Selective proteolytic digestion as a method for the modification of human HDL_3 structure

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Abstract Trypsin digestion of human high density lipoprotein (d 1.125–1.21 g/ml) on which the lysine residues have been masked with the reversible blocking group, 2,3,4,5-tetrahydrophthallic anhydride (THPA), was found to result in the fragmentation of the apoA-I component, but not the apoA-II component of this lipoprotein particle. Approximately 50–80% of the apoA-I polypeptide was found in a lipid-free fraction, while the residual apoA-I material plus the apoA-II moiety constituted a core particle that contained most of the original lipid. Immunological analysis indicated that such fragmentation did not affect the immunoreactivity of apoA-II, but that all immunoreactivity of apoA-I was lost within the first 30 min of trypsinization. By column chromatography and electron microscopy this core particle appeared identical in size with the untrypsinized THPA-modified HDL_3 material. Size analysis of the core particle peptides suggests that not all of the A-I molecules present on the HDL_3 are trypsinized to the same extent, which indicates possible nonequivalence of these peptide chains. Analysis of the amino acid composition revealed a somewhat greater proportion of hydrophobic residues in the lipid-bound fraction than in the lipid-free fraction. Analysis of tryptophan showed that almost all of this highly hydrophobic residue was found in the lipid-bound fraction; this suggests that lipid binding occurs preferentially in the more hydrophobic domains of the A-I molecule. Incubation of the core particle with intact apoA-I, obtained from either human or bovine HDL, showed that these proteins could be incorporated to regenerate an HDL_3 of selectively altered protein composition, compared to the original lipoprotein. It is concluded that some latitude is allowable in the surface/volume relationship in lipoproteins before reorganization of the particle is required; this might, for example, provide a mechanism whereby the HDL could serve in a storage role for the C apolipoproteins in plasma.—**Swaney, J. B.** Selective proteolytic digestion as a method for the modification of human HDL_3 structure. *J. Lipid Res.* 1983. **24:** 245–252.

Supplementary key words trypsinization • apoA-I • apoA-II

Human high density lipoproteins (HDL) contain two major protein constituents, termed apoA-I and apoA-II, while the HDL of many other species contain mainly the apoA-I and virtually no apoA-II. The primary and secondary structure of these proteins has been studied (1–4) but little is known of their tertiary structure, if any such exists in the HDL particle, or of the structural interdependence of these two proteins in the intact particle. Cross-linking studies have indicated, however, that these proteins reside in close proximity in the HDL (5).

One method for studying the structure of protein complexes is to perform proteolytic digestion and to determine if residual function remains with any fragments (6, 7). Camejo (8) applied this approach to the study of HDL and found that trypsinization released 70% of the Lowry-reactive material as lipid-free peptides; from this it was concluded that most of the protein was accessible to the aqueous environment. Since more recent studies have revealed the primary structure of these peptides, we decided to repeat these experiments in order to elucidate the specific domains of primary structure that possess lipid binding affinity. Tryptic cleavage of HDL_3, while desirable for its specificity, cleaves both the A-I and A-II proteins into many peptides that are generally too small to allow retention of lipid-binding properties. In order to restrict the number of cleavages, and thereby increase the average size of the fragments, we decided to reversibly mask the lysine residues. This approach had an additional and highly desirable advantage: since apoA-II contains no arginine residues, masking of lysine residues was found to protect this protein from tryptic cleavage. We reasoned that if apoA-II were left intact, this would significantly simplify the characterization of the products resulting from proteolysis. This report describes the application of this approach and physical characterization of the products, as well as our attempts to replace the degraded apolipoprotein with either homologous or heterologous intact apoA-I.

MATERIALS AND METHODS

Materials

HDL_3 (d 1.125–1.21 g/ml) was isolated from fresh human plasma by centrifugation and dialyzed against

Abbreviations: HDL, high density lipoproteins; THPA, 2,3,4,5-tetrahydrophthallic anhydride; SDS, sodium dodecyl sulfate; TPCK, tosyl phenylalanyl chloromethyl ketone.

