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Enzymatic hydrolysis of reconstituted dimyristoylphosphatidylcholine–apo A-I complexes

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Apolipoproteins share a common structural feature, their interaction with phospholipids. It is believed that amphipathic helical sequences enable apolipoproteins to bind to lipid bilayer and to form discoidal particles of defined dimensions. While the knowledge of the apo A-I sequence and secondary structure has been used to make predictions about its mode of association with lipids, the available experimental data necessary to propose a precise model of these discoidal structures are still limited. An important step in our understanding of these structures would be to identify the apolipoprotein lipid-associated domains. Proteolysis of apo A-I–DMPC reconstituted HDL (rHDL) and free apo A-I is used here to identify lipid-protected domains of apo A-I. Free cleaved peptides were separated from rHDL associated peptides by density gradient centrifugation. The lipid-associated peptides were further analyzed by SDS-PAGE and transferred by Western blot to a ProBlott membrane for sequencing. Cleavage occurred at residue 43 with proteinase K, 46 with trypsin and residue 47 or 48 with pronase. A large domain from about residue 45 to the C-terminal remains highly protected against hydrolysis eventhough it contains several bonds susceptible to proteolytic cleavage. No protected fragments were detected by SDS-PAGE after enzymatic cleavage of free apo A-I in identical experimental conditions.

Introduction

The structural assembly of circulating plasma lipoproteins is mainly due to the lipid-apolipoproteins interactions. The sequences of the plasma apolipoproteins are characterized by the occurrence of several internal repeats [1,2,4] consisting of amphipathic helical segments. The role of these amphipathic motives has been substantiated by the observation of amphipathic helical segments in the X-ray structure of the N-terminal domain of apo E [6]. This amphipathic character enables hydrophobic interaction of the non polar face of the helix with the phospholipid hydrophobic region and of the polar face, containing the charged residues, with the phospholipid headgroups or with water [3].

Reassembly of various phospholipids with apo A-I yields discoidal particles with defined stoichiometry [7–9]. These particles are quite similar to nascent HDL, which are transformed in vivo into mature HDL, through the action of lecithin cholesterol acyltransferase (LCAT) [10]. Segrest has proposed the first model describing the interaction of the amphipathic helices of the apolipoproteins with phospholipids [3]. In this model, these helices interact with a single bilayer liposome, causing the rupture of the phospholipid bilayer with the concomitant formation of 'bicycle-tyre' micelles. These micelles are 'naked' bilayers whose edges are lined by amphipathic helices. The axes of the helices in this model are parallel to the phospholipid bilayer. Infrared spectroscopy provided experimental evidence of a mainly parallel orientation of the α-helices and of the acyl chains [12,23]. Recently, Katz et al. [5] demonstrated that an amphipathic helix is present both at the 'edge' and 'faces' of the discoidal

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Abbreviations: Apo A-I, apolipoprotein A-I; DMPC, dimyristoylphosphatidylcholine; HCA, hydrophobic cluster analysis; HDL, high-density lipoprotein; PMSF, phenylmethylsulfonyl fluoride; rHDL, reconstituted high-density lipoprotein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

complexes. On the basis of the overall composition, size and length of the amphipathic helices, it has been proposed that adjacent helical segments of apo A-I are arranged antiparallel to each other and joined by β turns [8,12,13]. The discoidal apo A-I–lipid complexes have recently been mapped with antibodies [11]. The analysis of some of these epitopes provides a structural model, in which two structurally distinct regions appear in apo A-I. One is the N-terminal region, which contains a complex tertiary structure, and the other one is constituted of antiparallel amphipathic helices, separated from each other by a β-turn [11].

In order to locate the apo A-I helical segments in close contact with the phospholipid bilayer more precisely, discoidal reconstituted HDL (rHDL) were digested by proteinase K, trypsin and pronase. The digestion pattern was compared to that observed with free apo A-I. Proteinase K cleaves specifically after hydrophobic amino-acid residues and, therefore, has been used successfully to locate the transmembrane helical segments of bacteriorhodopsin [22]. Trypsin cleaves the polypeptidic chain after an arginine or a lysine residue and no specific activity has been associated to pronase.

