Structure of Human Apolipoprotein D: Locations of the Intermolecular and Intramolecular Disulfide Links†

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ABSTRACT: We have determined the primary structure of human apolipoprotein D (apoD) by aligning peptides derived from digestions by cyanogen bromide, trypsin, and chymotrypsin. Our results confirm the primary structure derived from cDNA [Drayna et al. (1986) *J. Biol. Chem. 261*, 16535–16539]. ApoD consists of 169 amino acid residues, including 5 cysteines. Tryptic peptide analysis indicated that Cys41 and Cys116 are joined by a disulfide bridge. Using a combination of manual Edman degradations and mass spectrometric analysis on a purified cluster of chymotryptic fragments, we identified an intramolecular disulfide bridge between Cys8 and Cys114 and an intermolecular bridge between Cys116 of apoD and Cys6 of apoA-II. In addition, sites of N-glycosylation were found at Asn45 and Asn78. Because apoD contains two intramolecular disulfide linkages and has a high content of proline to disrupt α-helical structures, formation of the amphipathic helical regions that characterize the other soluble apolipoproteins is unlikely. We conclude that apoD binds to lipoprotein surfaces through structures other than α-helices, such as disulfide links.

Low levels of plasma high-density lipoprotein–cholesterol (HDL–C)[1] are often associated with the presence of coronary artery disease (Miller & Miller, 1975). Although the mechanistic basis of this correlation is not known, it is thought to involve the facilitated transport of cholesterol from peripheral tissue to the liver by HDL. HDL contains several proteins, such as apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, and apoE, that spontaneously associate with phospholipids via amphipathic helical regions. These proteins, which are derived from genes with similar structures (Li et al., 1988), are also associated with the biochemistry and cell biology of HDL. ApoC-I and apoA-I activate lecithin:cholesterol acyltransferase (Soutar et al., 1975), apoE targets lipoproteins to the cell's surface receptors (Rall et al., 1982), and apoC-II activates lipoprotein lipase (LaRosa et al., 1972).

HDL contains another protein, apoD, which shares few of the structural and functional attributes of the apoA and apoC proteins. When apoD was first characterized by McConathy and Alaupovic (1973, 1976), it was considered a cholesteryl ester transfer protein, but that activity was later attributed to another protein (Morton & Zilversmit, 1981). A more recent finding shows that apoD is the same protein as GCDPF-24, a progesterone-binding protein in the cyst fluid of patients with breast gross cystic disease (Balbin et al., 1990). However, GCDPF-24 does not bind cholesterol. The lack of correlation between GCDPF-24 and cholesterol concentrations in cyst fluid suggests that sterols other than cholesterol and its esters are the ligands for apoD (Lea, 1988; Sanchez et al., 1992).

ApoD belongs to the $\alpha_{2\mu}$-globulin superfamily of proteins, which binds to monomeric hydrophobic ligands (Drayna et al., 1986; Peitsch & Boguski, 1990; Pevsner et al., 1988). In plasma, apoD circulates as a monomer or as a dimer linked by disulfide bridges to other proteins such as apoA-II (Weech et al., 1986; Camato et al., 1989; Blanco-Vaca et al., 1992). Although the primary structure of apoD is known from its cDNA sequence, the chemical states of its five cysteines are not. We have sequenced apoD and identified the sites of attachment of intramolecular and intermolecular disulfide bridges. On the basis of these data, a structural model of apoD is proposed.

MATERIALS AND METHODS

α-Chymotrypsin and trypsin treated with L-(tosylamino)-2-phenylethyl chloromethyl ketone (TPCK) were obtained from Worthington Biochemical Co. (Freehold, NJ). The source for acetonitrile for high-performance liquid chromatography (HPLC) was J. T. Baker (Phillipsburg, NJ). Vydac C18 columns (4.6 × 250 mm) for peptide purification were procured from the Separation Group (Hesperia, CA). The solvents and reagents for sequencing and amino acid analysis were purchased from Applied Biosystems (Foster City, CA), and those for Hewlett-Packard Amino-Quant amino acid analysis were brought from Hewlett-Packard (San Fernando, CA). Reagents and solvents for manual Edman degradation were obtained from Pierce (Rockford, IL). Other chemicals were of the highest available reagent grade.

