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THE CYANOGEN BROMIDE PEPTIDES OF THE APOPROTEIN OF LOW DENSITY LIPOPROTEIN (ApoB): ITS MOLECULAR WEIGHT FROM A CHEMICAL VIEW

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SUMMARY: After >95% cleavage of the apoprotein (apoB) of the low density lipoproteins with cyanogen bromide, the peptides produced are shown to be extensively aggregated in sodium dodecyl sulfate. Both high temperature and increased concentration (5%) of the detergent are necessary to shift the aggregated peptides from high molecular weight (>25,000) to lower molecular weight aggregates as seen on sodium dodecyl sulfate polyacrylamide gel electrophoresis. End group analyses of the cyanogen bromide digestion by automated sequencer techniques indicate the presence of five (5) methionines. With a known methionine content of 16 moles/100,000 g protein, the molecular weight of the apoprotein must be approximately 30,000.

The structure and function of plasma low density lipoproteins (1-3) (LDL)[3] continue to receive wide attention due to their role in the regulation of the cellular metabolism of cholesterol (4) and the direct relationship between LDL plasma cholesterol levels and the incidence of increased risk for coronary heart disease (5).

Although extensive metabolic and physical studies of LDL have been carried out, there is lack of agreement concerning the molecular weight of the apoprotein. Reported molecular weights have ranged from 8,000 to 255,000 (2). The apoprotein is insoluble in aqueous buffers and requires detergents (6,7) (i.e. sodium decyl sulfate, sodium deoxycholate), chemical modification (8) or other proteins (9) for solubilization. Even though the protein is

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[3]Abbreviations used: LDL, low density lipoproteins; apoB, the major apoprotein of low density lipoproteins; SDS, sodium dodecyl sulfate; CNBr, cyanogen bromide; Met, methionine; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoins; TMU, tetramethylurea; GLC, gas-liquid chromatography.

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soluble[4] in denaturants, both guanidine hydrochloride (10) and SDS (11) appear to promote the aggregation of the apoprotein.

In an attempt to characterize the apoprotein, we have extensively tested various solvents and physical techniques. Using a chemical approach (12) to the molecular weight enigma of apoB, in this manuscript we report our preliminary findings on the results obtained by CNBr digestion.

MATERIALS AND METHODS

Low density lipoproteins were isolated from a patient with type IIa hyperlipoproteinemia in the density range 1.025-1.050 g/ml by sequential ultracentrifugation. The apoprotein was delipidated by extraction with ether:ethanol (3:1, v/v) according to the procedures of Brown (13). The TMU soluble protein was less than 1% of the total apoprotein. By overloading SDS and urea PAGE gels, we were able to show that the small contaminant consisted of apoC proteins. Iodine-125 labelled apoB was prepared essentially as previously described by Mao et al. for apoA-II (14).

Cyanogen bromide digestions were carried out in 88% formic acid at room temperature for 50 hours. The protein concentration was 10 mg/ml and the ratio of CNBr to Met was 500:1. Digestions were quenched by dilution with 10 volumes of water, followed by lyophilization. The lyophilized material was redissolved at 10 mg/ml and pH 11.0 with NH_4OH. Digestions were fractionated on G-75 Sephadex (2.5 x 190 cm) in 0.1 M NH_4HCO_3 and monitored at 226 nm.

SDS-PAGE was essentially the method of Weber and Osborn (15). Densitometric scanning of gels was carried out on a Beckman Acta V at 570 nm. The amount of material present was estimated by the area under the traced peaks. This method was verified by slicing the gels and extracting the peptides followed by amino acid analyses to determine the absolute amount in each band. For ^{125}I-radiolabelled scans of the SDS gels, 1 mm slices were obtained and counted in a Packard γ-counter. Better than 90% recovery of the applied materials was achieved in each of the above techniques.

High temperature incubations of peptides in SDS were accomplished in sealed tubes which were flushed with prepurified nitrogen and then evacuated to about 50 torr. Production of new NH_2-terminal groups was monitored by fluorescamine analysis (16).

Automated runs were performed with a Beckman Sequencer Model 890B (updated) with the addition of either sequenal grade 0.5% SDS (17) or N-terminally blocked apocytochrome C, prepared by the method of Bonicel et al. (18). Thiazolinone or phenylthiohydantoin (PTH) derivatives were identified by back hydrolysis with 55% HI for 24 hours at 115° C and/or by GLC (19,20).