0.5 M sodium phosphate, pH 8.4, prior to use. Bovine apoA-I was isolated from apoHDL by gel filtration, as reported previously (9). The concentration of bovine apoA-I was determined by absorbance at 280 nm using $E_{1\ cm}^{0.1\%} = 1.25$ (10). TPCK-trypsin and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp, NJ. 2,3,4,5-Tetrahydrophthallic anhydride (THPA) was purchased from Aldrich Chemical Co., Kankakee, IL. Fluorescamine™ was purchased from Pierce Chemical Co., Rockville, IL. [^{14}C]Acetic anhydride was obtained from Amersham, Chicago, IL and was used to radiolabel bovine and human apoA-I as previously described (11).

Proteolysis

Primary amino groups on HDL_3 were blocked by reaction with THPA at alkaline pH, as described by Gibbons and Schachmann (12). The completeness of masking of these groups was monitored by reaction with Fluorescamine™, as described by Böhlen et al. (13). Trypsinization was initiated by the addition of 1% TPCK-trypsin (w/w), with a second addition after 5 hr. Aliquots were removed at various times and the digestion was halted by the addition of soybean trypsin inhibitor (1.0 g/2.0 g trypsin) and by freezing. Molecular weight analysis of these fractions was performed by SDS gel electrophoresis on acrylamide gradient slab gels as previously reported (14). In some cases, loss of antigenicity was evaluated by lowering the pH to 6, to remove the THPA blocking group (12), and performing immunodiffusion (15).

Gel filtration

Separation of the core particle from peptide fragments was effected by chromatography on a 1.6 × 95 cm column of Agarose A-5m (Bio-Rad Laboratories, Rockville Center, NY). The column effluent was monitored at 280 nm with an UA-5 absorbance monitor (Instrument Specialties Co., Lincoln, NE). The phospholipid content of collected fractions was determined by the method of Stewart (16). The core particle was identified as Peak II and the lipid-free peptides were contained in Peak III. The cholesterol content of these pools was determined enzymatically using the method of Allain et al. (17). Protein content of these fractions was determined by the method of Lowry et al. (18).

Peptide analysis

Molecular weight analysis of the peptides in the Peak II material was performed using the SDS-urea gel technique of Swank and Munkres (19). Samples to be analyzed were first delipidated with ethanol–ether 3:1 to eliminate zone distortion due to lipid.

Electron microscopy

Preparations of HDL_3 and the core particle as isolated by gel filtration were negatively stained with 2% sodium phosphotungstate, pH 7.2, on carbon- and parlodion-coated grids. Electron micrographs were obtained with a JEOL 100 CX electron microscope.

Amino acid analysis

Aliquots for analysis were dried in a vacuum desiccator hydrolyzed with 5.6 N HCL and quantitated on the Beckman 120C amino acid analyzer. In addition, tryptophan was determined by the method of Gaitonde and Dovey (20) in selected fractions.

Reconstitution of the core particle with apoA-I

Trypsinized HDL_3 or Peak II material obtained by Agarose A-5m chromatography was incubated at 37°C for 6 hr with ^{14}C-labeled bovine or human apoA-I. Following a 6-hr incubation at 37°C, this material was reapplied to the agarose A-5m column and aliquots from collected fractions were counted for radioactivity. The peptide composition of the fraction that eluted in the region of the core particle was analyzed by SDS gel electrophoresis.

Other methods

Secondary structure of native and unmodified HDL_3 was determined using a Cary model 60 spectropolarimeter with a circular dichroism attachment which was calibrated with (+)-camphor-sulfonic acid. Ellipticities were calculated using a mean residue weight of 115. Fluorescence was measured using a Perkin-Elmer 650-10S fluorescence spectrophotometer.