Apolipoprotein D Isolation. Human plasma was obtained from healthy fasting donors at The Methodist Hospital Blood Donor Center. The plasma lipoproteins were isolated by sequential flotation (Havel et al., 1955), and the HDL and very-high-density lipoproteins were delipidated. The apoD

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[1] Abbreviations: apoD, apolipoprotein D; cDNA, complementary DNA; D–AII, apolipoprotein D–apolipoprotein A-II heterodimer; FAB, fast atom bombardment; HDL–C, high-density lipoprotein–cholesterol; HPLC, high-performance liquid chromatography; MS, mass spectrometry; PTH, phenylthiohydantoin; RBP, retinol-binding protein; TFA, trifluoroacetic acid; TPCK, L-(tosylamino)-2-phenylethyl chloromethyl ketone; VLDL, very-low-density lipoprotein(s).

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and apoD–apoA-II heterodimers (D–AII) were then isolated from apoHDL on a hydroxyapatite column in 2 M urea and 1 mM K_2HPO_4 at pH 8.0 (Blanco-Vaca et al., 1992). D–AII, which was the major apoD-containing species found, was further purified by Mono Q chromatography using a linear gradient (0–1 M NaCl in 10 mM Tris/4 M urea, pH 7.4). Further attempts to isolate pure monomeric apoD produced only small amounts of apoD homodimer. Therefore, the D–AII was used for further sequence analysis and for the assignment of the disulfide bridges.

Enzymatic Cleavage and Peptide Isolation. D–AII (1 mg/mL in 0.1 M ammonium bicarbonate, pH 8.0) was digested by α-chymotrypsin and TPCK-treated trypsin at room temperature for 5 h. The liberated peptides were separated on a Vydac C18 column (4.6 × 250 mm) with a trifluoroacetic acid (TFA) buffer system (Yang et al., 1990; A: 0.1% TFA in water; B: 0.08% TFA in 95% acetonitrile and 5% water). The eluted peaks were collected and sequenced.

Rechromatography. A phosphate buffer system (A: 5 mM sodium phosphate buffer, pH 6.0; B: 90% acetonitrile and 10% A) was used to purify disulfide-containing and N-glycosylated peptides of D–AII. The column, flow rate, and HPLC system were the same as those described above.

Cyanogen Bromide Cleavage (Gross & Witkop, 1962). Five milligrams of D–AII was dissolved in 1 mL of 70% formic acid with excess cyanogen bromide (50-fold, w/w) and reacted overnight in the dark at room temperature. The hydrolysate was diluted with water (1:1) and concentrated to remove excess reagent. The cyanogen bromide fragments were separated by HPLC using a Vydac C18 column.

Sequence and Amino Acid Analysis. An Applied Biosystems gas phase sequencer 470A equipped with 120A phenylthiohydantoin (PTH) analyzer was used to sequence peptides derived from the chymotryptic and tryptic cleavages. A modified manual Edman degradation was used to analyze the D–AII peptide (Yang et al., 1986). The peptide was desalted and dissolved in pyridine/water (2:1) solution. Ten microliters of phenyl isothiocyanate was added to this solution, which was then incubated at 54 °C for 30 min. After extraction with *n*-heptane/ethyl acetate (2:1 v/v), the sample was dried and incubated with 50 μL of TFA at 54 °C for 15 min. After the second Edman cycle, the dried sample was subjected to mass spectrometric analysis.

Amino Acid Analysis. A Hewlett-Packard 1090 HPLC for Amino-Quant amino acid analysis was used. Pure peptides were hydrolyzed in the gas phase with 6 N hydrochloric acid and 0.25% phenol for 70 min at 150 °C. After hydrolysis, the samples were dried and prepared for amino acid analysis. Aliquots (10 μL) of hydrolyzed sample in 0.4 N borate buffer (pH 10.4) were automatically mixed in the injection loop with an additional 5 μL of buffer, 1 μL of *o*-phthalaldehyde, and 2 μL of 9-fluorenylmethyl chloroformate. The total volume was then injected into an ODS Hypersil (5 μL) HPLC column (2.1 × 200 mm) at 40 °C. With the commercially available standards and reagents, the amino acid composition of the isolated sample was obtained.

