Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 250-257

IDENTIFICATION OF SURFACE-EXPOSED SEGMENTS OF APOLIPOPROTEIN B-100 IN THE LDL PARTICLE

Patricia FORGEZ[†], Harry GREGORY[*], Janice A. YOUNG[*], Timothy KNOTT[°], James SCOTT[°], and M. John CHAPMAN

Groupe de Recherches INSERM sur les Lipoprotéines,
Pavillon Benjamin Delessert, Hôpital de la Pitié,
75651 PARIS Cedex 13, France

*Imperial Chemical Industries PLC, Pharmaceutical Division,
Alderley Park, MACCLESFIELD, Cheshire SK10 4TG, U.K.

°Molecular Medecine, Clinical Research Centre,
HARROW, Middlesex, U.K.

Received August 8, 1986

SUMMARY: The isolation and amino acid sequence of eleven peptides liberated by tryptic treatment from surface-exposed regions of apolipoprotein B-100 in the native low-density lipoprotein particle are described. These peptides represent eight segments in the sequence of the B-100 protein, one of which was localised to the amino-terminal thrombolytic fragment T4 (1297 amino acids), four to the T3 fragment (2052 residues) and three to the carboxyl-terminal fragment T2 (1287 residues). An exposed segment was identified on each side of the T2/T3 cleavage site, in close proximity to two segments enriched in basic amino acids (residues 3147-3157 and 3359-3367 respectively). The surface exposure of this region is consistent with its contribution to the putative apo-B,E receptor binding domain. Four of the eight tryptic segments contribute to regions of proline-rich clusters. Homology between the sequences of the tryptic peptides and those predicted by cDNA cloning was complete.

Apolipoprotein B-100 is the major protein constituent of the circulating low-density lipoproteins (LDL) and is of hepatic origin (1,2). The precise nature of this protein has remained elusive as a result of its unique physicochemical properties and marked susceptibility to proteolytic and oxidative degradation (2). It is however established that apo B-100 is a large polypeptide whose molecular weight has been estimated in the range of 350 to 550 KD (2, 3).

Two essential functions are fulfilled by apo B-100. Firstly, this protein stabilises the structural organisation of the LDL particle, and secondly, is responsible for the cellular recognition and catabolism of LDL by the

†To whom correspondence should be addressed.

0006-291X/86 $1.50

LDL (apo-B,E) receptor pathway (4, 5). The structure which underlies the specific binding of LDL to this receptor remains to be elucidated. The functional contribution of certain arginine and lysine residues to the binding site has been demonstrated by chemical modification (6, 7). Another approach to the determination of the receptor binding domain has involved modification of apo B-100 in the native LDL particle with enzymes of high substrate specificity. In this way, portions of the surface-exposed regions of apo B-100 may be removed (8-10), with marked effect on the receptor-binding affinity of the LDL particle (10-12).

By virtue of the molecular cloning of the cDNA for apolipoprotein B-100, large portions of its primary structure have become available (13-19). More recently, Scott and colleagues (20) have elucidated the complete sequence of the human protein. Despite such progress, these findings do not permit identification of the surface-exposed, hydrophilic regions of apo B-100 in the intact LDL particle.

With the aim of evaluating the relationship of apo B-100 structure to its function in the LDL particle, we have purified and characterized certain trypsin-accessible, surface-exposed segments of human apolipoprotein B-100, whose amino acid sequence and location in the intact protein is presented herein.

MATERIALS AND METHODS

Lipoprotein isolation: Human LDL (d 1.024-1.050 g/ml) were separated from normolipidemic serum by sequential flotation ultracentrifugation as previously described (21), and dialysed extensively in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA; mol. wt. cutoff 10,000-12,000) at 4°C against a solution containing 0.05 M NaCl, 5 mM NH_4HCO_3, 0.04 % EDTA, 10 mM NaN_3 and 0.001 % sodium merthiolate at pH 7.4. The protein moiety of such LDL contained > 98 % by weight of apolipoprotein B-100 (9).

