ersity Park Press, Baltimore, MD.
Lambert, J. M., & Traut, R. R. (1981) *J. Mol. Biol. 149*, 451–476.
Lambert, J. M., Jue, R., & Traut, R. R. (1978) *Biochemistry 17*, 5406–5416.
Langberg, S., Kahan, L., Traut, R. R., & Hershey, J. W. B. (1977) *J. Mol. Biol. 117*, 307–319.
MacKeen, L. A., Kahan, L., Wahba, A. J., & Schwartz, I. (1980) *J. Biol. Chem. 255*, 10526–10531.
O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell (Cambridge, Mass.) 12*, 1133–1142.
Pon, C. L., Brimacombe, R., & Gualerzi, C. (1977) *Biochemistry 16*, 5681–5686.
Shatsky, I. N., Evstafieva, A. G., Bystrova, T. F., Bogdanov, A., & Vasiliev, V. D. (1980) *FEBS Lett. 121*, 97–100.
Shih, C. T., & Craven, G. R. (1973) *J. Mol. Biol. 78*, 6651–663.
Sommer, A., & Traut, R. R. (1976) *J. Mol. Biol. 106*, 995–1015.
Stöffler, G., & Stöffler-Meilicke, M. (1981) in *International Cell Biology 1980–1981* (Schweiger, H. R., Ed.) pp 93–102, Springer-Verlag, Berlin and Heidelberg.
Stöffler, G., Balet, R., Kastner, B., Luhrmann, R., Stöffler-Meilicke, M., & Tishendorf, G. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 171–205, University Park Press, Baltimore, MD.
Stöffler-Meilicke, M., Stöffler, G., Odom, O. W., Zinn, A., Kramer, G., & Hardesty, B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5538–5542.
Strycharz, W. A., Nomura, M., & Lake, J. A. (1978) *J. Mol. Biol. 126*, 123–140.
Tolan, D. R., Lambert, J. M., Boileau, G., Fanning, T. G., Kenny, J. W., Vassos, A., & Traut, R. R. (1980) *Anal. Biochem. 103*, 101–109.
Traut, R. R., Lambert, J. M., Boileau, G., & Kenny, J. W. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 29–110, University Park Press, Baltimore, MD.
Van der Hofsted, G. A. J. M., Buitenhek, A., Ven den Elsen, P. J., Voorma, H. O., & Bosch, L. (1978) *Eur. J. Biochem. 89*, 221–228.
Van Duin, J., Kurland, C. G., Dondon, J., & Grunberg-Manago, M. (1975) *FEBS Lett. 59*, 287–290.
Weiel, J., & Hershey, J. W. B. (1981) *Biochemistry 20*, 5859–5865.
Wittmann, H. G., Littlechild, J. A., & Wittmann-Liebold, B. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 51–88, University Park Press, Baltimore, MD.

Solubilization of Low-Density Lipoprotein with Sodium Deoxycholate and Recombination of Apoprotein B with Dimyristoylphosphatidylcholine†

Mary T. Walsh* and David Atkinson‡

ABSTRACT: Apoprotein B (apoB) of human plasma low-density lipoprotein (LDL) (*d* 1.025–1.050 g/mL) has been solubilized with solid sodium deoxycholate (NaDC) above its critical micellar concentration. ApoB is isolated by gel-filtration chromatography as a mixed micellar complex of protein and detergent in high yield in a lipid-free form. A soluble apoB–dimyristoylphosphatidylcholine (DMPC) complex has been prepared by incubation of aqueous solutions of apoB–NaDC and DMPC–NaDC (2/1 w/w) at room temperature with detergent removal by extensive dialysis. A combination of gel chromatographic and density gradient fractionation of DMPC–apoB incubation mixtures demonstrates that a reasonably well-defined complex of DMPC and apoB is formed with a 4:1 w/w lipid:protein ratio. Negative-stain electron microscopy shows these particles to be single-bilayer phospholipid vesicles with a diameter of 210 ± 20 Å into which the apoB is incorporated. Circular dichroic spectra of NaDC-solubilized apoB show apoB to have similar conformation to that seen in the native LDL particle. However, apoB that has been complexed with DMPC exhibits more α-helix. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis shows a single band (apparent M_r 366 000) for apoB after solubilization, purification, and interaction with phospholipid. The behavior of apoB during its reassociation with phospholipid and the structural features of the DMPC–apoB particle are similar to those observed in the interaction of solubilized membrane proteins with lipid rather than that of other apolipoproteins.