Mass Spectrometric Analyses. The products of two stages of manual Edman degradation were analyzed by fast atom bombardment mass spectrometry (FAB MS) using a VG ZAB SEQ interfaced to an 11/250 data system (Fisons Instruments, Manchester, U.K.). The primary beam consisted of xenon atoms with kinetic energies of 8 keV. The sample matrix was a 1:1 mixture of thioglycerol and 2,2′-dithiodiethanol (2-hydroxyethyl disulfide) saturated with oxalic acid. The *m/z* range, 100–2500, was scanned at a rate of 10 s/decade. The "multichannel analyzer" mode was used to acquire data, and successive scans were accumulated into a single data file. An estimated 60 pmol of peptide was used for each analysis.

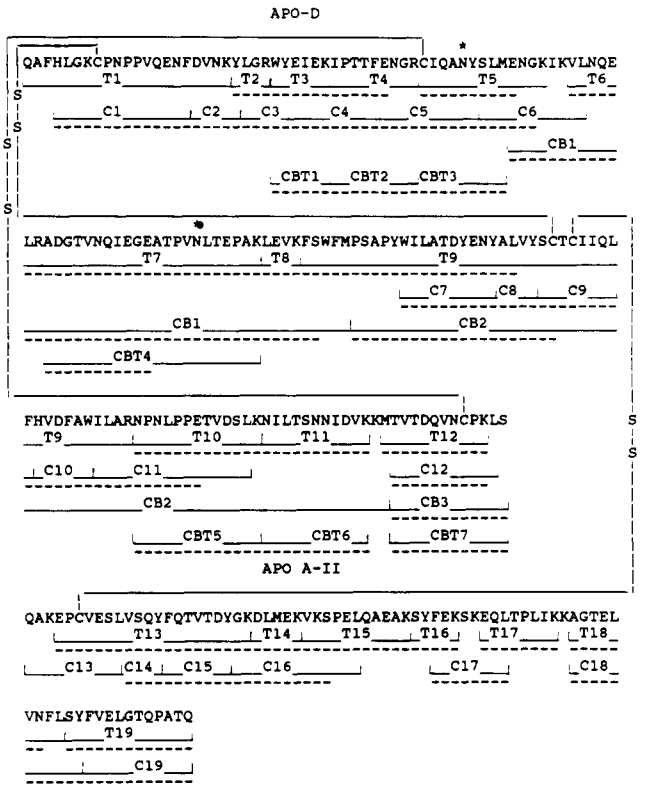

FIGURE 1: Primary structure of apolipoprotein D (apoD). Tryptic peptides, chymotryptic peptides, cyanogen bromide peptides, and cyanogen bromide-tryptic peptides are designated Tn, Cn, CBn, and CBTn, respectively. The N-glycosylated residues are designated by an asterisk. The cysteines connected by disulfide bridges are indicated by -S-S-. The dashed lines designate residues that were identified by gas phase sequence analysis.

Predictive Algorithms. The helical hydrophobic moments and hydrophobicities of 11-residue segments of apoD were calculated using the predictive algorithms of Eisenberg et al. (1982).

RESULTS

The amino acid sequence of apoD (Figure 1) was determined by aligning tryptic, chymotryptic, and cyanogen bromide peptides, as well as peptides cleaved by both trypsin and cyanogen bromide. Although the amino terminal of apoD is blocked by a pyroglutamine residue, the 12 tryptic peptides (T1–T12), 12 chymotryptic peptides (C1–C12), 3 cyanogen bromide peptides (CB1–CB3), and 7 cyanogen bromide-tryptic peptides (CBT1–CBT7) align the same sequence for apoD as that deduced from the sequencing of the cDNA clone (Drayna et al., 1987).