RESULTS

Time course of proteolysis

HDL_3 was reacted with THPA to block all exposed primary amino groups on the particle. The completeness of lysine modification was evaluated by the use of Fluorescamine™ (13), after which trypsinization was begun; in all cases the residual fluorescence after modification was less than 95% of the unmodified sample. In those cases where more residual fluorescence was observed, degradation of apoA-II was noted by the absence of a 17,000 molecular weight band in SDS gel electrophoresis. The time course was followed by stopping the reaction with soybean trypsin inhibitor at various times and freezing aliquots for quantitation of amino groups generated by proteolysis (13), for peptide size distribution by SDS gel electrophoresis, and for re-

sidual antigenicity by Ouchterlony plates (15). It was found, in agreement with Camejo (8), that liberation of amino groups reached a plateau at 18–24 hr. However, SDS gel electrophoresis (**Fig. 1**) indicates that no intact chains of apoA-I remain after 240 min of digestion. In some experiments it was found that no intact A-I was evident after as little as 30 min. Immunodiffusion analysis showed that antigenic reactivity of the trypsinized HDL_3 with anti-apoA-I appears to be lost within 30–240 min as well, but that apoA-II appears to retain its immunoreactivity even after 21 hr of trypsinization. Immunodiffusion experiments performed with and without removal of the THPA group showed similar precipitin band formation with each form of the protein.

Gel filtration

Preparative scale samples digested with trypsin for 24 hr were applied to a 2.5 × 95 cm column of agarose A-5m in order to separate the peptide fragments (Peak III) from the residual lipoprotein particle (Peak II), which we have termed the core particle (**Fig. 2**). The core particle is seen to elute from this column very similarly to the original HDL_3 material. Over seven separate proteolysis experiments the percentage of protein in the Peak II fraction, as determined by the method of Lowry et al. (18) and by amino acid analysis, ranged from 40–65% of the total HDL_3 protein applied to the column; in each experiment trypsinization was allowed to proceed for 21–24 hr and was presumed to be complete, based on time-course experiments described above. Assuming that approximately 75% of the HDL_3 protein is apoA-I, this implies that trypsinization releases about 45–80% of this protein.

This figure also shows the distribution of phospholipid in the eluate, and it is apparent that this lipid component is found exclusively with the core particle. Compositional analysis of the pooled fractions from A-5m chromatography yielded a value of 45–49% protein, 14–24% cholesterol, and 27–41% phospholipid in the Peak II material, as opposed to 93–95% protein, 3–5% cholesterol, and 3–4% phospholipid in the Peak III fraction. Both the original HDL_3 and the THPA-modified HDL_3 possessed a composition in the range of 57–63% protein, 20–27% phospholipid, and 9–17% cholesterol. Due to the dilute concentration of the Peak III fraction, the lipid concentrations observed may reflect mainly the lower limit of detection for these components rather than their actual presence in this fraction.

Analysis of the peptide distribution in the Peak II fraction by SDS gel electrophoresis is shown in **Fig. 3.** In addition to apoA-II, a number of peptide bands can be seen in the molecular weight range of 14,000 and below, with major bands at about 12,000, 8,000, and 6,000. Lower molecular weight bands could be present, but not visible, due to poor uptake of the Coomassie blue stain. Since at least 45% of the total A-I protein

