Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 210-216

APOLIPOPROTEIN B IS A GLOBULAR PROTEIN - MORPHOLOGICAL STUDIES BY ELECTRON MICROSCOPY

Diana M. Lee,[†‡] David L. Stiers[§], and Tina Mok[†]

[†]Lipoprotein and Atherosclerosis Research Program and [§]Electron Microscopy Laboratory, Oklahoma Medical Research Foundation, the [†]Department of Biochemistry and Molecular Biology, and the [§]Department of Anatomical Science, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

Received February 10, 1987

SUMMARY: Water-soluble apolipoprotein B was prepared from fresh plasma by quick isolation of low density lipoproteins and immediate delipidization under non-oxidative conditions. The denatured protein in 6 M guanidine•HCl was reduced and carboxymethylated, dialyzed through 6 M urea/preservatives and to 1% ammonium acetate/0.05% EDTA/0.13% ε-amino caproic acid, pH 7.3 under N_2 at 4°C. The morphological studies were carried out by electron microscopy with negative staining and freeze fracture. Both these techniques showed that apolipoprotein B is a globular protein with average diameter of 11.48 ± 1.25 nm (n=978). The M.W. of apolipoprotein B calculated from this particle size was comparable to that from amino acid sequence.

Apolipoprotein B (ApoB) is the major protein moiety of triglyceride-rich and low density lipoproteins (LDL). It is the major carrier for cholesterol and triglycerides in the circulation. ApoB is the ligand of LDL receptor in many cells (1,2), and it plays a key role in the regulation of cholesterol metabolism (1).

For decades, ApoB has been known as an insoluble protein in aqueous buffers after the removal of lipids from its lipoproteins. The general conception about ApoB is that it is probably fibrous-shaped so that the protein tends to adhere to each other in aqueous buffers and causes insolubility. Indeed, there was an early study showing ApoB solubilized in detergent sodium

The abbreviations used are: ApoB, apolipoprotein B; LDL, low density lipoproteins (d 1.006-1.063 g/ml); LDL_2, LDL of d 1.019-1.063 g/ml or its narrow density range of d 1.032-1.043 g/ml for present study; BHT, butylated hydroxytoluene; RCM-, reduced and carboxymethylated; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

‡To whom correspondence and reprint requests should be addressed.

0006-291X/87 $1.50

decyl sulfate had a long fibrillar appearance (3). Recent success in solubilizing ApoB in the absence of detergent (4-6) has provided the opportunities for examining the secondary structure of ApoB in aqueous buffers. It has been shown that ApoB consists of all three secondary structure components, i.e. α-helix, β-sheets and other random structure (6,7), just as these are all present in native lipoprotein B particles or in other common proteins. Most recently, the amino acid sequence of ApoB has been deduced from its complement cDNA sequence (8-10). It consists of 4536 amino acids with molecular weight of 512,937 daltons (8-10). It is probably the largest single polypeptide chain found in plasma. The sequence data also allow the prediction that the secondary structure of ApoB should consist of α-helix regions, β-pleated sheets, and random structure (8). Then, it is more likely that in the absence of denaturing agent or detergent, ApoB may be like other common proteins - being a spherical particle.

The purpose of this study was to examine the morphology of ApoB by electron microscopy with two different techniques, i.e. negative staining and freeze fracture.

MATERIALS AND METHODS

Collection of plasma. Plasma samples were collected by plasmapheresis from normolipidemic subjects after a 12 h overnight fast. Immediately after collection of a plasma sample, a preservative (100-fold) stock solution was injected into the plasmapheresis bag to yield final concentrations of the antibiotics, 500 units/ml penicillin-G, 50 μg/ml streptomycin sulfate, a proteinase inhibitor, 0.13% ε-amino caproic acid, and antioxidants, 0.1% EDTA and 0.05% reduced glutathione (5) (designated preservative D). In addition, from a freshly prepared 75% alcoholic preservative solution, a 200-fold dilution was made to yield in the plasma final concentrations of 25 μg/ml chloramphenicol and 50 μg/ml butylated hydroxytoluene (BHT) (11).