Tryptic Peptides. In order to locate the sites of N-glycosylation and disulfide linkage, the sequences of the purified tryptic peptides generated from D–AII were determined (Blanco-Vaca, 1992). Some peptides that coeluted in the first purification step required rechromatography (Figure 2A). Nine major fractions were isolated. By sequence and amino acid analysis, fractions 1–4 were identified as peptide T7 (residues 63–84), and fractions 5–9 were determined to be a disulfide peptide connecting peptides T5 (residues 41–53) and T12 (residues 157–167). Nineteen peptides were

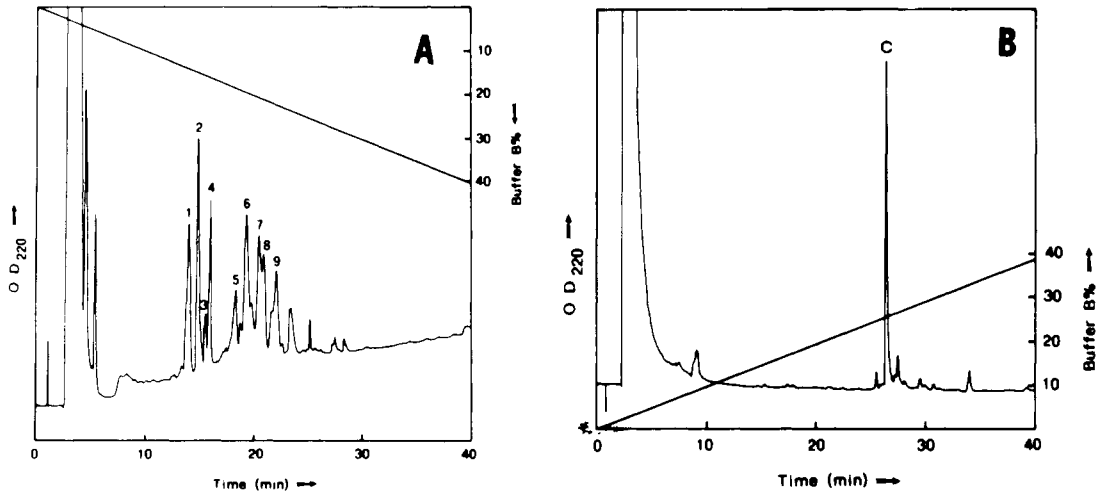

FIGURE 2: (A) Rechromatography of a tryptic fraction (23% B of TFA buffer system) on a Vydac C18 (4.6 × 250 mm) column with the phosphate buffer system. A linear gradient was run from 0% to 40% B. (B) Rechromatography of a chymotryptic fraction (29% B of TFA buffer system) on the same column. Conditions are the same as in (A). Peptide C, which contained two disulfide bridges, was purified.

Table 1: Amino Acid Composition of Chymotryptic Peptide C from the ApoD–ApoA-II Heterodimer

amino acid	number of residues: expected	found
Asx	2	2.4
Glx	6	5.8
Ser	2	1.9
Gly	1	1.1
His	1	0.9
Arg	0	0.0
Thr	1	1.0
Ala	1	1.1
Pro	4	4.8
Tyr	0	0.0
Val	2	2.3
Met	0	0.0
Cys-Cys	2	1.6
Ile	2	1.2
Leu	3	3.2
Phe	2	2.1
Lys	2	2.0
Trp	0	0.0

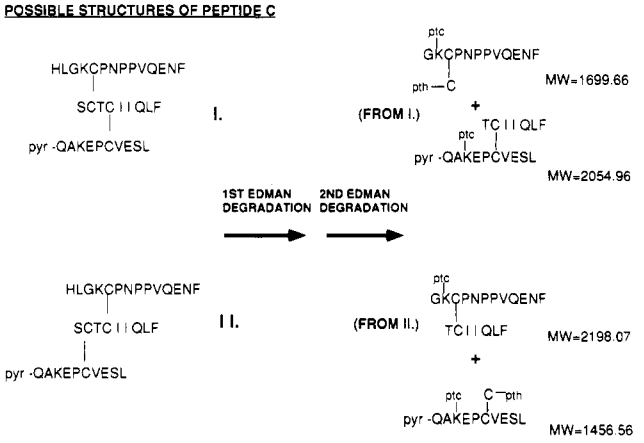

FIGURE 3: Two possible structures (I and II) of peptide C and four possible fragments after two cycles of Edman degradation are proposed. The molecular weight of each fragment is indicated.

isolated and identified from tryptic digestion of D–AII. The first 12 peptides (T1–T12) comprised apoD, and the remaining 7 peptides were components of apoA-II (Figure 1).

