Amino acid sequence of rabbit apolipoprotein E

Bo-rong Lee,* Jonathan M. Miller,* Chao-Yuh Yang,*,† Latha Ramdas,* Manlan Yang,* Joel D. Morrisett,*,† and Martha P. Mims[1,*]

Departments of Medicine* and Biochemistry,† Baylor College of Medicine and The Methodist Hospital, Houston, TX 77030

Abstract The complete amino acid sequence of rabbit apolipoprotein E (apoE) was determined by generating three sets of peptides using cyanogen bromide, endoproteinase AspN, and *Staphylococcus aureus* V8 protease to cleave the protein. Through twenty cycles of sequence analysis on the whole protein, glutamic acid was identified as the N-terminal residue of rabbit apoE; the C-terminus of the protein was identified as glutamine. Based on the sequence of 294 amino acid residues determined by protein structure analysis, the molecular weight of rabbit apoE was determined to be 33,684. The protein sequence differed from the cDNA inferred sequence in 19 positions, only one of which could be attributed to microheterogeneity. The corrected amino acid sequence of rabbit apoE shares 80% homology with the human apoE sequence, 4% greater homology than that inferred from the cDNA sequence. The great similarity in the amino acid sequences of human and rabbit apoE suggests that their physical and physiological properties may also be similar. This homology and the relative ease with which apoE is isolated from rabbit plasma make it possible to conduct some in vitro experiments with the rabbit apoprotein that would have direct relevance to human apoE, but would be difficult or impossible with the human counterpart because of the quantity of protein required. —**Lee, B-r., J. M. Miller, C-Y. Yang, L. Ramdas, M. Yang, J. D. Morrisett, and M. P. Mims.** Amino acid sequence of rabbit apolipoprotein E. *J. Lipid Res.* 1991. **32:** 165–171.

Supplementary key words apoE cDNA nucleotide sequence • homology with human apoE

Apolipoprotein E is a plasma glycoprotein of molecular weight ~34,000 (1). A major physiological function of apoE is to mediate the binding of apoE-containing lipoproteins to the LDL (B/E) receptors of liver and peripheral tissues (2). As a component of both hepatic VLDL and a specific class of HDL, apoE participates in the binding of triglyceride-rich VLDL to peripheral tissue and in the HDL-mediated redistribution of cholesterol. In addition, apoE is transferred to intestinally derived chylomicrons as they enter the bloodstream, and mediates binding of chylomicron remnants to a second hepatic receptor, the apoE receptor (3).

Human apoE contains 299 amino acids and appears to consist of two distinct structural domains joined by a hinge region (4, 5). The NH_2-terminal domain contains roughly 145 amino acids, is approximately 60% α-helical in solution, and retains both lipid-binding and receptor-binding characteristics. This domain is unusually resistant to denaturation with a free energy of stabilization of >8 kcal/mol. The COOH-terminal domain (residues 216–299) retains lipid-binding characteristics, but does not bind to the LDL receptor. Like the NH_2-terminal domain, the COOH-terminal domain is predicted to be highly α-helical, and it is this carboxyl terminal region that is postulated to take the major role in binding of apoE to lipoprotein surfaces.

ApoE has been identified and characterized in a number of animal species in which cholesterol feeding has been shown to alter markedly the plasma concentration of apoE and its distribution among different lipoprotein classes (6, 7). Rabbits are particularly sensitive to cholesterol feeding, and respond by producing an abundance of a large cholesteryl ester-rich VLDL from which considerable amounts of apoE can be easily isolated. Recently, a full length rabbit apoE cDNA has been isolated and sequenced (8). This cDNA analysis suggested that rabbit apoE is synthesized with an additional 18 amino acid prepeptide, and that mature rabbit apoE contains 293 amino acids and possesses 76% homology with human apoE. Our results, derived from protein sequencing of rabbit apoE, suggest a number of differences from the amino acid sequence inferred by the cDNA nucleotide sequence.

Abbreviations: apoE, apolipoprotein E; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; CN, cyanogen bromide; DMPC, dimyristoylphosphatidylcholine; CO, cholesteryl oleate.