The lipid-associated peptides were separated on a density gradient and run on a Tris-Tricine gel. Band sequencing provided evidence that cleavage occurred at residue 43 for proteinase K, 46 for trypsin and 47 or 48 for pronase. The 43–243, 46–243 and 47 or 48–243 domains are highly protected against enzyme cleavage, even though they contain bonds susceptible to proteolytic cleavage. The data strongly suggest a protection of eight or seven predicted helical segments associated to apo A-I. The data are discussed in terms of available models describing the mode of assembly of lipid and helices of apo A-I.

Materials and Methods

Materials

Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma (St. Louis, MO, USA). Pronase was obtained from Boehringer-Mannheim (Mannheim, Germany) and trypsin and proteinase K were from Sigma. Standard proteins were purchased from BRL Gibco.

Methods

Apolipoprotein A-I–DMPC complexes. Apo A-I was isolated from total HDL (d 1.08–1.21 g/ml) by delipidation and ion-exchange chromatography as described previously [14]. Synthetic apo A-I–DMPC complexes were prepared by incubation of sonicated vesicles of DMPC with apo A-I in a ratio of 2.5 : 1 (w/w) for 3 h at 24°C in 5 mM Tris-HCl (pH 7.6). The complexes were isolated by gel chromatography on a Superose 6 HR with an FPLC system (Waters) [15]. In the hydrolysis experiments, the top fractions of the peak corresponding to apo A-I–DMPC complexes in the chromatographic run were pooled. In these pooled fractions, the apo A-I concentration was measured by the Lowry method [18], using BSA as standard and phospholipids with a Boehringer Kit (Boehringer-Mannheim) [18].

Isolation of proteolytic fragments of apo A-I. Aliquots of isolated complexes containing 25 μg of apo A-I, were incubated with each proteolytic enzyme in a proteinase K (or trypsin)/apo A-I ratio of 5 : 100 (w/w) for 10, 20 or 60 min (for proteinase K) or for 15, 30, 60 or 120 min (for trypsin), at 37°C. For pronase digestion, the same procedure was applied except that the enzyme/protein ratio was 2 : 100 (w/w) and incubation

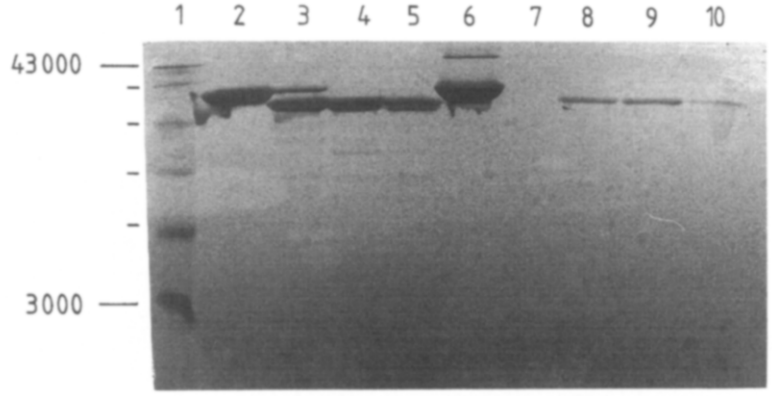

Fig. 1. Incubation of apo A-I–DMPC and free apo A-I with proteinase K. Separation of the hydrolyzed peptides after incubation with proteinase K (protein/enzyme ratio 100 : 5 (w/w)) on a Tricine gel [20]. lane 1, molecular-mass marker proteins; lane 2, apo A-I–DMPC complexes (25 μg apo A-I); lanes 3, 4, 5, apo A-I–DMPC (25 μg apo A-I) incubated with proteinase K (10, 20, 60 min); lane 6, native apo A-I (25 μg apo A-I); lane 7, native apo A-I (25 μg) incubated 60 min with proteinase K; lanes 8, 9, 10, apo A-I–DMPC hydrolyzed peptides after separation on a NaBr density gradient (10, 20, 60 min incubation with proteinase K).

