# ISOLATION AND CHARACTERIZATION OF THE CYANOGEN BROMIDE FRAGMENTS FROM APOLIPOPROTEIN A-II OF *MACACUS RHESUS* SERUM HIGH DENSITY LIPOPROTEINS: COMPARISON WITH THE HUMAN PRODUCT

Celina EDELSTEIN, Claudia NOYES and Angelo M. SCANU

*Departments of Medicine and Biochemistry, Pritzker School Medicine, University of Chicago, and The Franklin McLean Memorial Research Institute*, Chicago, Illinois 60637, USA*

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## 1. Introduction

We have previously shown that the serum HDL** of *Macacus rhesus*, when compared to human serum HDL, exhibits similar, though not identical chemical and physical properties [1, 2]. Of particular structural relevance was the observation that apo A-II, one of the two major polypeptides of HDL, is present in the rhesus as a monomer, contrary to the dimeric form occurring in man [3, 6]. This difference was found to be related to the replacement in the rhesus of cysteine by serine in position 6 from the $NH_2$-terminus [3, 4]. Amino acid data of apo-II indicated additional differences in the two animal species [3]. Based on this information, and on the potential significance of such studies from the evolutionary standpoint, an investigation was undertaken on the definition of the primary structure of rhesus apo A-II. We have isolated and characterized the cyanogen bromide fragments of both rhesus and human apo A-II. In this communication we present the results of such studies and compare them with information presented in the literature [6, 7].

* Operated by the University of Chicago for the U.S. Atomic Energy Commission.

** Abbreviations used: HDL, high density lipoproteins of *d* 1.063–1.21 g/ml; apo HDL, delipidated apoprotein from HDL; apo-II, one of the two major apoproteins of HDL – equivalent to fraction IV or apo LP Gln-II (12); CNBr, cyanogen bromide; PCA, pyrrolidone carboxylic acid; PCAse, pyrrolidone carboxylic acid peptidase; SCMC, S-carboxymethyl-cysteine.

## 2. Materials and methods

### 2.1. *Source and isolation of apo A-II*

Blood was obtained by plasmapheresis from a single male monkey (*Macacus rhesus*) maintained on a Purina Primate Chow low-fat diet. The serum HDL was isolated by ultracentrifugal flotation between solvent densities of 1.063 and 1.21 g/ml and tested for contamination by agarose gel electrophoresis and by immunodiffusion techniques [2]. The apoprotein apo HDL was obtained by delipidation [8] and then fractionated by DEAE-ion exchange column chromatography as previously described [3]. By such a procedure apo A-II was obtained in pure form and assessed by polyacrylamide gel electrophoresis, by its reactivity against monospecific antisera and by amino acid composition which was identical to that previously reported [3]. The isolation of apo A-II from human donors was carried out as described before [9].

### 2.2. *CNBr cleavage*

Cleavage at the methionyl residue of apo A-II was performed in 70% formic acid at room temperature. A 500-fold molar excess of CNBr to methonine was added, and the reaction was allowed to proceed in a sealed vessel under nitrogen for 48 hr [7]. Following incubation, the sample was diluted 10-fold with distilled water and the excess reagents were removed by lyophilization. The percentage of methionine that remained uncleaved never exceeded 20%, as checked by amino acid analysis.

### 2.3. *Chromatographic separation of CNBr fragments*

After treatment with CNBr, apo A-II was separated by gel filtration on a column (1.5 × 200 cm) of Sephadex G-50 (superfine; Pharmacia Fine Chemicals, Piscataway, N.J.). The equilibrating and eluting buffer was 0.05 M Tris–HCl-8 M urea, pH 8.2, pumped ascendingly at 6 ml/hr in a constant-temperature room kept at 8°C. The eluate was continuously monitored by absorbance at 280 nm on an ISCO optical unit (Instrumentation Specialities Co., Lincoln, Neb.). The eluted peptide fractions were desalted on a column (2.5 × 45 cm) of Sephadex G-10 equilibrated in 0.05 M *N*-ethyl morpholine–acetate buffer, pH 8.3. Purity of the isolated fractions was determined by amino acid analysis, polyacrylamide gel electrophoresis, and COOH- and $NH_2$-terminal analysis. These procedures were described previously [3]. The isolated peptides were lyophilized and stored at −20°C.