Chymotryptic Peptides. Chymotryptic digestion of D–AII generated peptides which were purified by a Vydac C18 column and a TFA buffer system. Sequence analysis on a chymotryptic fraction isolated from the TFA system on 29% B revealed two amino terminals. Rechromatography of this fraction produced a major peak (fraction C) possessing the same amino terminal as the original fraction (Figure 2B). Two amino acid residues were obtained from all cycles except cycles 2, 4, and 5, which appeared only once. On the basis of this information and the published results of tryptic digestion (Blanco-Vaca, 1992), fraction C appeared to be the disulfide peptide that linked the apoD loop and connected apoD with apoA-II. Amino acid analysis (Table 1) indicated that fraction C contained two cystines and covered the sequences of peptides C1 and C6 of apoD (residues 4–17 and 113–121) and peptide C9 of apoA-II (residues 1–10). Nineteen chymotryptic peptides were isolated and identified. Twelve peptides (C1–C12) were derived from apoD, and 7 (C13–C19) were derived from apoA-II (Figure 1).

Cyanogen Bromide Peptides. Two major cyanogen bromide fragments of D–AII were isolated by HPLC using a Vydac C18 column and TFA buffer system. Sequence analysis indicated that the first fraction, eluting at 41% B, corresponded to the peptide CB1 and included residues 50–93 of apoD; the second fraction, eluting between 45 and 50% B, contained the remainder of the apoD peptides and apoA-II linked by a disulfide bond. A second digestion with trypsin was performed, and the liberated peptides were purified by HPLC. Seven cyanogen bromide-tryptic peptides (CBT1–CBT7) were isolated and identified (Figure 1).

Disulfide Linkage within ApoD and between ApoD and ApoA-II. ApoD contains five cysteine residues. According to the results of tryptic and chymotryptic peptide analysis, Cys41 and Cys165 form a disulfide bridge (Figure 2A), while Cys8, Cys114, and Cys116 of apoD and Cys6 of apoA-II cluster to form two disulfide bridges. Based on the connection of these two disulfide bridges, two structures are possible for this complex (I and II, Figure 3). In order to determine which cysteine of apoD is bridged to Cys6 of apoA-II, two cycles of manual Edman degradation were applied to peptide C, and the reaction mixture was subjected to mass spectrometric analysis. The FAB mass spectrum (Figure 4) included a prominent ion of m/z 2056, which was consistent with the protonated fragment predicted as a product of Edman degradation of structure I; the complementary fragment was not found. Prominent ions (Figure 4) of m/z 1432 and 2131 (not observed in control studies involving Edman degradation

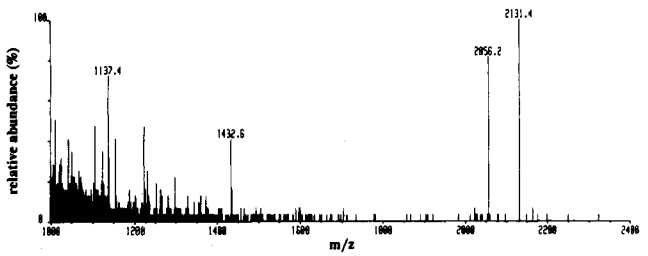

FIGURE 4: Fast atom bombardment mass spectrum of the products of two stages of Edman degradation of chymotryptic fragment C. The signal at m/z 2056.2 corresponds to the protonated form of one of the expected fragments from structure I (calculated m/z 2056.0); the complementary fragment was not observed. Neither of the fragments predicted from the alternative disulfide-bonded structure (II) was observed.

in the absence of the peptide) could not be assigned. Significantly, neither of the two predicted products of Edman degradation of the alternative disulfide-bonded structure (structure II) was observed during FAB MS. This result proves the linkage of Cys116 of apoD to Cys6 of apoA-II.

Location of N-Glycosylation Sites. The N-glycosylation sites of apoD were assigned on the basis of Marshall's rule (1974), which states that an asparagine followed by a variable amino acid followed by a serine or a threonine contains a carbohydrate. Two asparagines (residues 45 and 78) were identified as potential N-glycosylation sites in apoD. Gas phase sequence analysis and the HPLC pattern of peptides T5 and T7 in a phosphate buffer system confirmed these asparagines as N-glycosylation sites.