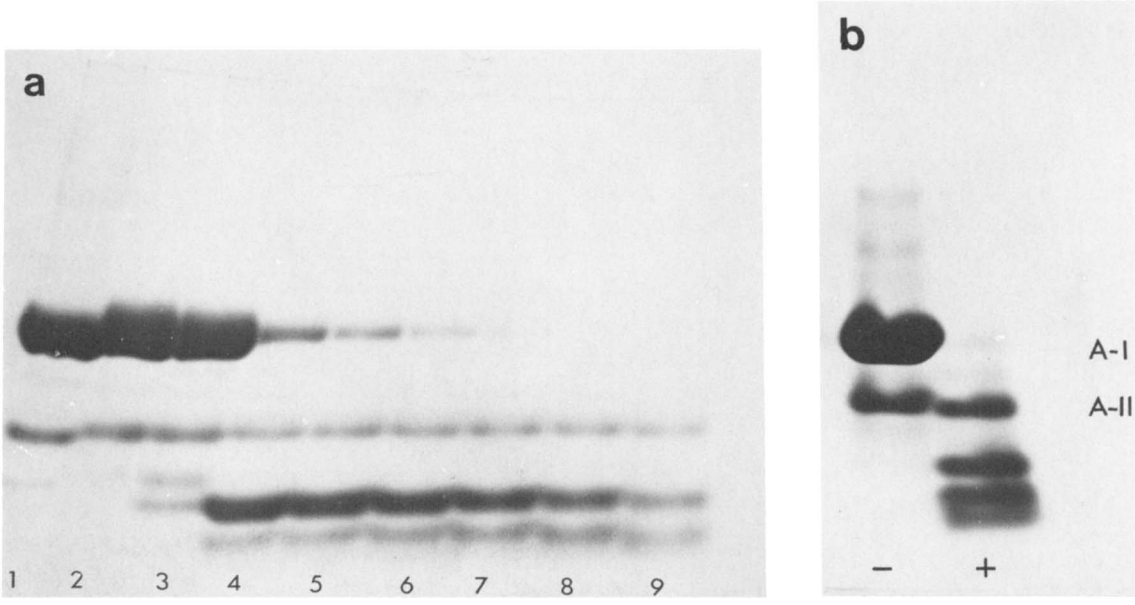

Fig. 1. Acrylamide gradient SDS slab gel electrophoresis of aliquots taken from the trypsinization of THPA-modified HDL_3. Panel a: 1, unmodified HDL_3; 2, THPA-modified HDL_3; 3–9, THPA-modified HDL_3 trypsin-treated for 0.4, 10, 20, 40, 80, 240 min, and 21 hr, respectively. After removal from the incubation, soybean trypsin inhibitor was added and the aliquot was frozen until used for electrophoresis. Panel b: THPA-HDL_3 before (−) and after (+) 18 hr of trypsin digestion; equal-sized aliquots containing 40 μg of protein were applied to each lane of this gel.

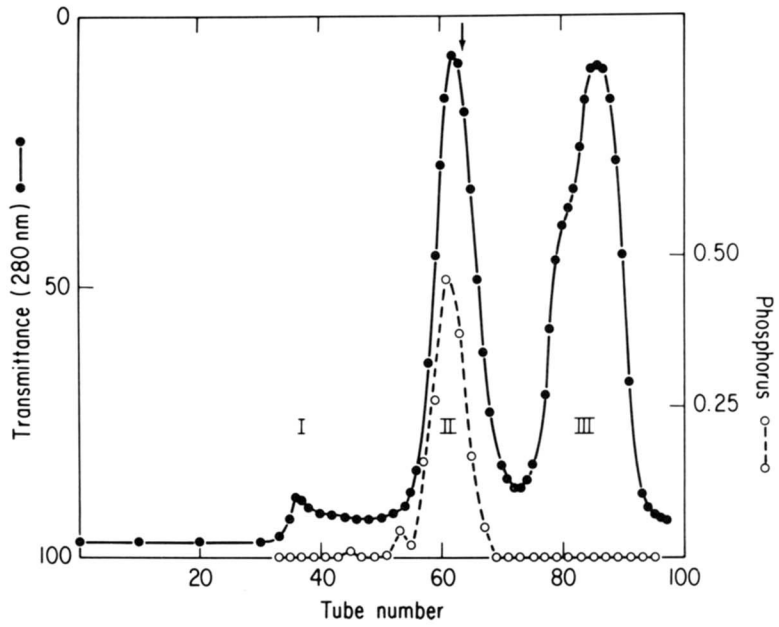

Fig. 2. Agarose A-5m column chromatography of trypsinized THPA-modified HDL_3. A sample containing 25 mg of protein was applied to a 2.5 × 95 cm column and the effluent was monitored at 280 nm (solid circles). Samples from each fraction were also analyzed for phosphorous, using the method of Stewart (16) (open circles). The elution position of native HDL_3 is indicated by the arrow. Fractions were collected every 15 min which contained 1.9 ml; chromatography was performed at room temperature.

in the HDL_3 is removed by trypsinization and recovered in Peak III, we had expected the sum of the fragments to amount to no more than 15,000 daltons. The finding that the major fragments sum to a significantly greater value than 15,000 implies that heterogeneity of A-I fragmentation has occurred, probably resulting from nonequivalence of the multiple copies of this protein on the HDL particle. Identification of the 17,000 molecular weight band as apoA-II, rather than as a fragment of apoA-I, is based upon the elimination of this band upon reduction or upon incomplete blockage of the lysine side chains with THPA prior to trypsinization.