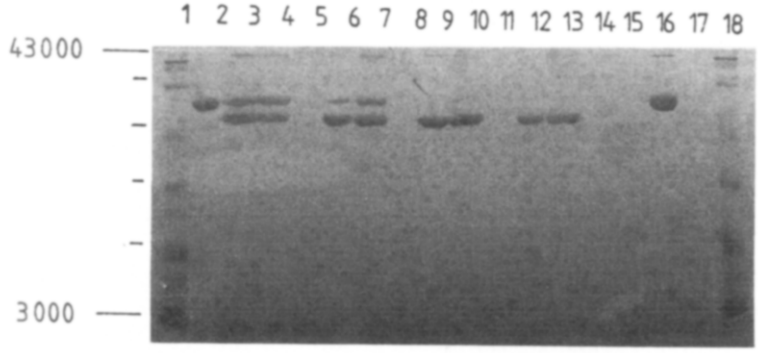

Fig. 2. Incubation of apo A-I–DMPC and free apo A-I with trypsin. Separation of the hydrolyzed peptides after incubation with trypsin (protein/enzyme ratio 100:5 (w/w)) on a Tricine gel [20]. lanes 1, 18, molecular-mass marker proteins; lane 2, apo A-I–DMPC complexes (25 μg apo A-I); lanes 3, 6, 9, 12, apo A-I–DMPC (25 μg apo A-I) incubated with trypsin (15, 30, 60, 120 min); lanes 4, 7, 10, 13, apo A-I–DMPC hydrolyzed peptides after separation on a NaBr density gradient (15, 30, 60, 120 min incubation with trypsin); lanes 5, 8, 11, 14, trypsin alone incubated at 37°C (15, 30, 60, 120 min); lane 16, native apo A-I (25 μg apo A-I); lane 17, native apo A-I (25 μg) incubated 15 min with trypsin.

was performed for 15, 30, 60 or 120 min. Enzymatic hydrolysis is stopped by addition of ethanolic PMSF solution at a final concentration of 3 mM in the sample. The proteinase K, pronase and trypsin digests were separated on a tricine gel system described by Schägger and von Jagow [17]. Gels were composed of a 10-cm long small pore running gel (16.5% T, 6% C), a 2-cm spacer gel (10% T, 3% C) and a 4-cm stacking gel (4% T, 3% C).

In order to distinguish bands corresponding to free peptides from those corresponding to lipid-associated peptides, the digestion products were fractionated on a NaBr density gradient (d 1.15 g/ml) at 55000 rpm for 66 h at 10°C. Fractions (0.3 ml) were collected from the bottom of the gradient. In each fraction, protein was monitored by Trp fluorescence and lipid content was determined with a commercially available kit (Boehringer-Mannheim) [18]. The fractions containing lipids and protein were pooled and separated on a Tricine gel as described above.

The molecular mass of the digests was estimated using low-molecular-mass standards, including ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), bovine trypsin inhibitor (6.2 kDa) and insulin (A and B chains, 3 kDa).

Microsequencing. The protected bands were directly transferred to a PVDF (polyvinilidene difluoride) proBlott™ membrane for 3 h at 24 V. Peptides were detected with a Coomassie blue solution (Coomassie blue 0.1%, methanol 40%, acetic acid 1%); the membrane was decolored with a 50% methanol solution. The N-terminal sequence of the peptides was determined by Edman degradation on an Applied Biosystems 477 A sequencer (pulsed-liquid sequenator).

Size of the complexes. The size of the apo A-I–phospholipid complexes before and after enzymatic hydrolysis was determined by electron microscopy. Apo A-I–phospholipid complexes, at a protein concentration of 150 μg/ml, were negatively stained with a 20 g/l solution of potassium phosphotungstate (pH 7.4). 7 μl of the samples was applied to Formvar carbon-coated grids and examined in a Zeiss EM 10C transmission electron microscope operating at 60 kV. Particle size was estimated by measuring 50 discrete particles/sample.