Isolation of low density lipoproteins (LDL_2). Narrow density range of LDL_2 (apparent d, 1.032 - 1.043 g/ml) were isolated immediately from the fresh plasma by a single-spin density gradient ultracentrifugation (12) and purified by reflotation in the presence of preservative D (5). Throughout the preparations, samples and reagents were always maintained at 4°C under N_2. The purified LDL_2 were free of albumin as demonstrated by immunochemical analysis with anti-albumin. Analysis of the protein moiety by electroimmunoassay (13-15) indicated that >98% of the protein in such LDL_2 preparations was ApoB.

Preparation of water-soluble ApoB. The LDL_2 samples were immediately delipidized with ethenol-ether (3:1,v/v) under N_2. The ether was purified and redistilled before use (5). The delipidization procedure and solubilization of ApoB were carried out according to the method of Lee et al (5) with minor

modifications: 1) antioxidants 0.02% BHT and 0.02% tryptamine were added to ethanol under N_2 (Singh and Lee, unpublished results), 2) the pH of the buffer solutions for 6 M guanidine·HCl and 6 M urea was adjusted to 7.3, and 3) the reduced and carboxymethylated (RCM-) ApoB in 6 M urea/0.05 M Tris/preservatives was diluted with the same urea buffer to approximately 0.1 mg/ml and dialyzed extensively against 1% ammonium acetate/0.05% EDTA/0.13% ε-amino caproic acid, pH 7.3, saturated with N_2 at 4°C.

Polyacrylamide gel electrophoresis (PAGE) in SDS. The ApoB in 6 M urea and in ammonium acetate were analyzed by 3.3% PAGE in SDS according to the method of Fairbanks et al (16) with modifications as described previously (17). About 10 μg of protein was applied onto each gel.

Electron Microscopy

A. Negative staining. In preparation for negative staining a sample of Apo B was diluted 1:5000 in 1% ammonium acetate buffer. A bacitracin-treated formvar coated grid was placed onto a drop of the diluted specimen for 2-3 minutes. The grid was then drained briefly by touching the edge to a piece of filter paper and then placed membrane side down onto a drop of 2% phosphotungstic acid, pH 5.5 After 2 minutes, the grid was picked up, drained with a piece of filter paper, air dried and then examined in a JEOL 1200 analytical electron microscope at 60 kV.

B. Freeze fracture. A small amount of sample (~0.1 μl) of ApoB solution was sandwiched between thin (0.1 mm) copper specimen support plates and cryofixed ultrarapidly by plunging into liquid propane at 85°K in a Reichert-Jung KF 80 immersion cryofixation system. Support plates containing the frozen sample were loaded under liquid nitrogen into a double replica holder and transferred to a Reichert-Jung Cryofract 190 freeze fracture unit. The samples were fractured near liquid nitrogen temperature and at 10^{-9} Torr. The fractured samples were shadowed with 1.2 nm Pt/carbon at an angle of 45° and followed by 9 nm carbon at a 90° angle. Collodion was used to stabilize the replicas (18) and 50 mesh gold grids, as described by Fetter and Costello (19), during the cleaning process. Grids onto which the replicas had adsorbed were picked up from below on formvar supporting films spread over platinum loops, allowed to dry, carefully removed from the platinum loops and the collodion then dissolved in methanol. All replicas were examined as above. In all micrographs of the freeze fracture replicas, the deposited metal is black and shadows white. Shadow direction is from below as indicated by arrows.

RESULTS AND DISCUSSION

The water-soluble ApoB in ammonium acetate showed a single band on SDS-PAGE (Fig. 1), suggesting the homogeneity in size. Its mobility was identical to that of ApoB in 6 M urea or in intact LDL_2, suggesting that during delipidization, solubilization, carboxymethylation and dialysis of ApoB, the size was not altered.

Figure 2 shows the electron micrographs of negatively stained ApoB particles. This figure provides evidence that ApoB is a globular protein. Freeze fracture of ApoB also confirmed that ApoB is a spherical particle (Fig. 3).