[1]To whom correspondence should be addressed at: The Methodist Hospital, MS A601, 6565 Fannin Street, Houston, TX 77030.

MATERIALS AND METHODS

Materials

Sequencing grade endoproteinase AspN was from Boehringer Mannheim Co. *Staphylococcus aureus* V8 protease, cyanogen bromide, sequencing reagents, and solvents were obtained from Pierce Chemical Co. Acetonitrile, methanol, and other solvents for high performance liquid chromatography (HPLC) were from Burdick and Jackson. Vydac C_{18}, Hypersil ODS, and Spherisorb ODS II columns were obtained from Cel Associates. Solvents and reagents for the Applied Biosystems 470 Gas Phase Sequencer, and 120PTH Amino Acid Analyzer were the products of Applied Biosystems. Other chemicals used were the highest reagent grade available.

Purification of rabbit apolipoprotein E

ApoE was isolated from the plasma of rabbits maintained on chow containing 2% cholesterol (ICN Biochemicals) by a modification of the method of Roth et al. (9) in which the VLDL apoproteins were solubilized in 50 mM ammonium bicarbonate buffer, pH 7.8, containing 1% decyl sodium sulfate. The protein had a molecular weight of approximately 34,000 as determined from SDS 7.5% polyacrylamide gel electrophoresis. ApoE was judged pure by its migration as a single band on both 7.5% and 12% SDS polyacrylamide gels.

Cyanogen bromide cleavage

Two mg of rabbit apoE was dissolved in 70% formic acid and a 50-fold excess (w/w) of cyanogen bromide and left to react overnight in the dark at room temperature (10). The mixture was then diluted with an equal volume of water and concentrated in a Speedvac concentrator to remove excess reagents. The cyanogen bromide fragments were separated by HPLC using a Vydac C_{18} column.

Endoproteinase AspN cleavage

Four hundred and fifty μg of rabbit apoE in 150 μl of 0.1 M ammonium bicarbonate, pH 8, was added to the original microfuge tube containing 2 μg of AspN protease, and allowed to react overnight at room temperature (11). The hydrolysates were then subjected to HPLC purification.

Staphylococcus Aureus V8 protease cleavage

Two mg of rabbit apoE dissolved in 0.5 ml of 0.1 M ammonium bicarbonate, pH 8.0, was digested with 40 μg of *S. aureus* V8 protease at room temperature overnight. The hydrolysates were then subjected to HPLC purification.

HPLC for peptide purification

Purification of peptide mixtures derived from chemical and proteolytic cleavage was performed at 50°C on a Waters HPLC system with a Vydac C_{18} column (4.6 × 250 mm) at a flow rate of 1.5 ml/min. A trifluoroacetic acid (TFA) buffer system (A: 0.1% TFA in water, v/v; B: 0.08% TFA in 95% acetonitrile and 5% water, v/v/v) was used for primary separation. Peptides were monitored at 220 nm. The eluents from each peak were collected manually and subjected to purity analysis. For rechromatography, a phosphate buffer system (A: 5 mM sodium phosphate, pH 6.0; B: 90% acetonitrile and 10% water) and a Shandon Hypersil ODS column (4.6 × 250 mm) were used (12).