Results and Discussion

Comparison of apo A-I–DMPC complexes (Fig. 1, lane 5) and free apo A-I (Fig. 1, lane 7), respectively, digested with proteinase K for 60 min, provides evidence of a protection against hydrolysis of the lipid-associated protein. The protected domain corresponds to a 24 kDa band (lanes 3–5), which appears at the expense of the 28 kDa band (non digested apo A-I).

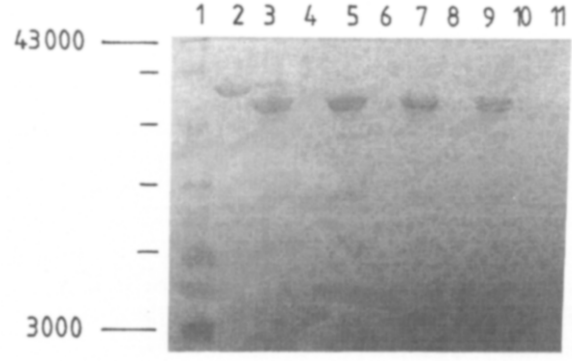

Fig. 3. Incubation of apo A-I–DMPC and free apo A-I with pronase. Separation of the hydrolyzed peptides after incubation with pronase (protein/enzyme ratio 100:2 (w/w)) on a Tricine gel [20]. lane 1, molecular-mass marker proteins; lane 2, apo A-I–DMPC complexes (25 μg apo A-I); lanes 3, 5, 7, 9, apo A-I–DMPC (25 μg apo A-I) incubated with pronase (15, 30, 60, 120 min); lanes 4, 6, 8, 10, pronase alone incubated at 37°C (15, 30, 60, 120 min); lane 11, native apo A-I (25 μg) incubated 15 min with pronase.

Low-molecular-mass bands disappeared after ultracentrifugation of the digests (lanes 8–10). It should be mentioned that after 15 min incubation (data not shown), in identical experimental conditions, free apo A-I is totally digested.

A 24 kDa band is also visible after 15 (lane 3), 30 (lane 6), 60 (lane 9) and 120 min (lane 12) trypsinolysis of the apo A-I–DMPC complex, with a concomitant disappearance of the 28 kDa spot; low-molecular-mass fragments are poorly resolved (Fig. 2). After ultracentrifugation, the only fragment which remains associated to phospholipids is the 24 kDa proteolytic fragment (lanes 4, 7, 10 and 13). Free apo A-I is totally digested after 15 min hydrolysis (lane 17).

In the apo A-I–DMPC complexes (Fig. 3), after 15 (lane 3), 30 (lane 5), 60 (lane 7) or 120 min (lane 9), a domain of apo A-I is protected against pronase hydrolysis (24 kDa), whereas the 28 kDa band (lane 2), corresponding to non-digested apo A-I, disappears after 30 min incubation (lane 5). It is worth noting that the 24 kDa band is resolved into two spots after 2 h incubation with pronase (lane 9). The 24 kDa band remains associated to lipids after ultracentrifugation (data not shown). No bands could be detected after 15 min pronase digestion (lane 11) of free apo A-I.

The 24 kDa bands corresponding to the three proteolysis experiments and obtained after ultracentrifugation of the digests, were transferred to a ProBlott™ membrane, revealed with Coomassie blue and microsequenced (Table I). The sequences corresponded to the C-terminal segment of apo A-I, starting at residue 43, 47 or 48 and 46 for hydrolysis with proteinase K, pronase and trypsin, respectively. Pronase digestion generated another proteolytic fragment which starts at

TABLE I

Apo A-I sequence

The eight predicted amphipathic helical segments are underlined [4,13]. Cleavage sites of the enzymes are indicated by arrows: 1, proteinase K; 2, trypsin; 3, pronase.