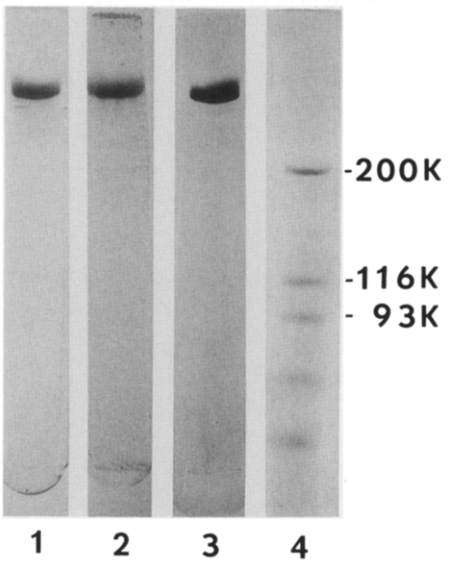

Fig. 1. Analysis of water-soluble ApoB on 3.3% SDS-PAGE. 1. RCM-ApoB in 1% ammonium acetate/0.05% EDTA/0.13% ε-amino caproic acid, pH 7.3. 2. RCM-ApoB in 6 M urea/0.05 M Tris/0.05% thiodiglycol/0.1% dithiothreitol/0.02% tryptamine/preservative D, pH 7.3. 3. Intact LDL_2 (d 1.032-1.043 g/ml). Each sample containing about 10 μg of ApoB was applied onto each gel. 4. Calibration protein standards, the top three bands being: myosin, β-galactosidase and phosphorylase B.

The average diameter measured from the micrographs of negatively stained particles was 11.48 ± 1.25 nm (n=978) and from the freeze fracture approximately 13 nm. These two measurements are comparable considering that in freeze fracture 1.2 nm Pt/carbon was added to the fractured ApoB. The variation in particle size was probably due to the varying depth of the particles from the surface.

From the average particle diameter, one can calculate the molecular weight of ApoB using Oncley's equation (20),

$$M.W = 0.317\ \bar{d}\ (Dia)^3$$

where Dia. is the diameter of the particle in A and $\bar{d}$ is the density of the particle and the reciprocal value of partial specific volume $\bar{v}$ of the protein. Based on the amino acid composition derived from the sequence of ApoB (8) and the $\bar{v}$ from each amino acid, the composite $\bar{v}$ for the protein moiety is 0.740 ml/g. Since ApoB is a glycoprotein, the carbohydrate contents in ApoB are: manose 1.56%, galactose 0.71%, glucosamine 1.50%, and sialic

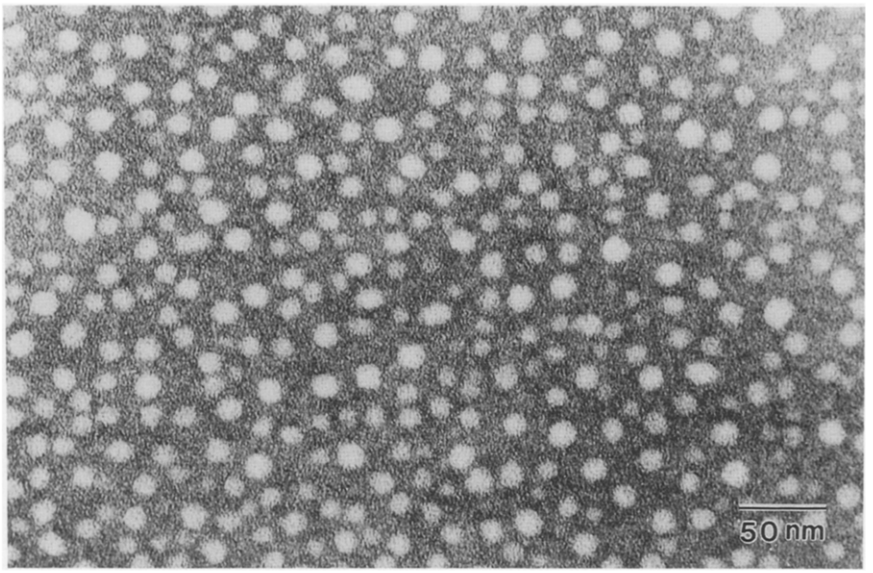

Fig. 2. Electron micrographs of negatively stained RCM-ApoB. The RCM-ApoB was dialyzed under N_2 and diluted with 1% ammonium acetate/0.05% EDTA/0.13% ε-amino caproic acid, pH 7.3 to a suitable protein concentration before application to a grid. The average diameter is 11.48 ± 1.25 nm SD (n=978). The original magnification factor was 208,000X.