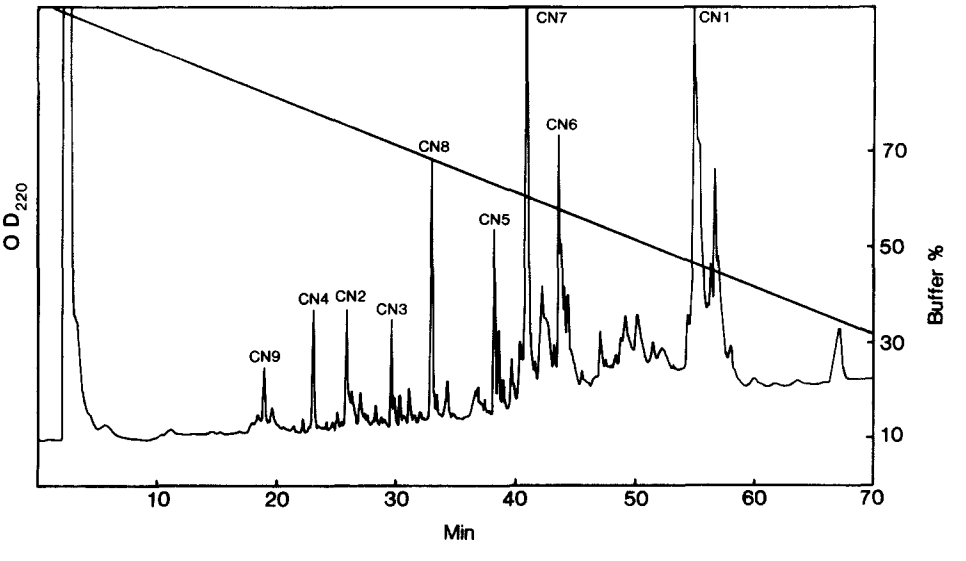

Fig. 1. Separation of cyanogen bromide peptides of rabbit apoE by HPLC using a Vydac C_{18} column and the TFA buffer system with a linear gradient running from 0 to 70% buffer B in 70 min.

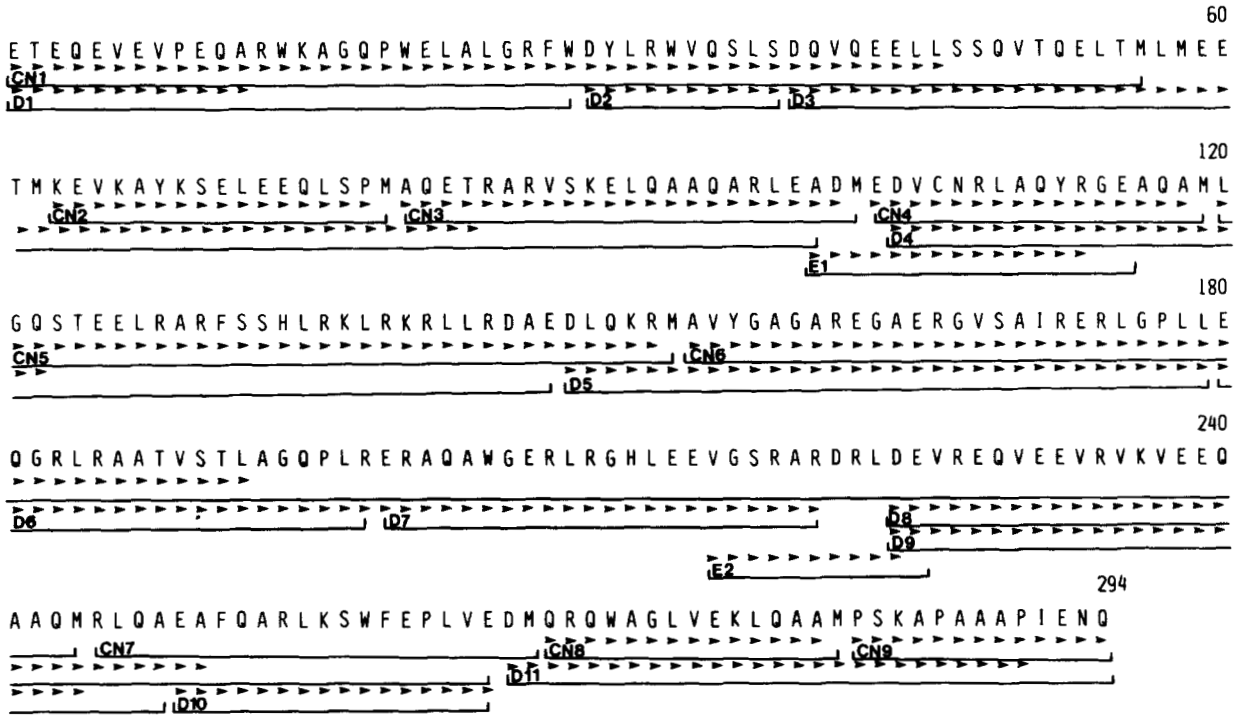

Fig. 2. Primary structure of rabbit apoE as deduced from overlapping cyanogen bromide (CN1–CN9), staphylococcal (E1, E2), and endoproteinase AspN (D1–D11) peptides. Arrows indicate the extent of sequencing of each peptide.

