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THE DISTINCTION BETWEEN THE EXPOSED REGIONS AND THE BURIED REGIONS OF APOPROTEINS IN HIGH DENSITY LIPOPROTEINS BY THEIR REACTIVITIES WITH PRONASE

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SUMMARY: Pronase digestion was used to study the surface disposition of apoproteins on high density lipoproteins. After digestion the average density of high density lipoproteins decreased from 1.14 to 1.10 g/ml. The immunoreactivities of apoproteins A-II, C-II, and C-III were completely destroyed, but 80% of the reactivity of ApoA-I was retained. Only 5-10% of ApoA-I reacts with anti ApoAI antisera in intact high density lipoproteins. The similar accessibility of ApoA-I to pronase and to antibodies suggests that pronase hydrolyzes only the exposed regions of protein moieties. Pronase may be an ideal probe for distinguishing the exposed regions of apoproteins in lipoproteins from those that are buried.

INTRODUCTION

The apoproteins of plasma lipoproteins have several functions. Apoproteins are carriers for the otherwise insoluble lipids in the circulation (1,2), they modulate enzymatic activities (3-6), interact with specific receptors in target tissues (7-9), and facilitate communication between lipoproteins (10,11). A clear understanding of the structural organization of apoproteins in human lipoproteins at the molecular level is essential for a clear understanding of lipoprotein metabolism, since those regions of the apoproteins which are "masked" probably interact with lipids, while the "exposed" regions probably interact with enzymes, tissues or other lipoprotein particles.

The lipid-binding regions of some apoproteins have been identified by reconstituting lipids with peptides obtained either by solid-phase peptide synthesis or by isolation of enzymatic fragments (12,13). The long term goal

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of our studies is the identification of lipid-binding and other interacting domains of apoproteins in lipoproteins. Our experimental approach is to hydrolyze the "exposed" regions of apoproteins in intact lipoproteins and to characterize the unaffected, "protected" segments. Here we report on the digestion of human high density lipoproteins (HDL) by pronase.

HDL isolated between the densities 1.063 and 1.210 g/ml (14), contains apoprotein A-I (ApoA-I) and apoprotein A-II (ApoA-II) which are similar to each other at their carboxyl terminal regions (15) and together account for ∿90% of the proteins in HDL. Other apoproteins in HDL are ApoC, ApoD and ApoF (16-19). The amino acid sequences of ApoA-I, ApoA-II, and the three ApoC's are known (20-27). We have previously shown that a large proportion of the antigenic sites of ApoA-I on intact HDL are inaccessible to anti ApoA-I antibodies, but that nearly all of the antigenic sites become accessible following the delipidation of HDL (8,29). By contrast, most of the antigenic sites of ApoA-II, ApoC-II, and ApoC-III in intact HDL are reactive (30,31). These findings suggest that most of the immunologically active sites of ApoA-I in HDL are "masked" whereas the immunologically active sites of the other apoproteins are "exposed". We now report on the accessibility of the immunologically active sites of HDL apoproteins to digestion by pronase.

MATERIALS AND METHODS

Human high density lipoproteins (d 1.09-1.19 g/ml) were isolated from the plasma of a normal 12-hour fasting male by ultracentrifugation (14). This "narrow" density range was used to avoid inclusion of any Lp(a) (32), or HDL_C (33). Salt was removed by dialysis against 1 mM EDTA-saline, 5 mM Tris-Cl, pH 8.1 (Tris EDTA). ApoA-I and ApoAII were purified by column chromatography (28,30).

The kinetics of the digestion of HDL with pronase were studied by incubation (HDL, 8 mg/ml, pronase 75 μg/ml in Tris EDTA) for different periods of time. An aliquot (100 μl was withdrawn and mixed with an equal volume of 20% tricholoric acetic acid (TCA). After incubation at 4° for one hour, TCA-soluble materials were obtained by centrifugation at 1,500 x g for 20 minutes. The ninhydrin reaction was carried out directly on supernates according to the methods of Moore and Stein (34), using glycine as a standard to calculate the amount of peptides and amino acids released.

Linear density gradients of d 1.050-1.185 g/ml were prepared from equal volumes (6.2 ml) of KBr (1 mM EDTA, pH 8.1). Samples (400 μl) were placed on

the tops of centrifuge tubes. Ultracentrifugation was at 35,000 rpm for 88 hours in a SW 40 rotor at 4°C with a Beckman Model L2-65B ultracentrifuge. Thirty-two fractions (0.4 ml each) were collected from the bottoms of tubes with a pump. The lipoprotein was located by protein determination. Since KBr interferes with protein determination, proteins obtained from density gradient ultracentrifugation were precipitated by deoxycholate-TCA (35) prior to Lowry determination.

Radioimmunoassays (RIA) of apoproteins were described elsewhere (28-31). ApoA-I immunoreactivity in HDL was determined both before and after delipidation of the HDL (28). Delipidation does not result in the losses of apoproteins. Digestion products of apoproteins were also examined by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (5-20% gradient slab gel, SDS-PAGE) (36).

RESULTS

To ascertain whether lipid-free apoproteins were completely digestible by pronase. Purified ApoA-I or ApoA-II (1 mg/ml) were incubated with pronase (10 μg/ml) at 37°C for 3 hours. At the end of incubation the incubation mixtures were diluted 5000-fold and analyzed by appropriate RIA's. All of the immunoactivities of both ApoA-I or ApoA-II were destroyed. On SDS-PAGE, no Coomassie blue staining materials were detected in gels containing digested samples, whereas the expected apoprotein bands were seen on untreated HDL controls. The results suggest that whole molecules of lipid-free ApoA-I and ApoA-II, including their immunologic sites, were digested by pronase.