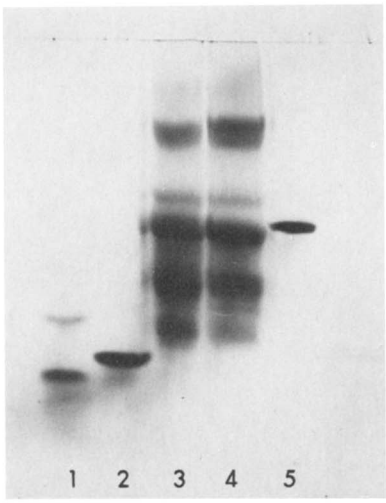

Fig. 3. Polyacrylamide gel electrophoresis on 12% acrylamide gels containing SDS and urea of insulin with (position 1) and without (position 2) reduction with dithiothreitol, two preparations of Peak II material from conventionally trypsinized material (positions 3 and 4, 40 mg each), and lysozyme (position 5). Lysozyme has a molecular weight of 14,400, unreduced insulin, 5700; reduced insulin, 2300 and 3480.

Electron microscopy

Since a significant portion of the protein surface is removed from the HDL_3 particle by trypsinization, we considered the possibility that fusion of particles might occur. Gel filtration experiments were not consistent with this possibility, but the gel filtration technique might not have sufficient resolving power to discriminate between fused and unfused particles, since even HDL_2 and HDL_3 are not separated by this procedure. When electron microscopy was employed (**Fig. 4**), however, it was found that the Peak II material from the A-5m column (core particle) closely resembled the original HDL_3 material, although in occasional fields small aggregates of particles were visualized. It was found that the diameter of the THPA-modified material was somewhat greater (101 ± 18 Å) than the native HDL_3 (85 ± 5 Å), but that trypsinization had no significant effect on the diameter of the modified particle (104 ± 15 Å). The difference in size between the native and THPA-modified form is presumed to result from artifactual