Low-density lipoprotein (LDL)[1] is the primary transport particle for cholesterol in the plasma (Brown & Goldstein, 1976) and is a microemulsion of nonpolar lipids, cholesteryl ester, and triglyceride, whose surface is stabilized by a monolayer of polar phospholipids and cholesterol, together with apoprotein B (apoB) (Nelson, 1979). ApoB functions as the ligand for the LDL receptor in cells of the peripheral tissue

† From the Biophysics Institute, Departments of Medicine and Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118. *Received September 8, 1982; revised manuscript received March 24, 1983.* This work was supported by U.S. Public Health Service Grants HL26335 and HL07291. This paper was written during the tenure of the Samuel A. Levine Fellowship from the American Heart Association for 1982–1983 to M.T.W., Greater Boston, Massachusetts Division 13-437-812.

‡ D.A. is an Established Investigator of the American Heart Association.

[1] Abbreviations: LDL, low-density lipoprotein; apoB, apoprotein B; cmc, critical micellar concentration; NaDC, sodium deoxycholate; DMPC, 1,2-dimyristoylphosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate; NaPTA, sodium phosphotungstic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; V_0, void volume; V_t, total volume, L:P, lipid to protein ratio.

0006-2960/83/0422-3170$01.50/0

(Basu et al., 1977; Mahley & Innerarity, 1978).

Little information is currently available on the chemical and physical properties of apoB mainly because of the insolubility of delipidated apoB in aqueous buffers in the absence of detergents or high concentrations of denaturants (Steele & Reynolds, 1979a; Morrissett et al., 1975; Smith et al., 1972). Therefore, most information concerning the lipid–protein interactions in LDL comes from observations on studies performed on the intact native lipoprotein (Dearborn & Wetlaufer, 1969; Forte & Nichols, 1972; Yeagle et al., 1977).

The solubilization of apoB and its reassembly with well-characterized lipids by use of either the surface-located polar lipids to give a partially recombined particle or polar and nonpolar lipids to give a totally recombined particle will provide a well-defined system in which to study the detailed interactions of apoB and lipids that stabilize the native LDL particle.

Delipidation of LDL has been accomplished in previous procedures by organic solvent extraction (Smith et al., 1972) or solubilization of the protein and lipid moieties with a variety of detergents, above their critical micellar concentrations. Detergent solubilization of LDL yields mixed micelles of apoB and detergent (Helenius & Simons, 1971; Steele & Reynolds, 1979a; Watt & Reynolds, 1980). Detergent may substitute for the naturally occurring lipid environment of the LDL particle to produce apoB–detergent complexes that are stable and soluble in aqueous buffers.

The choice of a detergent for solubilization of either an intrinsic membrane protein or apoB of LDL is quite empirical. However, several important factors must be considered. A high critical micellar concentration (cmc) is desirable if the detergent is to be rapidly removed by dialysis or displaced with another detergent or other lipids (Helenius et al., 1979; Razin, 1972). A second consideration is the type of detergent. Alkyl ionic detergents usually denature membrane proteins at the concentrations that are necessary to solubilize them (Helenius et al., 1979). Nonionic detergents can usually be used to solubilize membrane proteins in the native state since they do not, in general, denature proteins. These detergents are quite effective in disrupting lipid–lipid and lipid–protein interactions; however, they do not effectively disrupt protein–protein interactions (Furth, 1980). Therefore, aggregates may well form in the presence of this type of detergent. Bile salts, such as sodium cholate and sodium deoxycholate, are weak ionic detergents and have been widely used to solubilize intrinsic membrane proteins (Racker, 1979; Hwang & Stoeckenius, 1977), as well as apoB of LDL. As shown by Helenius & Simons (1971), sodium deoxycholate (NaDC) at pH 10 in sodium chloride–sodium carbonate buffer has a CMC of 2–3 mM and solubilizes apoB of LDL to produce a lipid-free protein–detergent complex.

On the basis of the method of Helenius & Simons (1971), we have developed a method to solubilize LDL and isolate apoB in a homogeneous, lipid-free form. In addition, a soluble, stable protein–phospholipid complex has been prepared between apoB and dimyristoylphosphatidylcholine (DMPC). The DMPC–apoB recombined particle has been characterized by gel-filtration chromatography and may be isolated in a homogeneous form by density gradient ultracentrifugation.