Enzymic treatment: Tryptic digestion of LDL (60-80 mg protein) was performed with TPCK-Trypsin (Worthington) as outlined earlier (9), with the exception that 5 mM CH_3COONH_4 was used as incubation buffer. The digest was initially fractionated into the protein-deficient, trypsinised LDL (T-LDL), trypsin and the liberated water-soluble peptides derived from LDL protein (T-peptides), by gel filtration chromatography on a column of Sephadex G75 (8).

Fractionation of tryptic peptides: The total, water-soluble peptides (T-peptides) eluted from the G75 column were pooled, lyophilised twice from distilled water, and solubilised in a 5 mM CH_3COONH_4 buffer at pH 7.2. These peptides were then fractionated on a Biogel P4 (90 x 1.6 cm) column equilibrated with the solubilisation buffer (9).

High performance liquid chromatography (HPLC): Peptides obtained by chromatography on Biogel P4 were further fractionated by HPLC on an Altex model 344 chromatography system (two model 112 pumps, a model 421 microprocessor system controller, a stirred-gradient mixing chamber, a model 210 sample injector, and a model 160 Altex detector).

Material in peaks II and III from the Biogel P4 chromatographic step (see Fig. 1) was pooled respectively, solubilised in 0.1 % trifluoroacetic acid (TFA; Baker) and sonicated two or three times at 50 W for 10 sec. The

dissolved peptides were loaded onto a 214 TP 104 Vydac column equilibrated with a 0.1 % TFA solution. Peptides were separated by the development of a gradient from 0 to 35 % in acetonitrile (Baker) containing 0.1 % TFA. The gradient was developed at a rate of 10 % per hour and at a flow rate of 1 ml/min. The final purification step for the majority of the peptides so obtained was carried out on a C18 HPLC column (Biorad Hi-Pore RP 18). Each peptide was eluted with a restricted acetonitrile gradient, this gradient starting and terminating within a range of ± 2.5 % of the acetonitrile concentration at which it was eluted from the 214 TP 104 Vydac column; such gradients were developed at a rate of 5 % per hour, and at a flow rate of 1 ml/min.

Peptide purity was verified by application of samples to a TSK ODS 120 T column and elution with a gradient of 0.1 % aqueous TFA to 0.1 % TFA/acetonitrile. Quantification of the amounts present was accomplished by comparison of the respective peak heights measured at 206 nm with that given by a low molecular weight standard.

Amino acid analysis: Amino acid analyses were carried out on acid hydrolysates of selected peptides using an LKB 4400 amino acid analyser, with a view to quantification. Small aliquots were analysed, which gave peaks near the limits of sensitivity of the instrument. The amino acid ratios so obtained were in good agreement with the subsequent sequence determinations.

Sequence determination: Sequencing was carried out using an Applied Biosystems gas phase sequencer Model 470 A with on-line HPLC identification of the phenylthiohydantoins in a manner similar to published procedures (22). Peptide quantities in the range 0.5-1.0 nmol (as determined by HPLC or amino acid analysis) were taken for sequencing. On this basis, initial yields were in the region of 25 %, with repetitive yields of 90 to 92 % thereafter.

RESULTS

With the aim of identifying water-soluble tryptic peptides from apo B-100 of maximal size, peptide material; eluting close to the excluded volume of the Biogel P4 column (Fig. 1) was taken for further purification and sequence determination. Thus the peptide mixtures corresponding to Biogel P4 peaks II and III were fractionated by reverse phase high performance liquid chromatography. Figure 2 shows a representative elution profile

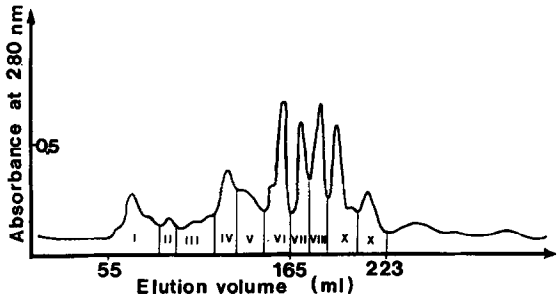

Figure 1. Elution profile of the fractionation of the mixture of T-peptides on a Biogel P4 column. Material in peaks II and III was pooled as indicated by the vertical lines; the eluate was monitored at 280 nm.