Sequence analysis

Both manual and automatic sequencing methods were used for structure determination. Short peptides and fragments for purity verification were determined according to the method of Chang, Brauer, and Wittman-Liebold (13). Thin-layer chromatography (13, 14) or an HPLC method (15) was used to identify amino acid residues derived from modified Edman degradation (13). The sequences of the intact protein and large peptide fragments were determined by a gas phase sequencer (Applied Biosystems) with an on-line PTH amino acid analyzer (16).

Amino acid analysis

Peptides were hydrolyzed in the gas phase in the Waters Hydrolysis apparatus with 6 N HCl and 0.25% phenol for 70 min at 150°C. After hydrolysis, the samples were dried and prepared for amino acid analysis using phenylisothiocyanate (17).

RESULTS

The sequence of apoE was determined by generating three sets of apoE peptides using cyanogen bromide, endoproteinase AspN, and *Staphylococcus aureus* V8 protease to cleave the protein. **Fig. 1** shows the separation of cyanogen bromide peptides of rabbit apoE, purified by HPLC using a Vydac C_{18} column. After peptides CN1 to CN9 were isolated, modified Edman degradation revealed that all CN fragments were in pure form. The primary structures of each peptide resulting from gas phase sequence analysis are recorded in **Fig. 2**. To obtain the sequence overlaps necessary for complete structure determination of rabbit apoE, a second cleavage of the protein was performed. Endoproteinase AspN, which cleaves at the NH_2-terminus of aspartic acid, was used. HPLC separation of the AspN peptides is shown in **Fig. 3**. Eleven peptides, D1 to D11, were purified and sequenced, and their structures are shown in Fig. 2. After the sequencing of the CN and AspN peptides, two regions, the sequence surrounding residue 102 and the residues between 221 and 223, required confirmation. To accomplish this, peptides E1 and E2 were isolated from the *S. aureus* V8 protease digestion after HPLC chromatography. As indicated in Fig. 2, peptide E1 contained residues 100 to 115 and E2 contained residues 215 to 225. The complete amino acid sequence of rabbit apoE was defined by aligning overlapping cyanogen bromide (CN), AspN (D), and staphylococcal (E) peptides. Twenty cycles of sequence analysis on the whole protein confirmed glutamic acid as

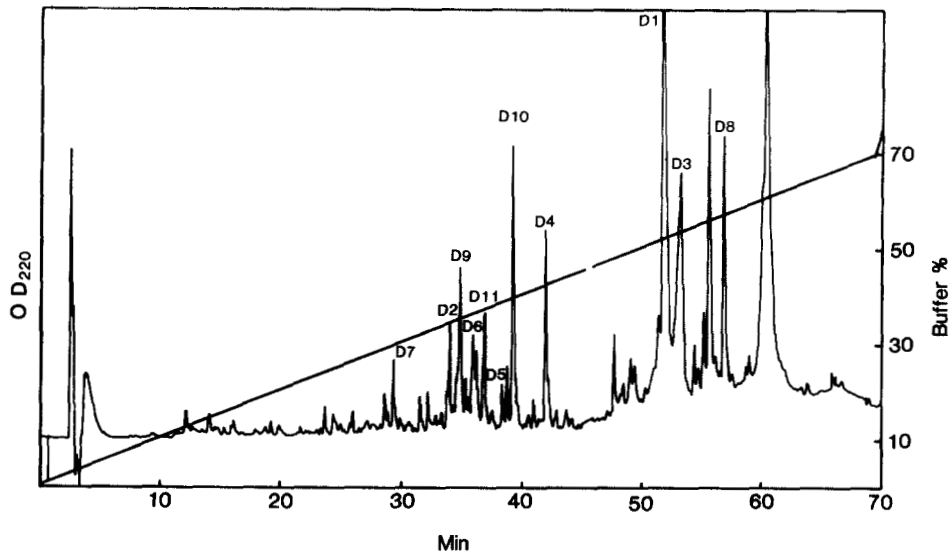

Fig. 3. Separation of endoproteinase AspN peptides using the Vydac C_{18} column and the TFA buffer system.