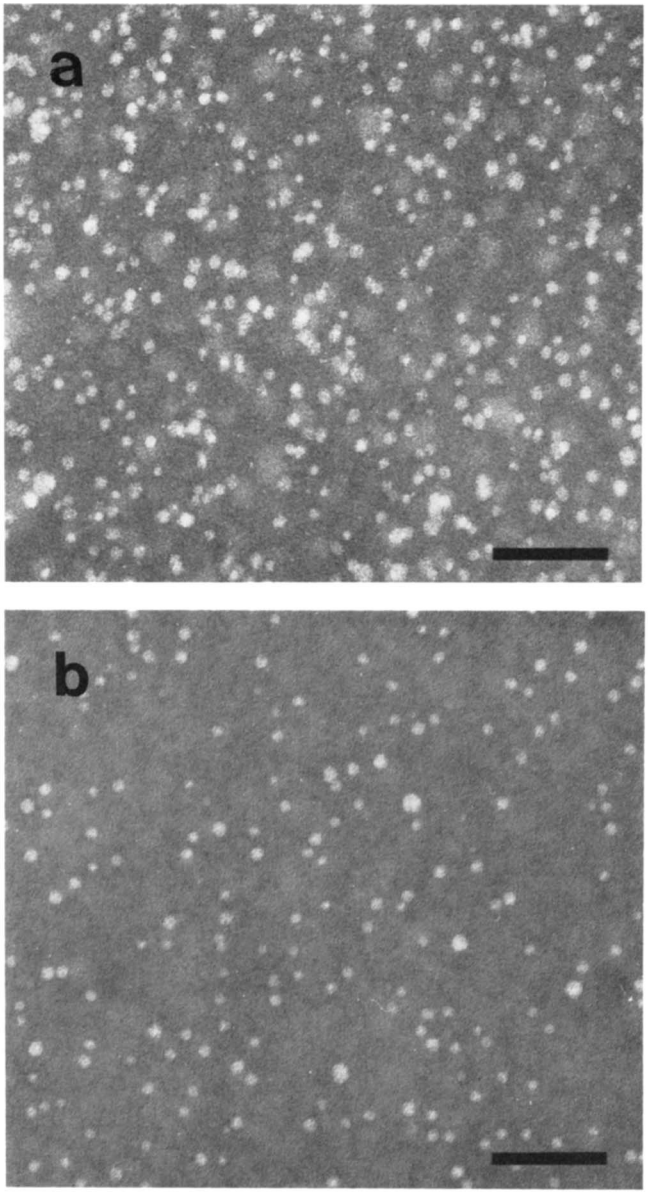

Fig. 4. Negative stain electron microscopy of THPA-treated HDL_3 (a) and Peak II material (b) isolated by gel filtration. Magnification is 150,000 diameters. The bar in the lower right corresponds to 1000 angstroms.

negative staining due to surface charge differences or to extension of these bulky groups into the aqueous environment.

Circular dichroism

Circular dichroism was performed on the original material as well as on the Peak II and Peak III material from the A-5m gel filtration procedure. Mean residue ellipticity values (deg cm^2/dmol) at 220 nm were as follows: native HDL_3, −19,500; THPA-modified HDL_3, −16,900; Peak II, −18,600. It would thus appear that the domains of A-I that remain bound to lipid (Peak II) are not preferentially enriched in helical structure, relative to the starting material. Peak III material gave baseline values of ellipticity at 220 nm, indicating a lack of significant secondary structure in the small, lipid-free peptides.

Amino acid analysis

Analysis of the amino acid composition (**Table 1**) of Peak II and Peak III material (lipid-bound and lipid-free, respectively) did not reveal substantial differences between these fractions, but did indicate a slight enrichment of hydrophobic residues (Phe, Tyr, Val) in the Peak II fraction and a relative depletion of certain hydrophilic residues (Arg, Asp, Glu). The small amounts of "C" peptides which were found in HDL_3 probably contributed to both fractions, but are believed not to have added a significant proportion of the amino acids that were measured.

Furthermore, quantitation of tryptophan by the technique of Gaitonde and Dovey (20) showed that 82–90% of the tryptophan from apoA-I was associated with the core particle. Thus, there does seem to be a relationship between hydrophobicity of the residue side chains and their lipid binding properties.

Reconstitution of the core particle with apoA-I

Since the core particle gave no indication of fusion by electron microscopy despite the loss of 40% or more of the surface protein, the possibility that intact

TABLE 1. Amino acid analysis of Peak II and Peak III fractions obtained by agarose A-5m column chromatography of trypsinized HDL_3 (25.2 mg applied)

	Total mg Recovered ± SEM[a]	
Amino Acid	Peak II[b]	Peak III[c]
Lysine	1.75 ± 0.06 (13.7)	0.79 ± 0.07 (6.2)
Histidine	0.04 ± 0.01 (0.29)	0.12 ± 0.03 (0.87)
Arginine	0.39 ± 0.03 (2.50)	0.46 ± 0.08 (2.9)
Aspartic acid	1.06 ± 0.04 (9.2)	0.71 ± 0.03 (6.2)
Threonine	0.78 ± 0.03 (7.7)	0.23 ± 0.01 (2.3)
Serine	0.83 ± 0.01 (9.5)	0.27 ± 0.01 (3.1)
Glutamic acid	3.01 ± 0.07 (23.3)	1.77 ± 0.03 (13.7)
Proline	0.39 ± 0.06 (4.0)	0.17 ± 0.01 (1.8)
Glycine	0.36 ± 0.03 (6.3)	0.19 ± 0.01 (3.3)
Alanine	0.59 ± 0.01 (8.3)	0.33 ± 0.02 (4.6)
Valine	0.95 ± 0.04 (9.6)	0.28 ± 0.01 (2.8)
Methionine	0.08 ± 0.01 (0.6)	0.10 ± 0.01 (0.8)
Isoleucine	0.17 ± 0.01 (1.5)	0.07 ± 0.01 (0.7)
Leucine	1.83 ± 0.06 (16.2)	0.97 ± 0.07 (8.6)
Tyrosine	0.67 ± 0.03 (4.1)	0.21 ± 0.01 (1.3)
Phenylalanine	0.80 ± 0.01 (5.4)	0.18 ± 0.01 (1.2)
Tryptophan[d]	0.27 (1.4)	0.06 (0.3)

[a] Number in parentheses corresponds to μmoles of each amino acid.
[b] Average of 15 determinations.
[c] Average of eight determinations.
[d] Average of two determinations.