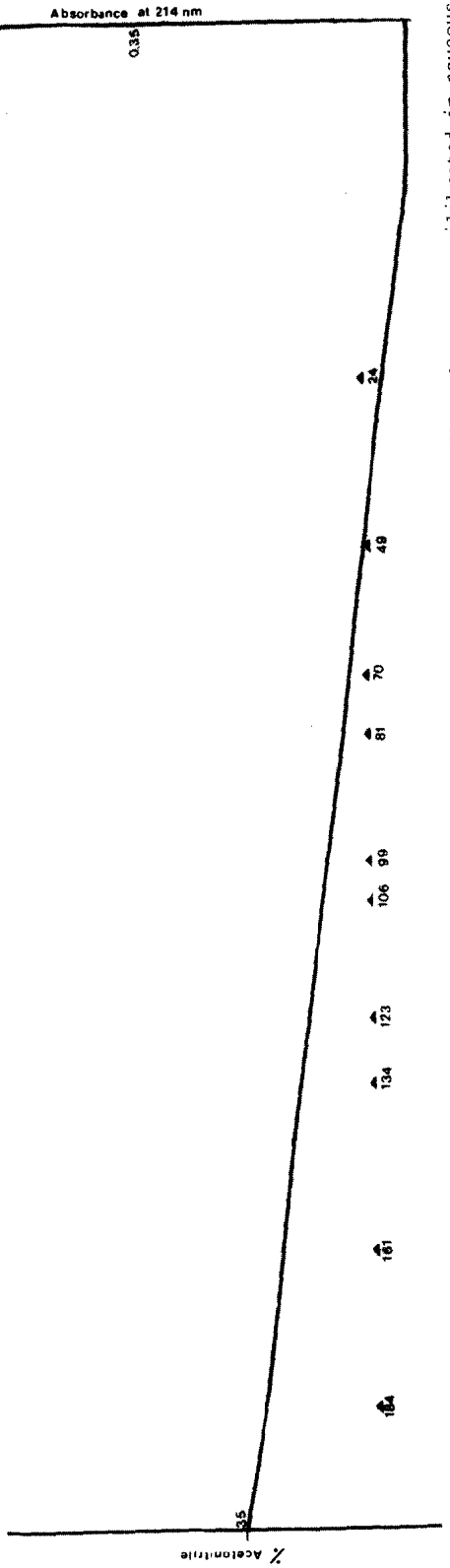

Figure 2. Reverse phase HPLC separation of material in T-peptide peak III on a Vydac C4 column. The column was equilibrated in aqueous 0.1 % TFA solution and developed with an acetonitrile gradient (0 to 35 % in 210 min) at a flow rate of 1 ml/min. The eluate was monitored at 214 nm. Peptide peaks denoted by arrows were taken individually for further purification and sequencing. Some 2-3 mg of peptide material were applied to the column.