DISCUSSION

We have determined the primary structure of apoD and the location of its disulfide links by direct protein sequencing and mass spectrometric analysis. The complete sequence was obtained from a sequence analysis of the cleavage products of trypsin, chymotrypsin, and cyanogen bromide. The results of this analysis confirm the data derived from the cDNA–deduced sequence. ApoD contains 169 amino acid residues in a single polypeptide chain, including 5 cysteines that are available to form disulfide links. The odd number of cysteines on apoD ensures that at least one cysteine is not involved in an intramolecular disulfide bond. Our evidence shows that four cysteines are involved in intramolecular disulfide links and that the remaining one is free or associated with another protein.

Peculiar qualities of apoD and apoA-II made identification of the sites of the disulfide linkages difficult. The amino terminals of both apoD and apoA-II are blocked by pyroglutamate residues, and two of the cysteines in apoD, Cys114 and Cys116, appear in the same peptides that are linked to apoA-II. According to our sequence analysis of tryptic and chymotryptic disulfide peptides, Cys6 of apoA-II is connected to either Cys114 or Cys116 of apoD. The FAB MS analysis of peptide C after two cycles of manual Edman degradation clearly shows that Cys6 of apoA-II is connected to Cys116 of apoD (Figures 3 and 4).

Blanco-Vaca et al. (1992) reported that plasma apoD was a component of all lipoprotein subclasses and that a large fraction of the apoD formed homodimers or heterodimers with other cysteine-containing proteins. The most abundant apoD heterodimer found contained apoA-II. In this report, we demonstrate that the disulfide linkage is between Cys6 of apoA-II and Cys116 of apoD. Intramolecular disulfide linkage

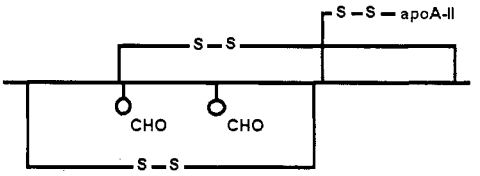

FIGURE 5: Primary structure of the D–AII heterodimer. Both amino terminals of the apoproteins are blocked by pyroglutamate. Two N-glycosylated carbohydrates are located on Asn45 and Asn78 of apoD. Intramolecular disulfide linkages connect Cys41 to Cys165 and Cys8 to Cys114 in apoD; Cys116 of apoD and Cys6 of apoA-II are linked to form a heterodimer.

is mediated by a disulfide isomerase in the rough endoplasmic reticulum. Thus, these intramolecular links are likely to be conserved among all heterodimers of apoD with other proteins. The remaining free sulfhydryl at Cys116 is the probable site of disulfide linkage with other plasma apolipoproteins. The disulfide links between apoD and monomeric apoA-II, which is known to bind lipids (Massey et al., 1981a,b), could contribute to the association of apoD with HDL. However, the presence of free apoD in HDL and other lipoproteins proves that disulfide linkage is not the only determinant of lipoprotein binding. Based on the locations of inter- and intramolecular disulfide linkages, a model for the apoD–apoA-II heterodimer is proposed (Figure 5).

Unlike most other water-soluble apolipoproteins, apoD belongs to a family of hydrophobic ligand carrier proteins termed lipocalins. This gene family contains at least 40 proteins with homologous sequences, including α-acid glycoprotein, β-lactoglobulin, insecticyanin, retinol-binding protein, and bilin-binding protein (Drayna et al., 1986; Peitsch & Boguski, 1990). The three-dimensional structures of four lipocalins have been determined. In each case, the polypeptide chain is folded into an eight-stranded β-barrel that is open at the top and closed at the bottom. All members of this family are thought to contain this structure, which carries hydrophobic ligands within the barrel. On the basis of similarities in the relative positions of cysteine residues and other sequences contributing to tertiary structure, Peitsch and Boguski (1990) have proposed that apoD is most closely related to insect biliverdin-carrying protein, insecticyanin, and bilin-binding protein. Chemical analysis shows that the disulfide bonding patterns are those predicted in the Peitsch–Boguski model, but one of the major loops at the entrance to the β-barrel in apoD differs from that of insecticyanin by the substitution of several hydrophobic residues in the side chain. In their model, Peitsch and Boguski (1990) suggest that this region, along with hydrophobic residues on another adjacent loop, may mediate the association of apoD with HDL, with the entrance of the binding pocket being close to the lipoprotein surface. The most significant inference from this model is that Cys116, which forms the disulfide link with apoA-II, is in this region. Thus, within the context of the Peitsch–Boguski model of apoD, Cys116 is both free and accessible to the surrounding aqueous phase that contains other free cysteines that may react with Cys116.

