrasts with the enhanced uptake of neutrophil elastase-modified LDL, compared to native LDL, by human monocyte-derived macrophages observed by Polacek, Byrne, and Scanu (42). The unique interactions of elastase-modified LDL with macrophages may be related to the increased size of this digested lipoprotein as assessed by nondenaturing GGE (42). In contrast with elastase-modified LDL, the mobility on nondenaturing GGE of DS-precipitated LDL is unaltered from that of the LDL control (fig. 2B).

Yokoyama et al. (12) have recently utilized 5 kDa dextran sulfate immobilized on cellulose beads as a specific sorbent of apoB-containing lipoproteins for plasmapheresis in the treatment of familial hypercholesterolemia. These investigators reported that two patients had successful removal of LDL and VLDL without the loss of HDL or other major plasma components. Since it is known that 5 kDa dextran sulfate in soluble form promotes the activation of factor XII by kallikrein (43), a high local density of surface-bound contact factors attained by immobilization of the low molecular weight dextran sulfate could result in significant contact activation. This potential effect should be evaluated in the future.

We wish to thank Denise Polacek, Celina Edelstein, and Gunther M. Fless for their useful discussions and advice during the course of these studies. We also thank Ms. Sherra McIntyre and Ms. Barbara Kass for their help in preparing this manuscript. This work was supported by grant HL-18577 from the National Institutes of Health. A portion of this work was presented at the meeting of the American Society of Biological Chemists, Philadelphia, PA, June 8–12, 1987. The abstract has been published (13).

Manuscript received 26 July 1988.

REFERENCES

1. Gofman, J. W., F. T. Lindgren, and H. Elliot. 1949. Ultracentrifugal studies of lipoproteins of human serum. *J. Biol. Chem.* **179:** 973–979.
2. Fless, G. M., C A. Rolih, and A. M. Scanu. 1983. Application of gradient gel electrophoresis to the study of serum lipoproteins and apolipoproteins of rhesus monkeys. *In* CRC Handbook of Electrophoresis. L. A. Lewis and H. K. Naito, editors. CRC Press, Inc., Boca Raton, FL. Vol. 4: 17–31.
3. Rudel, L. L., J. A. Lee, M. O. Morris, and J. M. Felts. 1974. Characterization of plasma lipoproteins separated and purified by agarose-column chromatography. *Biochem. J.* **139:** 89–95.
4. Cheung, M. C., and J. J. Albers. 1982. Distribution of high density lipoprotein particles with different apoprotein composition: particles with A-I and A-II and particles with A-I but no A-II. *J. Lipid Res.* **23:** 747–753.
5. McConathy, W. J., E. Koren, H. Weiland, E. M. Campos, D. M. Lee, H. U. Kloer, and P. Alaupovic. 1985. Evaluation of immunoaffinity for isolating human lipoproteins containing apolipoprotein B. *J. Chromatogr.* **342:** 47–66.
6. Fielding, P. E., and C. J. Fielding. 1986. An apo-E-free very low density lipoprotein enriched in phosphatidylethanolamine in human plasma. *J. Biol. Chem.* **261:** 5233–5236.
7. Burstein, M., and P. Legmann. 1982. Precipitation of serum lipoproteins by anionic polyelectrolytes and bivalent cations. *Monogr. Atheroscler.* **11:** 17–39.
8. Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–595.
9. Bachorik, P. S., and J. J. Albers. 1986. Precipitation methods for quantification of lipoproteins. *Methods Enzymol.* **129:** 78–100.
10. Doi, Y., and T. Hishida. 1981. Lecithin-cholesterol acyltransferase from human plasma. *Methods Enzymol.* **71:** 753–767.
11. Morton, R. E., and D. B. Zilversmit. 1982. Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* **23:** 1058–1067.
12. Yokoyama, S., R. Hayashi, M. Satani, and A. Yamamoto. 1985. Selective removal of low density lipoproteins by plasmaphresis in familial hypercholesterolemia. *Arteriosclerosis.* **5:** 613–622.
13. Byrne, R. E., and A. M. Scanu. 1987. Proteolysis of apolipoprotein B is present after isolation of plasma low density lipoproteins by precipitation with sulfated polyanions. *Fed. Proc.* **46:** 2094 (abstract).
14. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. 1. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
15. Scanu, A. M. 1966. Forms of human serum high density lipoprotein protein. *J. Lipid Res.* **7:** 295–306.
16. Morita, T., H. Kato, S. Iwanaga, K. Takada, T. Kimura, and S. Sakakibara. 1977. New fluorogenic substrates for α-thrombin, factor Xa, kallikreins, and urokinase. *J. Biochem.* **82:** 1495–1498.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
18. Markwell, M. A. K., S. N. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipid samples. *Anal. Biochem.* **87:** 206–210.
19. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46:** 83–88.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.

21. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **46:** 4350–4354.
22. Hawkes, R., E. Niday, and J. Gordon. 1982. A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* **119:** 142–147.
23. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
24. Fless, G. M., C. A. Rolih, and A. M. Scanu. 1984. Heterogeneity of human plasma Lp[a]: isolation and characterization of the lipoprotein subspecies and their apoproteins. *J. Biol. Chem.* **259:** 11470–11478.
25. Mandle, R. J., Jr., and A. P. Kaplan. 1977. Hageman factor substrates: human plasma prekallikrein: mechanism of activation by Hageman factor and participation in Hageman factor-dependent fibrinolysis. *J. Biol. Chem.* **252:** 6097–6104.
26. Griffin, J. H. 1979. Preparation of intrinsically labelled kinins. *In* Kinins. II. S. Fujii, H. Moriya, T. Suzuki, editors. Plenum Publishing, New York. 39–51.
27. Kurachi, K., and E. W. Davie. 1977. Activation of human factor XI (plasma thromboplastin antecedent) by factor XIIa (activated Hageman factor). *Biochemistry.* **16:** 5831–5839.
28. Kane, J. P., D. A. Hardman, and H. E. Paulus. 1980. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. *Proc. Natl. Acad. Sci. USA.* **77:** 2465–2469.
29. Cardin, A. D., K. R. Witt, J. Chao, H. S. Margolius, V. H. Donaldson, and R. L. Jackson. 1984. Degradation of apolipoprotein B-100 of human plasma low density lipoproteins by tissue and plasma kallikreins. *J. Biol. Chem.* **259:** 8522–8528.
30. Hardman, D. A., A. Gustafson, J. W. Schilling, V. H. Donaldson, and J. P. Kane. 1986. Scission of human apolipoprotein B-100 by kallikrein: characterization of the cleavage site. *Biochem. Biophys. Res. Commun.* **137:** 821–825.
31. Protter, A. A., D. A. Hardman, K. Y. Sato, J. W. Schilling, M. Yamanaka, Y. J. Hort, G. C. Hjerrild, and J. P. Kane. 1986. Analysis of cDNA clones encoding the entire B-26 region of human apolipoprotein B. *Proc. Natl. Acad. Sci. USA.* **83:** 5678–5682.
32. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Human apoB: complete protein sequence and identification of structural domains. *Nature.* **323:** 734–738.
33. Yang, C-Y., S-H. Chen, S. H. Gianturco, W. A. Bradley, J. T. Sparrow, M. Tanimura, W-H. Li, D. A. Sparrow, H. DeLoof, M. Rosseneu, F-S. Lee, Z-W. Gu, A. M. Gotto, Jr., and L. Chan. 1986. Sequence, structure, receptor-binding domains, and internal repeats of human apolipoprotein B-100. *Nature.* **323:** 738–742.
34. Cardin, A. D., C. A. Price, N. Hirose, M. A. Krivanek, D. T. Blankenship, J. Chao, and S. J. T. Mao. 1986. Structural organization of apolipoprotein B-100 of human plasma low density lipoproteins. *J. Biol. Chem.* **261:** 16744–16748.
35. Byrne, R. E., D. Polacek, J. I. Gordon, and A. M. Scanu. 1984. The enzyme that cleaves apolipoprotein A-II upon in vitro incubation of human plasma high density lipoprotein-3 with blood polymorphonuclear cells is an elastase. *J. Biol. Chem.* **259:** 14537–14543.
36. Polacek, D., R. E. Byrne, G. M. Fless, and A. M. Scanu. 1986. In vitro protolysis of human plasma low density lipoproteins by an elastase released from human blood polymorphonuclear cells. *J. Biol. Chem.* **261:** 2057–2063.
37. Kettner, C., and E. Shaw. 1981. Inactivation of trypsin-like enzymes with peptides of arginine chloromethyl ketone. *Methods Enzymol.* **80:** 826–842.
38. Gjonnaess, H. 1972. Cold promoted activation of factor VII. I. Evidence for the existence of an activator. *Thromb. Diath. Haemorrh.* **28:** 155–168.
39. Laake, K., A. M. Vennerod, and G. Haugen. 1974. Cold promoted activation of factor VII in human plasma: studies on the associated acyl-arginine esterase activity. *Thromb. Res.* **4:** 769–785.
40. Czendlik, C., B. Lämmle, and F. Duckert. 1985. Cold promoted activation and factor XII, prekallikrein and C-1 inhibitor. *Thromb. Haemost.* **53:** 242–244.
41. Yamamoto, M., S. Ranganathan, and B. A. Kottke. 1985. Structure and function of human low density lipoproteins. *J. Biol. Chem.* **260:** 8509–8513.
42. Polacek, D., R. E. Byrne, and A. M. Scanu. 1988. Modification of low density lipoproteins by polymorphonuclear cell elastase leads to enhanced uptake by human monocyte-derived macrophages via the low density lipoprotein receptor pathway. *J. Lipid Res.* **29:** 797–808.
43. Griep, M. A., K. Fujikawa, and G. L. Nelsestuen. 1986. Possible basis for the apparent surface selectivity of the contact activation of human blood coagulation factor XII. *Biochemistry.* **25:** 6688–6694.