TABLE I: AMINO ACID SEQUENCES OF TRYPTIC PEPTIDES FROM SURFACE-EXPOSED REGIONS OF APO B-100 IN THE NATIVE LDL PARTICLE

	[a]	[b]	Mol.wt.	
Tp	24	1008-1016	1093	(Lys)-Phe-Val-Thr-Gln-Ala-Glu-Gly-Ala-Lys
Tp	123	2091-2106	2263	(Lys)-Leu-Pro-Gln-Gln-Ala-Asn-Asp-Tyr-Leu-Asn-Ser-Phe-Asn- ? - Glu-Arg
TP	70	2091-2098	1073	..... (Trp)
Tp	49	2485-2493	1309	(Lys)-Phe-Arg-Glu-Thr-Leu-Glu-Asp-Thr-Arg
Tp	99	2679-2685	954	(Arg)-Ile-Ser-Leu-Pro-Asp-Phe-Arg
Tp	161	3218-3236	2415	(Arg)-Thr-Phe-Gln-Ile-Pro-Gly-Tyr-Thr-Val-Pro-Val-Val-Asn-Val-Glu-Val-
TP	81	3237-3242	905	.....
Tp	134	3224-3232	1162	Ser-Pro-Phe-Thr-Ile-Glu-Met-Ser-Ala-Phe-(Gly-Tyr-Val-Phe-Pro-Lys)
Tp	184	3265-3275	1145	(Arg)-Val-Pro-Ser-Tyr-Thr-Leu-Ile-Leu-Pro-(Ser-Leu-Glu-Leu-Pro-Val-Leu-His-Val-Pro-Arg)
Tp	59	3828-3841	1695	(Lys-Ile-Ala-Asp-Phe-Glu-Leu-Pro-Thr)-Ile-Ile-Val-Pro-Glu-Gln-Thr-Ile-Glu-Ile-Pro-Ser- ? - Lys (Ile)
Tp	106	4080-4094	2026	(Arg)-Asn-Leu-Gln-Asn-Asn-Ala-Glu-Trp-Val-Tyr-Gln-Gly-Ala-Ile-Arg

[a] The fraction in which the respective tryptic peptide (Tp) was eluted upon HPLC (see profil in Fig.2) is denoted on the left, with the exception that Tp 59 was eluted from Biogel P4 Peak II.

[b] Corresponding residue numbers in the complete amino sequence of apo B-100 (20). Residues underscored with dotted lines indicate sequences found in partial tryptic peptides. Residues within parentheses () have been taken from the complete primary structure (20).

for components of peak III; a wide range in hydrophobicity was evident, with all peptides being eluted within an acetonitrile concentration range of 0 to 31 %. Peptides denoted by arrows (Fig. 2) and denoted Tp 24, Tp 49, Tp 70, Tp 81, Tp 99, Tp 106, Tp 123, Tp 134 and Tp 184 were taken for further reverse phase purification on a C 18 column and subsequent sequencing.

The amino acid sequences of these surface-exposed, tryptic peptides are presented in Table 1, together with the residue numbers derived from homologous regions identified in the complete sequence of human apo B-100; this sequence was recently obtained by molecular cloning of the cDNA (13, 20). The molecular weights of the majority of these peptides fall in the range of 1000 to 2000 Daltons (Table 1). Eight of the eleven peptides (including partial peptides) result from amino-terminal cleavages at arginine or lysine residues, i.e. Tp 24, 49, 70, 99, 106, 123, 161 and 184. By contrast, three peptides result from amino-terminal cleavages at hydrophobic residues, i.e. Tp 59, Thr-Ile; Tp 81, Phe-Thr and Tp 134, Gly-Tyr. Six peptides (Tp 24, Tp 49, Tp 59, Tp 99, Tp 106 and Tp 123) result from tryptic cleavage at peptide bonds which are carboxy-terminal to lysine or arginine residues, while the remaining five again reflect cleavages which have predominantly occurred at hydrophobic peptide bonds (Tp 70, Tyr-Leu; Tp 81, Phe-Gly; Tp 134, Glu-Val; Tp 161, Phe-Thr and Tp 184, Pro-Ser).

Two partial peptides were identified, Tp 134 corresponding to nine internal residues of Tp 161, and Tp 70 corresponding to the eight amino-terminal residues of Tp 123 respectively. Two contiguous peptides were

also detected, and notably Tp 161 whose nineteen residues are situated amino-terminal in the native protein to the seven amino acids of Tp 81, giving a total exposed segment of some 26 residues.