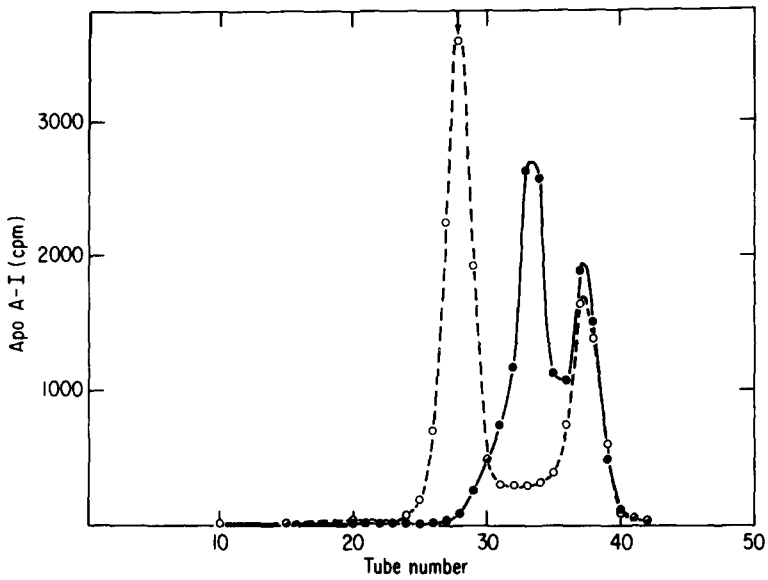

Fig. 5. Agarose A-5m column chromatography of ^{14}C-labeled bovine apoA-I alone (● —— ●) or ^{14}C-labeled bovine apoA-I incubated with Peak II material (○ – – – ○); equal amounts of the A-I protein were applied in each case (2 mg) to a 1.6 × 90 cm column. The elution of the Peak II material alone is indicated by the arrow. The last peak coincides with the elution position of salt and appears to be [^{14}C]acetic acid which was incompletely removed from the protein. ApoA-I alone was found to elute at tube 33. The column was eluted with 0.045 M NaCl, 0.030 M $NaHCO_3$, pH 8.5; fractions were collected every 20 min and contained 4.5 ml.

apoA-I could be added back to the core particle was explored. In one experiment 0.50 mg of Peak II material was incubated with 0.42 mg of ^{14}C-labeled bovine apoA-I and applied to an agarose A-5m column (**Fig. 5**). The apoA-I material was found to be quantitatively taken up by the core particle (9,700 cpm was lost from the apoA-I region and 10,200 cpm appeared in the Peak II region). SDS gel electrophoresis showed the material that eluted in the Peak II region possessed both apoA-I and apoA-II; the relative content of small peptides could not be evaluated by this procedure. When 0.50 mg of the Peak II material was incubated with 0.49 mg of ^{14}C-labeled human apoA-I, 0.34 mg of the human A-I protein (70%) was taken up by the core particle. This indicates that the amount of apoA-I that can be taken up by the core particle is limited to approximately the amount of material lost from the original HDL_3, and that the core particle may have somewhat less affinity for human apoA-I than for the bovine protein.

DISCUSSION

The human HDL particle presented a unique opportunity to perform selective enzymatic degradation of only one of the two major protein constituents of this lipoprotein while leaving the other intact. This situation arises from the fortuitous absence of arginine residues in the apoA-II protein, allowing us to protect this protein from tryptic cleavage by a reversible modification of the lysine residues. It was found that 45–80% of the apoA-I protein was released as lipid-free peptides, and that virtually all of the original lipid remained in a core particle with the intact apoA-II and residual apoA-I fragments.

Concurrent with degradation of the A-I polypeptide chain is a loss of reactivity with antibodies directed against this protein, as measured by double immunodiffusion, with a complete loss of antigenic activity after 21 hr of tryptic digestion. This suggests that the antigenic sites on this protein are in the domains that are cleaved by trypsin or are dependent upon residues in these areas to produce a three-dimensional antigenic site.