the N-terminal residue of rabbit apoE (18). Sequencing of CN9 revealed glutamine by the thirteenth cycle, and amino acid analysis revealed that CN9 contained 3 proline, 4 alanine, 2 glutamic acid, 1 serine, 1 lysine, 1 aspartic acid, and 1 isoleucine residue. From this information and the manner in which cyanogen bromide cleaves a protein (on the C-terminal side of a methionine residue), it was deduced that glutamine was the C-terminal amino acid. Based on the sequence of 294 amino acid residues determined by protein structure analysis, the molecular weight of rabbit apoE was calculated to be 33,684.

DISCUSSION

We have directly determined the amino acid sequence of rabbit apoE. This protein sequence differs from that in-

TABLE 1. Differences between amino sequences determined directly from the apoE protein and indirectly from the cDNA sequence (8)

Amino Acid Position	Amino Acid Derived from cDNA Sequence	Amino Acid Derived from Protein Sequence	Explanation for Difference
68	threonine	tyrosine	error in reproducing amino acid sequence from correct cDNA sequence
83	histidine	threonine	CAC→ACC possible transposition of AC
87	leucine	valine	CTG→GTG
93	valine	alanine	GTG→GCG
97	none	arginine	no explanation
95	glycine	glutamine	GGC→CAA possible microheterogeneity
128	alanine	arginine	128 129 130 128 129 130
129	arginine	alanine	GCG CGC GCG→CGC GCG CGC
130	alanine	arginine	possible frameshift
171	valine	isoleucine	GTC→ATC
177	serine	proline	TCG→CCG
178	arginine	leucine	CGG→CTG
181	arginine	glutamine	CGG→CAG
186	valine	alanine	GTC→GCC
190	glycine	serine	GGC→AGC
195	arginine	glutamine	CGG→CAG
224	asparagine	aspartic acid	AAC→GAC
235	alanine	valine	error in reproducing amino acid sequence from correct cDNA sequence
242	proline	alanine	CCG→GCG

ferred from the cDNA sequence in 19 positions. Two of these differences, at positions 68 and 235, were the result of errors in reproducing the amino acid sequence from the correct codon sequence. The remaining differences can be attributed to the transposition of two nucleotides within a codon, to a single nucleotide change within a codon, to a single nucleotide shift within the reading frame, and possibly to microheterogeneity (**Table 1**). We have also found an additional amino acid, arginine, at position 97 which was not detected in the cDNA deduced sequence. This corrected amino acid sequence of rabbit apoE shares 80% homology with the human apoE sequence; 4% greater homology than inferred from the cDNA sequence.

Fig. 4 compares the primary structure of rabbit apoE with that of human apoE3, the most common human isoform, and the one with which the rabbit protein is most homologous. The rabbit protein contains five fewer amino acids than the human protein. Like the human protein, rabbit apoE contains a single cysteine residue (position 106), and 7 tryptophan residues (positions 14, 20, 28, 33, 204, 258, 270) at positions analogous to those in the human sequence. The putative receptor-binding sequence (residues 134–144 in the rabbit sequence) is also identical in both proteins.