1	Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp Leu Ala
16	Thr Val Tyr Val Asp Val Leu Lys Asp Ser Gly Arg Asp Tyr Val
	1 2
31	Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys Gln Leu Asn Leu Lys
	3 3
46	Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu
	3
61	Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu
76	Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met Ser Lys Asp Leu
91	Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe Gln
106	Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys Val Glu
121	Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu His
136	Glu Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp
151	Arg Ala Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro
166	Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala
181	Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys
196	Ala Thr Glu His Leu Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala
211	Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val Leu Glu Ser Phe
226	Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr Lys Lys Leu
241	Asn Thr Gln

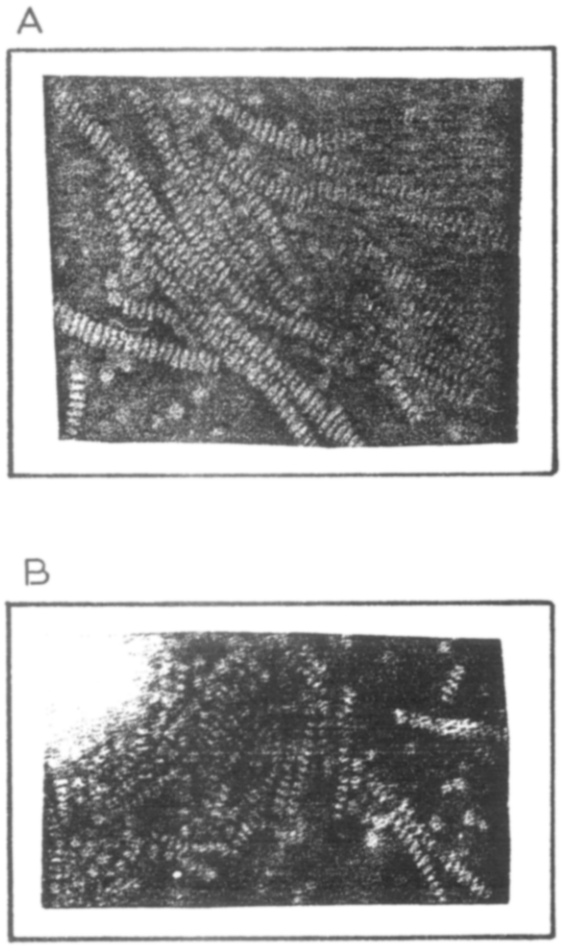

Fig. 4. Electron micrographs of negatively stained apo A-I–DMPC complexes before (A) and after (B) digestion with trypsin for 60 min.

residue 65. However, we cannot exclude that some residues (5 to 10) at the C-terminus might be digested, due to the precision on the determination of the molecular mass on Tricine gels. However, it seems unlikely that residues belonging to the last α-helix of apo A-I (222–239) were digested due to the high protection of the other seven helical repeats.

Fig. 4 shows the electron-microscopic photographs of the complexes before (Fig. 4A) and after (Fig. 4B) hydrolysis with trypsin (60 min). The diameter of the non-digested particles is 125 ± 19 Å. The discoidal complexes, incubated for 60 min with trypsin, also appear as stacked discs on Fig. 4B, with a 95 ± 15 Å diameter.

From these data, we can conclude that the interaction of apo A-I with DMPC protects an apo A-I domain running from about residue 45 to the C-terminal. This protection of apo A-I is probably not only associated to a change in the apo A-I conformation following its interaction with the lipids, which could perhaps decrease the reactivity of the cleavage sites, but rather reflects a direct protection of apo A-I lipid-bound domains. Indeed, it is well-known that the apo A-I secondary structure is not dramatically modified when associated to the lipids, as the α-helical structure is a major secondary structure in the lipid-free, as well as in the lipid-bound state [19,20]. Furthermore, we intentionally used, in this work, three proteinases with very different specificities. Most of their cleavage sites are located in the amphipathic domains which are precisely expected to be associated to the phospholipid bilayer: for example, among the 37 Lys and Arg contained in the apo A-I sequence, 27 are located in amphipathic regions and will favor the trypsin action. The hydrophobic amino-acids present in the amphipathic stretches will favor the proteinase K action too. Finally, all the proteinases digest apo A-I totally in its lipid-free state and cleave in the same 43–48 amino-acid region, when incubated with apo A-I–DMPC complexes.