### 2.4. *Other procedures*

PCAse, kindly provided by Dr. R.E. Fellows of Duke University, was used to unblock amino terminus of one of the CNBr fragments [3]. The released PCA was identified as its silylated derivative by gas–liquid chromatography as previously described [3]. Molecular weights were estimated from amino acid analysis. Protein concentrations were determined by the Lowry procedure [10] and by amino acid analysis. In the case of human apo A-II, reduction and alkylation [5] were carried out after cleavage with CNBr, and the products were chromatographed as described above. Sequencing was performed on an automatic Beckman Model 890 Sequencer. The released amino acids were detected as their phenylthiohydantoin and silylated derivatives by gas–liquid chromatography (Beckman GC Model 45) and by thin layer chromatography using the solvent system of Jeppson and Sjöquist [11].

## 3. Results

### 3.1. *Macacus rhesus*

Fig. 1 shows the elution profile of CNBr-treated apo A-II from a Sephadex G-50 column in the presence of 8 M urea. Three major peaks were eluted, designated as B, C and D. A fourth minor peak, A, which was recovered in less than 1% yield, will not be considered here. Peak B was the fraction that resisted CNBr cleavage as assessed by amino acid data and $NH_2$- and COOH-terminal analyses. Peaks C and D represented the two CNBr fragments.

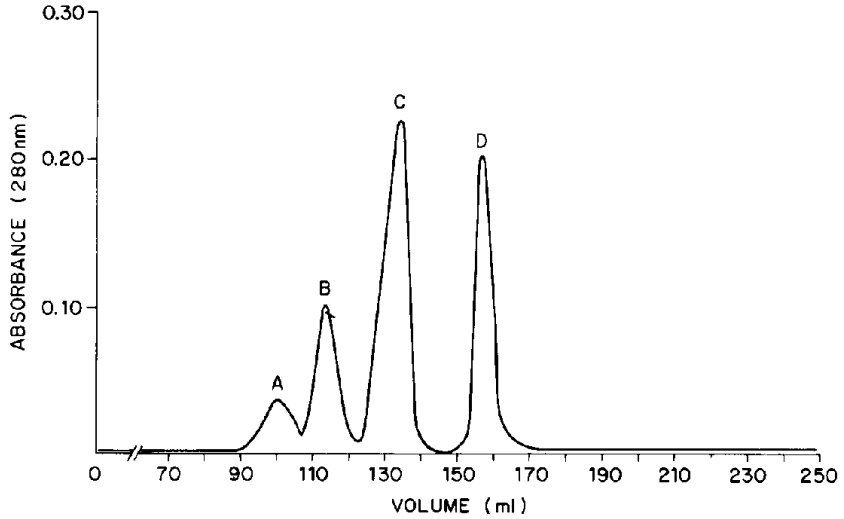


Fig. 1. Sephadex G-50 chromatography of CNBr-cleaved rhesus and reduced and alkylated human apo A-II. The conditions were: column dimensions, 1.5 × 200 cm; eluting buffer, 0.05 M Tris–HCl, 8 M urea, pH 8.0, at 8°C; amount of sample, 15–20 mg protein; flow rate, 6 ml/hr.