Upon evaluation of the relative contents of hydrophobic, hydrophilic and charged residues in the eight segments which have been sequenced, it was apparent that all the constituent peptides, with the exception of Tp 184, contained at least one and up to five charged amino acids. Half of the eight segment contained predominantly polar residues, while half were primarily of hydrophobic nature. Only one segment was highly hydrophilic (Tp 49, residues 2485-2493), seven of its nine residues being polar and five possessing a charge; indeed a charge pair (Arg-Glu) was present.

DISCUSSION

Apolipoprotein B-100 plays a fundamental role in determining the intravascular metabolism and tissue degradation of the LDL particle. Such functions are assured in large part by the surface-exposed regions of apo B-100, which may interact with diverse structures such as enzymes, transfer proteins, cellular membranes, components of the extracellular matrix and specific lipoprotein receptors. The eight segments described herein, representing a total of 105 residues, correspond to regions of apo B-100 in LDL which are surface-exposed and apparently free of interaction with both lipids and protein, given their ready liberation into the aqueous phase upon proteolysis. Most of these segments (four) are present within thrombin fragment T3 (residues 2091-2106, 2485-2493, 2679-2685 and 3218-3242), while three are found within T2 (residues 3265-3275, 3828-3841 and 4080-4094) and only one (residues 1008-1016) in T4, the N-terminal fragment. These findings are consistent with the surface exposure of the T2/T3 junction region (residues 3249-3250), since segments 3218-3242 and 3265-3275 are situated on either side of this thrombin cleavage site. Furthermore, these latter segments are located within the loop defined by cysteine residues n° 3167 and 3297 at either side of the T2/T3 site, the segment of residues 3218-3242 occurring in the middle of this loop (20).Both of these segments may indeed contribute to the overall conformation of the putative apo-B,E receptor binding domain postulated to involve residues 3147-3157 and 3359-3367 (20). In addition, segments 3218-3242 and 3265-3275 are found within a proline-rich cluster spanning residues 3102 to 3305, a region of high amphipathic β-sheet potential, and are themselves enriched in this amino acid (11.5 and 22.2 mole % respectively). The peptides corresponding to residues 2679-2685 and 3828-3841 also fall within proline-rich regions (20).

An essentially complete homology was found between the amino acid sequences of our various tryptic peptides and those predicted by cDNA cloning (20).

Comparison of the sequences of the exposed segments with those of other apolipoproteins in a protein database revealed a limited homology between residues 2485-2493 in apo B-100 and residues 14-23 of human apo-CI (23), in which five of seven residues were shared.

In conclusion, our present findings constitute a first step towards identification of functional domains in apo B-100 as a component of the intact LDL particle.

ACKNOWLEDGMENTS

This research was supported by grants from ICI-Pharma and from the Ministere de la Recherche et de la Technologie (MRT n° 85.T.0860). P.F. gratefully acknowledges the award of a fellowship from ICI-Pharma. We thank Mr. M. Anselme, Dr D.C.N. Earl and Dr A. Rossi for continued support and helpful discussion, Mr. I. Willshire and Miss S Goulinet for excellent technical assistance and Mrs M. Tassier for manuscript preparation.