RESULTS AND DISCUSSION

Cyanogen bromide cleavage of apoB proceeds to better than 95% as determined by loss of Met and the production of homoserine and homoserine lactone. The reaction mixture is soluble at pH 11.0 in NH_4OH at concentrations up to

[4]Soluble is a relative term in working with apoB solutions and is used here to refer to materials which do not sediment under low speed centrifugation (5000 rpm for 30 minutes).

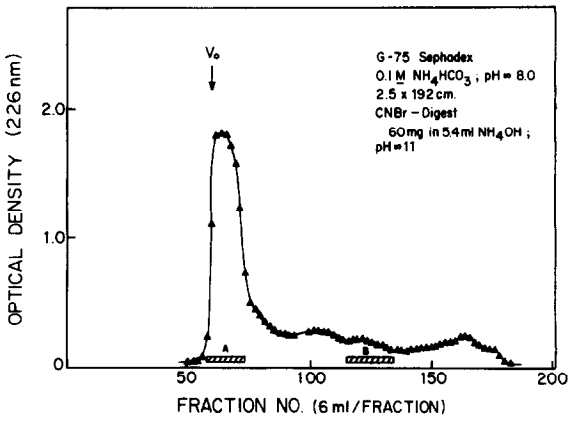

Figure 1: Elution profile of CNBr digestion on G-75 Sephadex in 0.1 M NH_4HCO_3 (pH 8.0).

approximately 10 mg/ml. The elution profile (Fig. 1) of the CNBr-peptides on Sephadex G-75 shows a major peak in the void volume with smaller peaks present throughout the included volume. Despite absorbance seen after fraction 150, these fractions contained no peptide material as determined by pooling this entire area, lyophilizing, and analyzing for amino acid content. The absorbance was probably due to excess CNBr and/or formic acid. SDS-PAGE was used to analyze the fractions isolated from the Sephadex G-75 column. The peptide pattern for both the whole apoB cyanogen bromide digestion as well as the void volume peak (area A) from the G-75 column shows 10 bands ranging from 99,000 to about 7,000 molecular weight (Fig. 2A). The 7,000 molecular weight CNBr peptide (Fig. 2B) seen in the void volume is also found in the included volume (area marked B on Fig. 1). This finding indicates that the CNBr peptides of apoB are highly aggregated. The gel patterns of other included areas of the G-75 column (not shown) also show SDS-PAGE banding patterns which contained similar, but with lesser amounts, of the high molecular aggregates. The use of denaturants (i.e. 6 M urea, 6 M Gdn•HCl, etc.) or detergents (1% SDS, sodium deoxycholate, Triton X-100, etc.) did not change the basic elution profiles of the filtration chromatography.

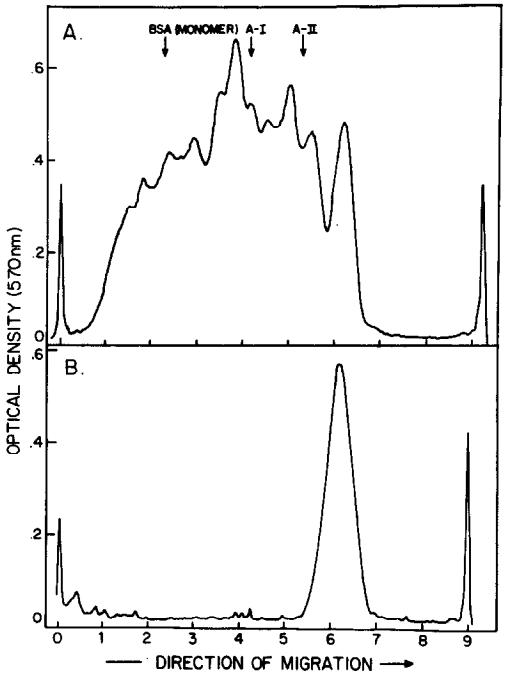

Figure 2: Densitometric tracings at 570 nm of 7.5% SDS-acrylamide gels of (A) the void volume material pooled from area A, Figure 1 and (B) included material pooled from area B, Figure 1.

In an attempt to quantitate the amount of material in each area of the gel bands, the gel was cut into corresponding pieces. The peptide material was extracted from the gel slice into SDS and hydrolyzed to determine the total amino acids present in each area. From the peptide distribution (Fig. 3), it can be estimated that the area corresponding to the 7,000 MW species is about 18% of the total material placed on the gels.

Since we found from the G-75 Sephadex chromatograph and SDS-PAGE analyses that the 7,000 MW CNBr-peptide was aggregated, we suspected that all of the CNBr-peptides were aggregated. If this were the case then there were even fewer CNBr peptides than initially indicated by SDS-PAGE. Two experiments were performed which support this assumption.