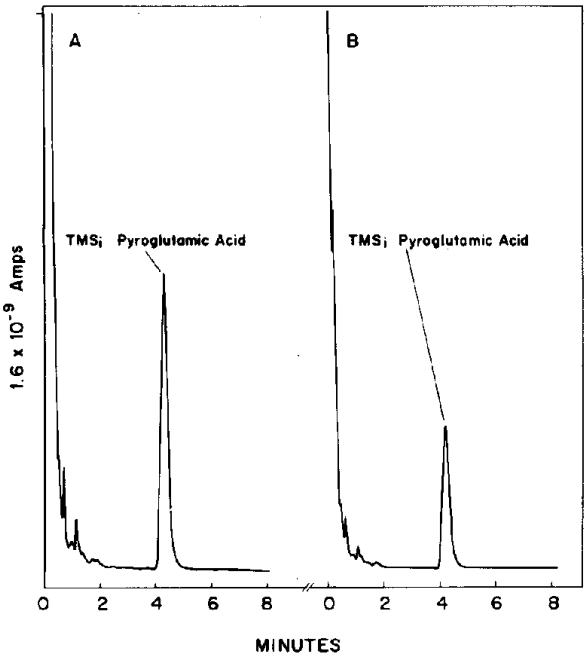


Fig. 2. Gas–liquid chromatographic tracings of trimethylsilyl pyroglutamic acid. A, Standard; B, PCAse-treated CNBr fragment D from rhesus apo A-II.

Fragment C had 52 amino acid residues, a mol.wt. of 5917, and the $NH_2$-terminal sequence: Glu–Lys–Val–Lys–Ser–Pro.... By carboxypeptidase A-digestion, the COOH-terminal residue was glutamine, which is the same as in the parent molecule [3, 4]. In addition, this fragment contained no homoserine, homoserine lactone, methionine or isoleucine, and had a single residue of arginine.

Fragment D had 26 amino acid residues and a mol.wt. of 2950. The $NH_2$-terminus was blocked. PCAse digestion released PCA, which was detected as its silylated derivative by gas–liquid chromatography (fig. 2). Edman's degradation of PCAse-treated fragment D gave the following partial sequence: PCA–Ala–Glu–Glu–Pro–Ser–Val.... Fragment D also contained 0.82 moles of homoserine and homoserine lactone per mole of peptide, indicating that it was the $NH_2$-terminal CNBr peptide.

The overall information on the two CNBr fragments, C and D, summarized in table 1, permitted thier alignments as indicated in fig. 3.

### 3.2. *Man*

The elution profile of the CNBr-treated, reduced and carboxymethylated apo A-II was the same as observed with the corresponding rhesus product (fig. 1). The two human CNBr fragments has properties identical to those reported by Lux et al. [6, 7] and differed from the rhesus fragments as summarized in table 1 and fig. 3.

## 4. Discussion

The present studies have shown that CNBr cleavage of the single methionine residue of *Macacus rhesus* apoprotein II yields two peptides which differ in size and composition and are readily separable from each other by column chromatography. In addition, methionine was found to occupy position 26 from the $NH_2$-terminus; this was also the case for the human apo A-II

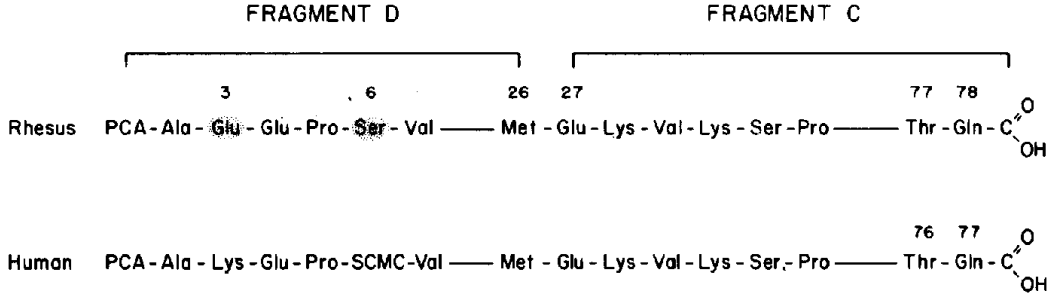


Fig. 3. Alignment of peptides of rhesus and human apo A-II derived from the results of the present study.