REFERENCES

1. Havel, R.J., Goldstein, J.L., and Brown, M.S. (1980) Metabolic Control and Disease, (Bondy, P.K., and Rosenberg, L.E., eds.) pp. 393-494, Saunders, Philadelphia.
2. Kane, J.P. (1983) Ann. Rev. Physiol. 45, 637-650.
3. Elovson, J., Jacobs, J.C., Schumaker, V.N., and Puppione, D.L. (1985) Biochemistry 24, 1569-1578.
4. Brown, M.S., and Goldstein, J.L. (1983) J. Clin. Invest. 72, 743-747.
5. Brown, M.S., and Goldstein, J.L. (1986) Science 232, 34-46.
6. Weisgraber, K.H., Innerarity, T.L., and Mahley, R.W. (1978) J. Biol. Chem. 253, 9053-9062.
7. Mahley, R.W., Weisgraber, K.H., and Innerarity, T.L. (1979) Biochim. Biophys. Acta 575, 81-89.
8. Chapman, M.J., Goldstein, S., and Mills, G.L. (1978) Eur. J. Biochem. 87, 475-488.
9. Chapman, M.J., Millet, A., Lagrange, D., Goldstein, S., Blouquit, Y., Taylaur, C.E., and Mills, G.L. (1982) Eur. J. Biochem. 125, 479-489.
10. Tikkanen, M.J., Dargar, R., Pfleger, B., Gonen, B., Davie, J.M., and Schonfeld, G. (1982) J. Lipid Res. 23, 1032-1038.
11. Chapman, M.J., Loisay, F.Y.M., Forgez, P., and Cadman, H. (1985) Biochim. Biophys. Acta 835, 258-272.
12. Chapman, M.J., Innerarity, T.L., Arnold, K.S., and Mahley, R.W. (1984) Latent Dyslipoproteinemias and Atherosclerosis (De Gennes, J.L. et al., eds.) pp. 93-99, Raven Press, New York.
13. Knott, T.J., Rall, S.C., Innerarity, T.L., Jacobson, S.F., Urdea, M.S., Levy-Wilson, B., Powell, L.M., Pease, R.J., Eddy, R., Nakai, H., Byers, M., Priestley, L.M., Robertson, E., Rall, L.B., Betsholtz, C., Shows, T.B., Mahley, R.W., and Scott, J. (1985) Science 230, 37-43.
14. Mehrabian, M., Schumaker, V.W., Fareed, G.C., West, R., Johnson, D.F., Kirchgessner, T., Lin, H.C., Wang, X., Ma, Y., Mendiaz, E., and Lusis, A.J. (1985) Nucleic Acids Res. 13, 6937-6952.
15. Protter, A.A., Hardman, D.A., Schilling, J.W., Miller, J., Appleby, V., Chen, G.C., Kirsher, S.W., McEnroe, G., and Kane, J.P. (1986) Proc. Natl. Acad. Sci. USA 83, 1467-1471.
16. Law, S.W., Lackner, K.J., Hospattankar, A.V., Anchors, J.M., Sakaguchi, A.Y., Naylor, S.L., and Brewer, H.B.Jr. (1985) Proc. Natl. Acad. Sci. USA 82, 8340-8344.

17. Shoulders, C.C., Myant, N.B., Sidoli, A., Rodriguez, J.C., Cortese, C., Baralle, F.E., and Cortese, R. (1985) Atherosclerosis 58, 277-289.
18. Carlson, P., Olofsson, S.O., Bondjers, G., Darnfors, C., Wiklund, O., and Bjursell, G. (1985) Nucleic Acids Res. 13, 8813-8826.
19. Deeb, S.S., Motulsky, A.G., and Albers, J.J. (1985) Proc. Natl. Acad. Sci. USA 82, 4983-4986.
20. Knott, T.J., Pease, R.J., Powell, L.M., Wallis, S.C., Rall, S.C.Jr., Innerarity, T.L., Blackhart, B., Taylor, W.R., Marcel, Y., Milne, R., Johnson, D., Fuller, M., Lusis, A.J., McCarthy, B.J., Mahley, R.W., Wilson-Levy, B., and Scott, J. (1986) submitted.
21. Chapman, M.J., and Goldstein, S. (1976) Atherosclerosis 25, 267-291.
22. Hunkapiller, M.W., Hewick, R.M., Dreyer, W.J., and Hood, L.E. (1983) Meth. Enzymol. 91(i), 399-413.
23. Shulman, R.S., Herbert, P.N., Werhrly, K., and Fredrickson, D.S. (1975) J. Biol. Chem. 250, 182-190.