Densitometric traces of a CNBr reaction mixture before and after heating with 5% SDS at 110° in an evacuated sealed tube are shown in Fig. 4. No new

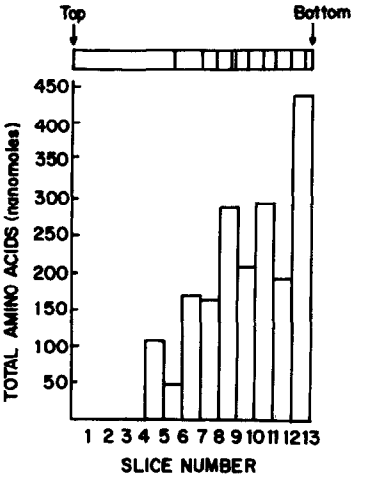

Figure 3: SDS-acrylamide gel (7.5%) of CNBr peptides. Gels were sliced as indicated, peptides extracted into SDS and the amount determined by amino acid analyses.

end terminals were produced under these conditions, as monitored by changes in fluorescamine reactivity. Under these conditions, higher molecular weight aggregates, i.e.>25,000 are converted to smaller species, <20,000 MW. In a similar experiment, ^{125}I-labelled apoB was digested with CNBr and the peptides were incubated in 5% SDS as described above. The gels were then sliced and counted. With this very sensitive measure, no high molecular weight aggregates were present (>25,000 MW) and 45% of the total ^{125}I counts were found co-migrating with the tracking dye.

These experiments suggest that after complete CNBr digestion of apoB, there remain only a few extensively aggregated CNBr peptides, the exact number of which cannot be obtained from the PAGE experiments. The number of CNBr-peptides was determined by end-group analyses in a Beckman automated sequencer in the presence of 0.5% SDS. The relative molar quantities of the end terminals (corrected for carry over) produced at each of the three steps in the sequencer are shown in Table I. PTH derivatives were determined by GLC and back hydrolysis in 55% HI to the corresponding amino acids. From

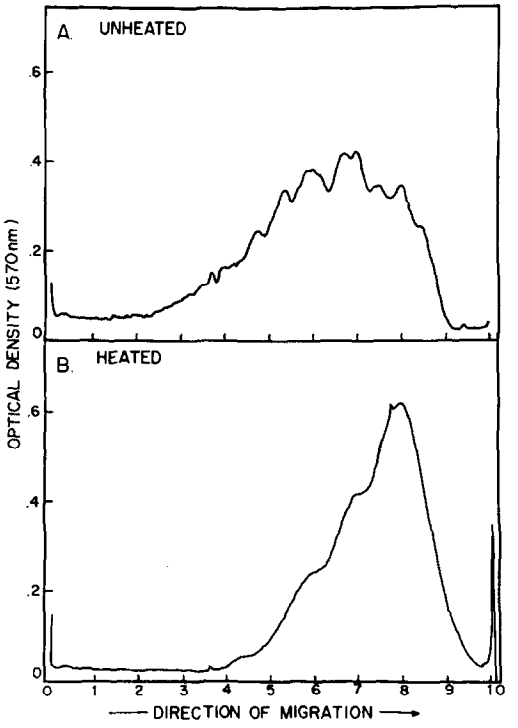

Figure 4: Densitometric tracings at 570 nm of SDS-acrylamide gels of (A) unheated CNBr peptides incubated in 5% SDS and (B) CNBr peptides heated at 110° C in 5% SDS.

these results it can be determined that six CNBr peptides have been produced which correspond to five Met residues in the original protein. This experiment also verifies our initial observation that the CNBr peptides are aggregated extensively even in the presence of SDS.

Based on these findings, we conclude that the apoprotein is aggregated in denaturants such as 6 M to 8 M guanidine hydrochloride, in which a molecular weight of 255,000 has been reported (10) and even more highly aggregated in the SDS micelle (11). Furthermore, we have established from the CNBr fragmentation that the molecular weight of apoB must be in the range of 25,000 to 35,000.

TABLE I

CNBr N-Terminals After N Steps on Sequencer[a,b]

Step (N=)	1	2	3
Asp	0.99	.47	1.0
Glu	1.32	0.62 (1)	1.2
Gly	1.09	.13	.7
"Ala"[c]	0.90	2.6 (3)	
Leu	0.71	1.7	1.1
Lys	0.99	.07	1.0
Val	--	--	--
Phe	--	--	1.0
Total Residues	6	6	6

[a]Carried out in presence of 0.5% SDS in spinning cup.
[b]Relative molar amounts from back hydrolysis with 55% HI.
[c]Serine not distinguishable from alanine in back hydrolysis.

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