The digestion of intact HDL by pronase at 37°C (measured as generation of TCA-soluble ninhydrin-positive compounds) reached a plateau within 3 hours, when approximately 24% of the HDL protein had been digested (Table 1). After

TABLE I

THE PROPORTIONS OF HDL APOPROTEINS' ANTIGENIC SITES ACCESSIBLE TO PRONASE AND TO ANTIBODY

	Protein	ApoA-I	ApoA-II	ApoC-II	ApoC-III
			%		
% Accessible to Pronase	24*	20	99.7	99+	99.4
% Accessible to Antibody*	N.A.	5-10	99+	99+	99+

For pronase experiments, apoproteins were determined on pronase-treated and untreated HDL incubation mixtures which were diluted and delipidated. Results are given as treated (mass) x 100 ÷ untreated (mass). For antibody experiments, delipidated and non-delipidated HDL were assayed. Results are non-delipidated (mass) x 100 ÷ delipidated (mass).
*Determined as ninhydrin reactive TCA soluble materials.
N.A. - not applicable

3 hours of pronase treatment, the hydrated density of HDL (by density gradient equilibrium sedimentation) decreased from 1.14 g/ml to 1.10 g/ml, confirming a selective loss of HDL protein.

The apparent apoprotein contents of HDL were determined by RIA after digestion of intact HDL with pronase (37°C for 3 hours). Incubation mixtures were diluted (>1000-fold) for the ApoAII, ApoC-II and ApoC-III assays, and delipidated and diluted (5000-fold) for ApoA-I assays. Twenty percent of the immunoreactivity of ApoA-I in intact HDL was destroyed by the action of pronase, while 99% of the immunoreactivities of ApoA-II, ApoC-II, and ApoC-III were destroyed (Table 1).

To assess the availability of apoproteins to antibodies in undigested, undelipidated HDL the apparent apoprotein contents of apoproteins were assayed in intact and in delipidated HDL. Results are given as mass of apoprotein in intact HDL x 100 ÷ mass in delipidated HDL (Table 1). The unavailability of ApoA-I to anti ApoAI antisera and the accessibility of ApoAII, ApoC-II and ApoC-III are similar to those reported previously (26-28). When the proportions of apoprotein immunoreactivities destroyed by pronase (line 1, Table 1) are compared with the accessibility of apoproteins to antibodies (line 2), it is seen that all of the regions accessible to antibody in intact HDL have been destroyed by pronase.

The pronase-treated and untreated HDL were also analyzed by SDS-PAGE (Fig. 1). The electrophoretic migration of some ApoA-I was not affected by pronase treatment, but several peptides also appeared which moved between ApoA-I and ApoA-II. These are likely to be derivatives of ApoA-I since they were too big to be the fragments of ApoA-II. A peptide moving slightly ahead of ApoA-II was also consistently observed.

DISCUSSION

The structures involved in the interactions between the various components of heterologous macro molecular complexes have been studied first by exhaustive

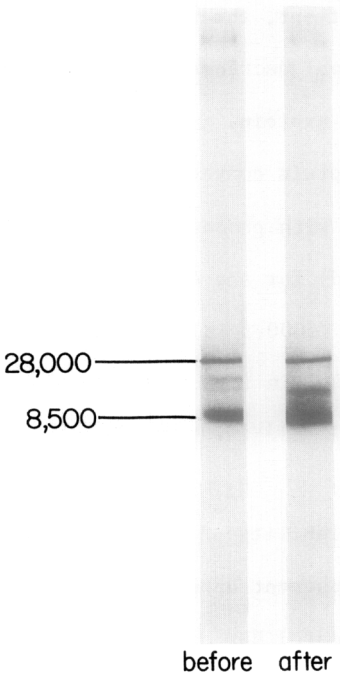

Fig. 1 The analyses of HDL and pronase-treated HDL by SDS-PAGE. Each gel contains about 20 μg of protein.

enzymatic hydrolysis of the "exposed" regions, and then by the isolation and characterization of "protected" fragments. For example, the lactose operator, which is protected by its repressor from the action of deoxyribonuclease, was isolated, and its sequence elucidated using this approach (36). The ribosome-binding region of bacteriophage R17 RNA in polyribosome complex, which is resistant to ribonuclease digestion, was also sequenced by this approach (37).

In order to be certain that pronase digested only the exposed regions of apoproteins in HDL, we compared the accessibility of HDL apoproteins to antibodies with their accessibility to pronase. The RIA detects only the exposed immunologically active regions of apoproteins in intact HDL, but following delipidation all of the immunological sites of apoproteins are detected (28-31). By comparing the apparent contents of apoproteins in intact and in delipidated HDL, we could estimate what proportion of the immunologically active sites of apoproteins are exposed. The proportion of apoprotein immunoreactivity destroyed by pronase was obtained by comparing the immunoreactivities of delipidated

undigested HDL with delipidated digested HDL. It turned out that all of the immunologically active sites of apoprotein in intact HDL were destroyed by pronase. The next step, the isolation of the "protected" regions of apoproteins from pronase digested lipoproteins and the identification of their linear sequences should yield interesting information on the lipid binding regions of the apoproteins.

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