Table 1
Chemical properties of CNBr fragments from rhesus apo A-II and reduced and alkylated human apo A-II.

| Amino acid | Rhesus | | | | Human | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CNBr fragments | | | Apo A-II | Apo A-II | CNBr fragments | | | Apo A-II | CNBr fragments | |
| | C | D | C + D | | R & A | C | D | C + D | R & A | C-III | C-IV |
| | This study | | | | Ref. [9] | This study | | | Refs. [6, 7] | | |
| | Residues/mole* | | | | | | | | | | |
| Aspartic** | 2 | 2 | 4 | 4 | 3 | 1 | 2 | 3 | 3 | 1 | 2 |
| Threonine*** | 4 | 2 | 6 | 6 | 6 | 4 | 2 | 6 | 6 | 4 | 2 |
| Serine*** | 3 | 3 | 6 | 6 | 6 | 4 | 2 | 6 | 6 | 4 | 2 |
| Glutamic** | 11 | 6 | 17 | 17 | 16 | 11 | 5 | 16 | 16 | 11 | 5 |
| Proline | 3 | 1 | 4 | 4 | 4 | 3 | 1 | 4 | 4 | 3 | 1 |
| Glycine | 1 | 1 | 2 | 2 | 3 | 2 | 1 | 3 | 3 | 2 | 1 |
| Alanine | 5 | 1 | 6 | 6 | 5 | 4 | 1 | 5 | 5 | 4 | 1 |
| Valine | 4 | 3 | 7 | 7 | 6 | 3 | 3 | 6 | 6 | 3 | 3 |
| Methionine | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| Isoleucine | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 |
| Leucine | 6 | 2 | 8 | 8 | 8 | 6 | 2 | 8 | 8 | 6 | 2 |
| Tyrosine | 2 | 2 | 4 | 4 | 4 | 2 | 2 | 4 | 4 | 2 | 2 |
| Phenylalanine | 3 | 1 | 4 | 4 | 4 | 3 | 1 | 4 | 4 | 3 | 1 |
| Lysine | 7 | 1 | 8 | 8 | 9 | 7 | 2 | 9 | 9 | 7 | 2 |
| Arginine | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Carboxymethylcystine | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| Homoserine<br>Homoserine lactone | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| Histidine | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tryptophan | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total number of residues | 52 | 26 | 78 | 78 | 77 | 51 | 26 | 77 | 77 | 51 | 26 |
| Molecular weight† | 5917 | 2950 | 8867 | 8867 | 8690 | 5710 | 2950 | 8690 | 8690 | 5710 | 2950 |
| $NH_2$-terminal | Glu | PCA | – | PCA | PCA | Glu | PCA | – | PCA | Glu | Unre. |
| COOH-terminal | Gln | Unre. | – | Gln | Gln | Gln | Unre. | – | Gln | – | – |

* The values were adjusted to the nearest integer.
** The value represents the sum of aspartic acid and asparagine or glutamic acid and glutamine.
*** Corrected for loss during hydrolysis.
† From amino acid composition.
R & A = reduced and alkylated.

monomer, a finding corroborating previous reports [6, 7]. The apo-II monomers from rhesus and man were previously shown to contain nearly the same number of amino acid residues, 78 and 77, respectively [3]; the current study now indicates that, upon CNBr cleavage, both monomers yield a minor (26 residues) and a major (52 and 51 residues, respectively) fragment, the minor one occupying the $NH_2$-terminal region of the parent molecule and the major, the COOH-terminal portion (fig. 3). In spite of the observed structural analogies, chemical differences in apo A-II were observed between rhesus and man, and these differences were reflected in both of the CNBr fragments. The minor peptide (fragment D) was of particular interest: in the rhesus, serine occupied position 6 from the blocked $NH_2$-terminus, whereas in man this residue was replaced by cysteine (table 1 and fig. 3). This observation, which supports previous results ob-

tained with the intact rhesus apo A-II [3], accounts for the existence of apo A-II monomers in rhesus HDL and of dimers in human HDL [2, 4, 6, 7].

The chemical differences which have been observed between the apo A-II of rhesus and man (more work in this direction is in progress in the laboratory) are of relevance not only from the viewpoint of HDL structure but also from an evolutionary aspect. *Macacus rhesus* and man occupy a distinct position in the phylogenetic tree; thus, a chemical comparison of their lipoprotein polypeptides may permit an assessment of the evolutionary distance of these two animal species. In this context, it is of interest that the baboon, which belongs to the same family as the rhesus monkey, has, like the latter, apo A-II monomers in its HDL class [3]. The chimpanzee, the closest ancestor to man, in turn, exhibits apo A-II dimers [13].

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