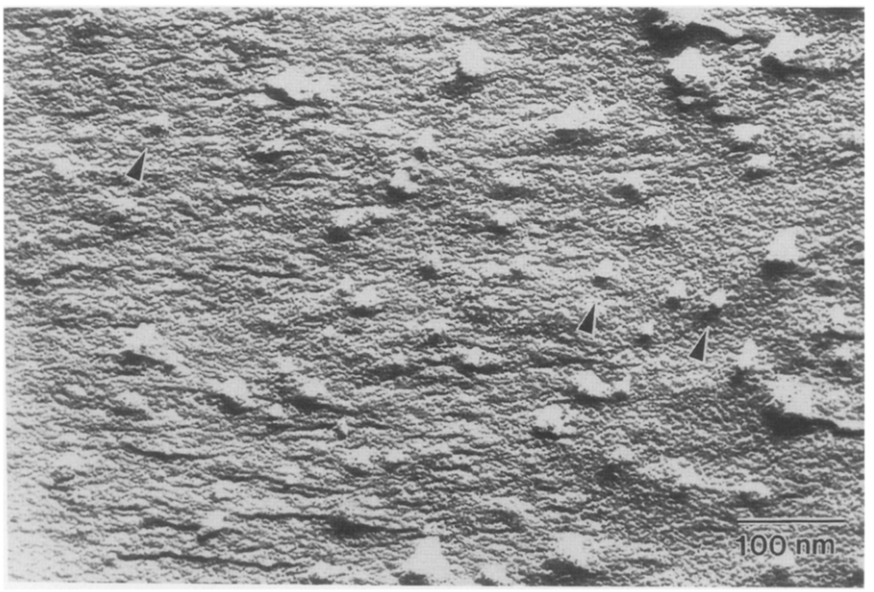

Fig. 3. Electron micrographs of RCM-ApoB by freeze fracture. The RCM-ApoB was dialyzed in the same buffer as above. Preparation of freeze fracture of the sample was described in the Methods. The average diameter is approximately 13 nm. The original magnification factor was 137,000X.

acid 1.05% (21). The $\bar{v}$ for each of these components is 0.613, 0.613, 0.666 and 0.584 ml/g, respectively (22). Therefore the weighted $\bar{v}$ for the glycoprotein moiety of ApoB is calculated to be 0.734 ml/g. Substituting the reciprocal value of 0.734 into $\bar{d}$ and 114.8 A into Dia., the M.W. of ApoB is calculated to be 653,000 daltons. This value is fairly comparable to the 513,000 daltons obtained from amino acid sequence (8-10). If 4.82% of carbohydrate content is added to the mass of the published value, the theoretical M.W. of the glycoprotein would be 539,000 daltons.

This study demonstrates that delipidization of LDL and denaturation of ApoB in guanidine or in urea is a reversible process. After the removal of the denaturing agents, ApoB is able to refold back to a globular shape as all water-soluble proteins do. That is, if the critical amino acid residues are not altered or cleaved during the processes, the thermodynamic driving force is able to direct the protein chain to refold in such a manner that the hydrophobic side chains are expelled from water and folded inward and the hydrophilic side chains are folded outward toward water. This finding is consistent with our earlier observation that after dialysis of ApoB from guanidine solution through urea, passing to aqueous buffers, ApoB is able to regain all its α-helical content originally present in the native lipoprotein B particles (7). Refolding of the ApoB chain in aqueous buffers was also observed by fluorescence spectroscopic studies where we found that all of the tryptophan residues were exposed to the surface when ApoB was in 6 M guanidine·HCl, or in 6 M urea, but some of the tryptophan residues turned into a partially, and some into a totally, buried environment upon the complete removal of the denaturants (23).