Our data are in agreement with those of Massey et al. [24], who compared the kinetics of tryptic hydrolysis of apo A-II in solution and in association with

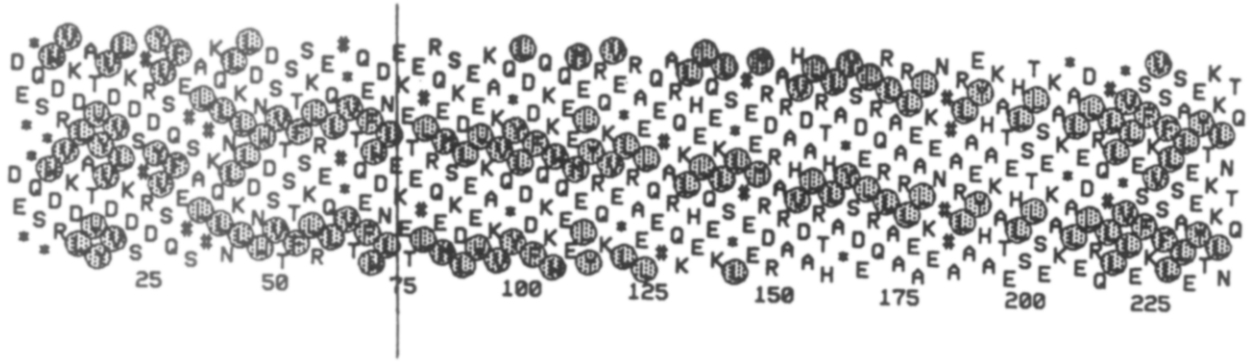

Fig. 5. 2-D α-helical patterns of Hydrophobic Cluster Analysis for apo A-I sequence. The sequence is written on a classical α-helix (3.6 amino acids per turn) smoothed on a cylinder. To make the 3-D representation easier to handle, the cylinder is then cut parallel to its axis and unrolled. As some adjacent amino acids are separated by the unfolding of the cylinder, the representation is duplicated to restore the full connection of each amino acid. The hydrophobic residues (VLIMYW) are circled and hatched; Pro is represented by * and Gly by #. The vertical line separates the N-terminal domain (1–70) from the rest of the sequence.

phospholipids. None of the tryptic cleavage sites were shown to be as susceptible to hydrolysis in the lipid-associated protein as in solution.

Our proteolytic data are in agreement with a structural model of the apo A-I–DMPC complex made of eight amphipathic apo A-I helical segments surrounding the lipid discoidal structure [8]. However, after 2 h incubation of the apo A-I–DMPC complex with pronase, an additional band, corresponding to the 65–243 C-terminal domain of apo A-I, appeared. This higher susceptibility of the first helical repeat suggests that it is not as well protected against enzyme action as the other seven helical repeats. The higher susceptibility of this helical stretch to proteolysis can be explained in terms of a weaker association with the lipid acyl chains and for instance, an interaction of the first helical segment with the lipid–water interface can not be excluded.

Furthermore, the 1–65 N-terminal domain is structurally quite different from the rest of the molecule as proposed by Marcel et al. [11] in a model based on the analysis of apo A-I epitopes. In two very recent papers, Sparks et al. also proposed that the N-terminal one third domain of apo A-I may have a 'modulable' conformation, which seems to depend on the size and the shape of the apo A-I–phospholipid complex [25,26]. The 'hydrophobic cluster analysis' (HCA), developed by Gaboriaud et al. [21], confirms this separation of apo A-I into two structural domains. Briefly, this method is based on an α-helical two-dimensional representation of protein sequences, where the size, shape and composition of clusters of hydrophobic residues are analyzed and compared. The N-terminal domain of apo A-I, delimited by a line (ending at about the 70th residue), shows obviously a more heterogeneous distribution of the hydrophobic clusters (hydrophobic residues are circled and hatched) than the 70–243 C-terminal of the protein, which is representative of a highly amphipathic helical domain (Fig. 5).

Such an organization is in agreement with a model in which seven amphipathic helices per apo A-I, 18 amino-acids long, oriented parallel to the lipid acyl chains, surround the discoidal disc [6] and are protected against the proteolytic cleavage, whereas the 1–65 N-terminal domain remains accessible to the enzyme action.

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