Analysis of the core particle (Peak II) shows that there is a higher proportion of hydrophobic to hydrophilic residues in this peak, compared with the lipid-free peptides (Peak III), and that the core particle is especially enriched in tryptophan. This suggests that there are specific domains within A-I that possess high lipid affinity because of their greater hydrophobic character. Although lipid binding is thought to occur at regions of alpha helix (21), the core particle did not show a dramatic increase in helix, perhaps because of the loss of helical structure near the points of cleavage. Considerable efforts were made to purify individual peptides from the core particle for further characterization, but these efforts were unsuccessful because of extensive ag-

gregation of the peptides following removal of lipid. From the position of the arginine residues in the amino acid sequence of A-I, we expected the apoA-I to be cleaved into a maximum of 17 peptides ranging in size from 200 to 4000 daltons. However, Peak III yielded a complex mixture of at least 25 peptides and Peak II was found by SDS gel electrophoresis to contain several more (Fig. 3). Especially surprising was the fact that the major peptides in Peak II were not only significantly larger than predicted, indicating protection of multiple bonds by lipid, but also that the sum of these fragments accounted for a higher proportion of the A-I molecule than we would logically expect, based on the amount of A-I remaining in the Peak II fraction. These complications point to the probability that the multiple A-I chains on the HDL_3 particle are not digested equally by trypsin, most likely because of nonequivalence in environment of the A-I chains. Such nonequivalence is not altogether unexpected, since it has been reported that some, but not all, A-I chains may be removed from HDL by low concentrations of guanidine hydrochloride (22).

One important principle which has recently been recognized is that metabolically-induced alterations in the ratio of surface-to-core lipoprotein components provides a mechanism for restructuring lipoproteins and creating new ones (23). Thus, lipase degradation of triglyceride-rich lipoproteins is thought to result in the loss of excess surface components that are recovered in the HDL. The question arises whether a loss of surface components, as with tryptic degradation, would also cause a restructuring of the lipoprotein particle due to a surface/volume imbalance.

Our results from gel filtration (Fig. 2) and electron microscopy (Fig. 4) suggest that this need not be the case, since a significant amount of protein was lost from the surface without the occurrence of a noticeable amount of fusion. We believe, therefore, that there may be considerable latitude allowed in the surface-to-volume relationship, which may be due to lateral compressibility of the phospholipid components (24). This property might, for example, explain the apparent ability of HDL to take up or release C apolipoproteins during the course of lipoprotein metabolism in plasma. Similar results were reported by Ritter and Scanu (25), who found that incubation of HDL_3 with blood leukocytes results in the proteolytic digestion of A-II exclusively, but that the resultant particle was indistinguishable in size from the starting material; however, less protein was lost from the HDL particle by their procedure than by ours. Thus, our studies on the selective proteolysis of apoA-I in HDL provide an interesting counterpoint to the studies of Ritter and Scanu (25), where A-II is selectively degraded.

Another means of modulating the composition of HDL, as reported by Lagocki and Scanu (26), involves the addition of human apoA-II to canine HDL (which contains primarily apoA-I) to generate hybrid molecules in which the A-II replaces some or all of the A-I molecules. Taken together with our results, these studies suggest that the structure of human HDL is not dictated to any significant extent by specific protein-protein interactions between A-I and A-II, although these may occur.

The role of A-II in the regulation of lecithin:cholesterol acyl transferase (LCAT) activity remains obscure, although it has been reported that this protein inhibits the activation of the LCAT enzyme by A-I (27). Since A-II displaces A-I from such complexes (26), this enzyme inhibition may merely reflect competition of these two apolipoproteins for the water:lipid interface. One promising means for studying the individual roles of these apolipoproteins in the LCAT reaction could be to utilize our A-II-containing core particle, reconstituted with various amounts of A-I (Fig. 5), in the enzymatic reaction. Thus, the ability to manipulate the structure of plasma lipoproteins, when coupled with metabolic approaches, may aid in understanding the roles of the A-I and A-II proteins, both in maintaining the structural integrity of the human HDL and in determining the metabolic fate of this particle.

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