For several reasons, it is unlikely that apoD binds lipids using the amphipathic helical regions which characterize the other soluble apolipoproteins. First, proline, an amino acid known to disrupt α-helices, accounts for 7% of the total peptide structure of apoD. Second, the two intramolecular disulfide links interrupt four sites on the protein and thereby place additional restrictions on the occurrence of long amphipathic helical regions. Third, analysis of the primary structure of

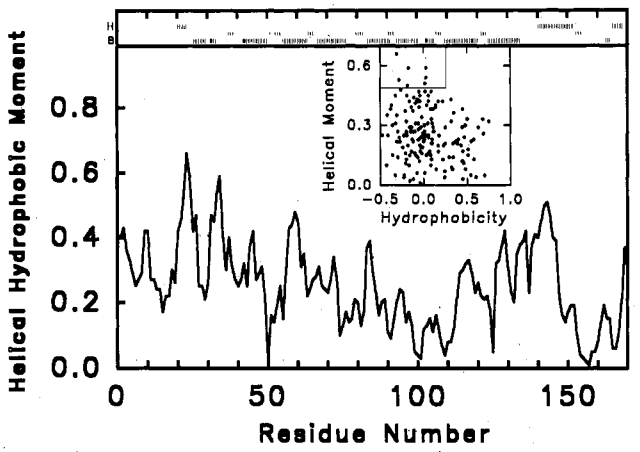

FIGURE 6: Inferred structure of apoD. The uppermost panel shows the distribution of α-helical (H) and β-strand (B) structures along the sequence. Within the panel, the locations of predicted reverse turns (t) and β-bulges (B) are also shown. The lower panel shows the sequence dependence of the helical hydrophobic moment. The insert to the lower panel contains a plot of the helical hydrophobic moment versus the hydrophobicity. The small box in the upper left-hand side of the insert is the region which contains the moments and hydrophobicities that are characteristic of "surface-seeking peptides".

apoD using the helical hydrophobic moment algorithm (Eisenberg et al., 1982) reveals no 11-residue regions with calculated moments comparable to those of the soluble plasma apolipoproteins (Figure 6). The mean value of the hydrophobic moments of the 11-residue segments of apoD (0.26) is far less than the range that includes most of the soluble apolipoproteins (0.5–1.0). Because of their low hydrophobic moments and hydrophobicities (Figure 6, insert), nearly all of the analyzed segments of apoD lie outside the region of the moment versus hydrophobicity plot assigned to "surface-associating peptides" (Eisenberg et al., 1982; Pownall et al., 1983, 1986). Finally, using the homology of apoD and insecticyanin at the level of their primary structures and the three-dimensional structure of the latter protein, the secondary structure of apoD has been inferred (Peitsch & Boguski, 1990). As shown in the uppermost panel of Figure 6, much of the inferred structure of apoD is composed of β-strands and not α-helices. Therefore, apoD does not appear to have distinct lipid-binding regions that are composed of amphipathic helices; instead, it must bind to the lipoprotein surface through other determinants, including disulfide links.

Peitsch and Boguski (1990) also predict the glycosylation of Asn45 and Asn78 in apoD. According to their model, both sites are at the surface of the protein and remote from the entrance to the binding pocket. In general, Asn45 is only partially conserved within the lipocalin family. The conservation of this residue in retinol-binding protein (RBP) is unexpected, since apoD and RBP have different disulfide bridge patterns. Insecticyanin, which has similar disulfide bridges, does not contain either of these sites. Among the lipocalins, Asn78 is a glycosylation site unique to apoD. The importance of glycosylation and the specific sequence position is not known.

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