From the results of the present study, we may predict that the de novo synthesized ApoB is a water-soluble globular protein prior to its association with lipids.

ACKNOWLEDGEMENTS

We would like to express our appreciation to Gerald Daley for the excellent technical assistance and to the staff of Word Processing for typing this manuscript. This work was supported in part by grant HL-23181 from the

National Heart, Lung, and Blood Institute of the National Institutes of Health, and by the resources of the Oklahoma Medical Research Foundation.

REFERENCES

1. Goldstein, J.L., and Brown, M.S. (1977) Ann. Rev. Biochem. 46, 897-930.
2. Dashti, N., Wolfbauer, G., Koren, E., Knowlee, B., and Alaupovic, P. (1984) Biochim. Biophys. Acta 794, 373-384.
3. Gotto, A.M., Levy, R.I., Rosenthal, A.S., Birnbaumer, M.E., and Fredrickson, D.S. (1968) Biochem. Biophys. Res. Commun. 31, 699-705.
4. Huang, S.S., and Lee, D.M. (1979) Biochim. Biophys. Acta 577, 424-441.
5. Lee, D.M., Valente, A.J., Kuo, W.H., and Maeda, H. (1981) Biochim. Biophys. Acta 666, 133-146.
6. Cardin, A.D., Witt, K.R., Barnhart, C.L., and Jackson, R.L. (1982) Biochemistry 21, 4503-4511.
7. Singh, S., and Lee, D.M. (1986) Biochim. Biophys. Acta 876, 460-468.
8. Chen, S-H., Yang, C-Y., Chen, P-F., Setzer, D., Tanimura, M., Li, W-H., Gotto, A.M., Jr., and Chan, L. (1986) J. Biol. Chem. 261, 12918-12921.
9. Knott, T.J., Wallis, S.C., Powell, L.M., Pease, R.J., Lusis, A.J., Blackhart, B., McCarthy, B.J., Mahley, R.W., Levy-Wilson, B., and Scott, J. (1986) Nuc. Acids Res. 14, 7501-7503.
10. Law, S.W., Grant, S.M., Higuchi, K., Hospattankar, A., Lackner, K., Lee, N., and Brewer, H.B., Jr. (1986) Proc. Natl. Acad. Sci. USA 83, 8142-8146.
11. Lee, D.M., and Alaupovic, P. (1986) Biochim. Biophys. Acta 879, 126-133.
12. Lee, D.M., and Downs, D. (1982) J. Lipid Res. 23, 14-27.
13. Curry, M.D., Gustafson, A., Alaupovic, P., and McConathy, W.J. (1978) Clin. Chem. 24, 280-286.
14. Curry, M.D., McConathy, W.J., Fesmire, J.D., and Alaupovic, P. (1980) Biochim. Biophys. Acta 617, 503-513.
15. Curry, M.D., McConathy, W.J., Alaupovic, P., Ledford, J.H., and Popovic, M. (1976) Biochim. Biophys. Acta 439, 413-425.
16. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
17. Lee, D.M., Koren, E., Singh, S., and Mok, T. (1984) Biochem. Biophys. Res. Commun. 123, 1149-1156.
18. Sommer, J.R., Wallace, N.R., and Junker, J. (1980) J. Ultrastruct. Res. 71, 126-142.
19. Fetter, R.D., and Costello, M.J. (1986) J. Microsc. 141, 277-290.
20. Oncley, J.L. (1963) in Brain Lipids and Lipoproteins, and the Leucodystrophies, Folch-Pi, J., and Bauer, H., eds., pp. 1-17, Elsevier Publishing Co., New York.
21. Huang, S.S. (1977) Ph.D. Dissertation, The University of Oklahoma.
22. Gibbons, R.A. (1966) in Glycoproteins, Their Composition, Structure and Function, Gottschalk, A., ed., pp. 29-95, Elsevier Publishing Co., Amsterdam.
23. Singh, S., and Lee, D.M. (1983) Int. J. Biol. Macromol. 5, 243-248.