It has been shown previously that the liver secretes apoE primarily as sialo-apoE, and that the protein is desialylated in plasma (18). Sialylation of human apoE arises from the attachment of a sialylated carbohydrate chain at single threonine residue (19). This residue corresponds to position 188 in the rabbit apoE sequence, and is conserved. Rabbit apoE also contains 2 asparagine residues (positions 107 and 293), which are possible sites for N-glycosylation; however, it lacks the amino acid sequence Asn-X-Ser (Thr) which is characteristic of N-glycosylation. Thus sialylation of rabbit apoE could occur by O-glycosylation in a manner similar to that observed for the human protein. Wernette-Hammond et al. (20) have demonstrated that when sialylated human apoE is subjected to amino acid sequence analysis, the yields of threonine 194 were substantially reduced as compared to the yields of threonine determined from sequencing other apoE peptides and to the yield of threonine 194 determined in unsialylated apoE. Sequence data on peptide D6, which contains threonine 188 and a second threonine

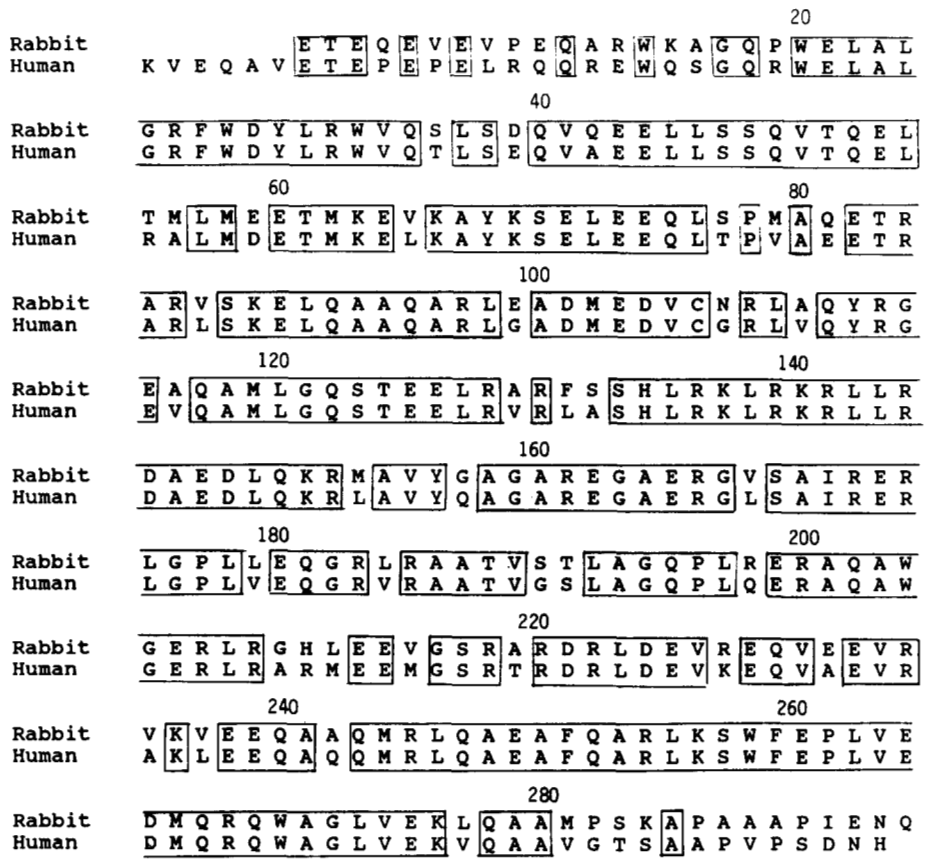

Fig. 4. Comparison of the amino acid sequence of rabbit apoE with that of human apoE3. The numbers correspond to the amino acid positions of rabbit apoE. Boxes enclose amino acids that are identical in both rabbit and human apoE. The amino acid sequence for human apoE is taken from Rall et al. (1).

at position 191, demonstrated that the yields of threonine 188 were greater than for threonine 191 (as might be expected since the yields decrease with each successive amino acid in a peptide), suggesting that threonine 188 of rabbit plasma apoE may not be O-glycosylated.

Clusters of nonhomologous sequence are found at the COOH- and NH_2-termini; the remaining differences are grouped in clusters of no more than three amino acids scattered throughout the protein. Other than the regions of nonhomology at the carboxyl and amino termini, the distribution of hydrophobic and charged amino acids is quite well conserved, suggesting that rabbit apoE probably folds in a manner similar to the human protein with distinct COOH- and NH_2-domains.

The abundance of apoE in rabbit plasma, and the relative ease with which it is isolated from the source, make it possible to conduct some in vitro experiments with the rabbit apoprotein that would be difficult or impossible with the human counterpart. The great similarity in the amino acid sequences of the two proteins suggests that their physical and physiological properties may be similar as well. Indeed, Mahley et al. (21) have reported that recombinant human apoE, with biological activity identical to that of naturally ocurring human apoE3, accelerated the clearance of rabbit plasma lipoproteins. Conversely, we have observed that rabbit apoE/DMPC disks, and DMPC/CO microemulsions to which rabbit apoE has been added competed very effectively with ^{125}I-labeled human LDL for binding, uptake, and degradation by human fibroblasts (22). For example, at 4°C, 0.17 μg/ml of rabbit apoE/DMPC gave nearly 70% (69.1%) displacement of ^{125}I-labeled LDL (0.5 μg/ml) from the receptors as compared to a 61% displacement by human LDL at 2.5 μg/ml. This result correlated very well with similar measurements by Innerarity, Pitas, and Mahley (23) which demonstrated that, at 4°C, 0.09 μg/ml of human apoE/DMPC complexes gave a 50% displacement of ^{125}I-labeled LDL (2.0 μg/ml) from the fibroblast LDL receptor as compared to a 50% displacement by human LDL at 4 μg/ml. Thus, like human apoE/DMPC complexes, rabbit apoE/DMPC complexes exhibited enhanced binding activity as compared to the apoB-containing LDL. Hence it appears that many experiments conducted with rabbit apoE will have direct relevance to human apoE.

We thank Billy Touchstone for technical assistance, Susan Kelly for graphic arts, and Rosetta Ray for manuscript preparation. This investigation was supported by a Specialized Center of Research in Atherosclerosis (HL-27341) (J. D. M. and C-Y. Y.), by Texas Heart Association Grant-in-Aid 88G-205 (M. P. M.), by American Heart Association Grant-in-Aid 870863 (C-Y. Y.), by a grant from The Methodist Hospital (C-Y. Y.), and by a grant from the Robert A. Welch Foundation (Q-837) (J. D. M.).

Manuscript received 13 August 1990 and in revised form 2 October 1990.

REFERENCES

1. Rall, S. C., Jr., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E: the complete amino acid sequence. *J. Biol. Chem.* **257:** 4171–4178.
2. Innerarity, T. L., and R. W. Mahley. 1981. Enhanced binding by cultured human fibroblasts of apoE-containing lipoproteins as compared with low density lipoproteins. *Biochemistry.* **17:** 1440–1447.
3. Mahley, R. W., D. Y. Hui, T. L. Innerarity, and K. H. Weisgraber. 1981. Two independent lipoprotein receptors on hepatic membranes of dog, swine, and man. Apo-B,E and apo-E receptors. *J. Clin. Invest.* **68:** 1197–1206.
4. Wetterau, J. R., L. P. Aggerbeck, S. C. Rall, Jr., and K. H. Weisgraber. 1988. Human apolipoprotein E3 in aqueous solution. I. Evidence for two structural domains. *J. Biol. Chem.* **263:** 6240–6248.
5. Aggerbeck, L. P., J. R. Wetterau, K. H. Weisgraber, C. C. Wu, and F. T. Lindgren. 1988. Human apolipoprotein E3 in aqueous solution. II. Properties of the amino and carboxyl terminal domains. *J. Biol. Chem.* **263:** 6249–6248.
6. Gidez, L. I., P. S. Roheim, and H. A. Eder. 1965. Effect of diet on the cholesterol ester composition of liver and of plasma lipoproteins in the rat. *J. Lipid Res.* **6:** 377–384.
7. Mahley, R. W., K. H. Weisgraber, T. Innerarity, H. B. Brewer, and G. Assmann. 1975. Swine lipoproteins and atherosclerosis, changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. *Biochemistry.* **14:** 2817–2823.
8. Hao, Q., T. Yamin, T. Pan, S. Chen, B. Chen, P. A. Kroon, and Y. S. Chao. 1987. Isolation and characterization of a full-length rabbit apolipoprotein E cDNA. *Atherosclerosis.* **66:** 125–130.
9. Roth, R. I., R. L. Jackson, H. J. Pownall, and A. M. Gotto. 1977. Interaction of plasma "arginine-rich" apolipoprotein with dimyristoyl phosphatidylcholine. *Biochemistry.* **16:** 5030–5036.
10. Gross, E., and B. Witkop. 1962. Nonenyzmatic cleavage of peptide bonds: the methionine residues in bovine pancreatic ribonuclease. *J. Biol. Chem.* **237:** 1856–1860.
11. Drapeau, G. R. 1980. Substrate specificity of a proteolytic enzyme isolated from a mutant of *Pseudomonas fragi*. *J. Biol. Chem.* **255:** 839–840.
12. Yang, C-Y., E. Pauly, H. Kratzin, and N. Hilschmann. 1981. Chromatography and rechromatography in high-performance liquid chromatography of peptide mixtures: the complete primary structure of an immunoglobulin L-chain of K-type, subgroup I (Bence-Jones protein den). *Hoppe-Seyler's Z. Physiol. Chem.* **362:** 1131–1146.
13. Chang, J. Y., D. Brauer, and B. Wittman-Liebold. 1978. Microsequence analysis of peptides and proteins using 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate/phenylisothiocyanate double coupling method. *FEBS Lett.* **93:** 205–214.
14. Yang, C-Y. 1979. The separation of the 4-[4-(dimethylamino)-phenylazo]phenylthiohydantoin derivatives of leucine and isoleucine on polyamid thin-layer sheets in the picomol range. *Hoppe-Seyler's Z. Physiol Chem.* **360:** 1673–1675.
15. Yang, C-Y., and S. J. Wakil. 1984. Separation of dimethylaminoazobenzenethiohydantoin amino acids by high-performance liquid chromatography at low picomole concentrations. *Anal. Biochem.* **137:** 54–57.
16. Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer. 1981. A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* **256:** 7990–8025.
17. Yang, C-Y., and F. I. Sepulveda. 1985. Separation of

phenylthiocarbamyl amino acids by high-performance liquid chromatography on Spherisorb octadecylsilane columns. *J. Chromatogr.* **346:** 413–416.
18. Zannis, V. I., J. L. Breslow, T. R. SanGiacomo, D. P. Aden, and B. B. Knowles. 1981. Characterization of the major apolipoproteins secreted by two human hepatoma cell lines. *Biochemistry.* **20:** 7089–7096.
19. Rall, S. C., K. H. Weisgraber, T. L. Innerarity, and R. W. Mahley. 1985. The carbohydrate moiety of human plasma apolipoprotein E. *Circulation* **72:** 111–143.
20. Wernette-Hammond, M. E., S. J. Lauer, A. Corsini, D. Walker, J. M. Taylor, and S. C. Rall, Jr. 1989. Glycosylation of human apolipoprotein E: the carbohydrate attachment site is threonine 194. *J. Biol. Chem.* **264:** 9094–9101.
21. Mahley, R. W., K. H. Weisgraber, M. M. Hussain, B. Grienman, M. Fisher, T. Vogel, and M. Goreck. 1989. Intravenous infusion of apolipoprotein E accelerates clearance of plasma lipoproteins in rabbits. *J. Clin. Invest.* **83:** 2125–2130.
22. Mims, M. P., M. R. Soma, and J. D. Morrisett. 1990. Effect of particle size and temperature on the conformation and physiological behavior of apolipoprotein E bound to model lipoprotein particles. *Biochemistry.* **29:** 6639–6647.
23. Innerarity, T. L., R. E. Pitas, and R. W Mahley. 1979. Binding of arginine-rich (E) apolipoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254:** 4186–4190.