Experimental Procedures

Materials

All chemicals were standard reagent grade unless otherwise indicated. Iodoacetamide, 2-[[tris(hydroxymethyl)methyl]-amino]ethanesulfonic acid (Tes), Amberlite XAD-2, and dinitrophenyl amino acid standards were purchased from Sigma (St. Louis, MO). Sodium deoxycholate (NaDC) was purchased from Calbiochem-Behring (La Jolla, CA) and twice recrystallized from 80% ethanol. Sodium [^{14}C]deoxycholate was purchased from Amersham (Arlington Heights, IL) and was judged to be 99% radiopure when chromatographed on silica gel G in 65/25/4/1 v/v chloroform–methanol–water–acetic acid. Sepharose CL-4B and all chromatographic columns were products of Pharmacia Fine Chemicals (Uppsala, Sweden). High molecular weight protein standards and reagents for polyacrylamide gel electrophoresis [acrylamide, ammonium persulfate, *N*,*N*′-methylenebis(acrylamide), sodium dodecyl sulfate ($NaDodSO_4$), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine] were electrophoresis purity grade, purchased from Bio-Rad (Richmond, CA), and were used without further purification. 1,2-Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma (St. Louis, MO). Thin-layer chromatography of DMPC on silica gel H plates (Analtech, Neward, DE) in 65/25/4 v/v chloroform–methanol–water showed only one spot as detected by sulfuric acid charring. DMPC was stored desiccated at −20 °C, under nitrogen and used without further purification. All solvents were reagent grade and redistilled prior to use. 1-Fluoro-2,4-dinitrobenzene was purchased from Eastman Organic Chemicals (Rochester, NY). Aquasol was purchased from New England Nuclear (Boston, MA).

Methods

Lipoprotein Isolation. Plasma was obtained from freshly drawn blood from normal human volunteers. Low-density lipoprotein was isolated by repetitive ultracentrifugation between salt densities of 1.025 and 1.050 g/mL by the addition of solid KBr (Havel et al., 1955). Isolated LDL was washed by ultracentrifugal flotation through an overlayering solution of d = 1.050 g/mL KBr. All centrifugation was performed at 55 000 rpm in a Beckman L8-70 ultracentrifuge in a 70 Ti rotor for 16 h at 4 °C. Purity of LDL from other lipoprotein fractions was verified by agarose electrophoresis (Noble, 1968) by staining with Oil Red O.

LDL was dialyzed for 24 h against 3–4-L changes of 0.1 M sodium bicarbonate, pH 8.6. By incorporation of the modification of Steele & Reynolds (1979a,b) and Watt & Reynolds (1980), the two free sulfhydryls per 250 000 g of protein were alkylated with a 1.5 molar excess of iodoacetamide at room temperature in the dark with gentle stirring for 2 h (Hirs, 1967; Gurd, 1967). The reaction was terminated, and excess reagent was removed by dialysis for 24 h against 3–4 L changes of 20 mM Tes–0.15 M sodium chloride, pH 7.4 at 4 °C. LDL was then dialyzed against 3–4 L changes of 0.05 M sodium chloride–0.05 M sodium carbonate, pH 10 (hereafter referred to as standard buffer), for 24 h.

Analytical Methods. Protein was quantitated by the Lowry (Lowry et al., 1951) protein method utilizing the modified procedure of Markwell et al. (1978), which incorporates 1% $NaDodSO_4$ in both bovine serum albumin standards and samples. Lipid phosphorus was assayed according to the method of McClare (1971). Cholesterol was measured by the method of Rudel & Morris (1973).

LDL Solubilization and ApoB Recovery. A total of 1.10 g of solid recrystallized NaDC was added slowly to LDL (20 mg of protein) in a 2.0-mL volume of standard buffer, and the sample was stirred gently for 3 h at room temperature in the dark. The clear orange viscous solution was then applied to a calibrated (V_0, Blue Dextran 2000; V_t, tryptophan) 2.5 × 40 cm column of Sepharose CL-4B and eluted downward at room temperature by passing the equilibrating buffer (standard buffer plus 10 mM NaDC, pH 10) over the column.

Approximately 3-g fractions were collected and analyzed for protein, phosphorus, and carotenoids (OD_{485}). Lipids were quantitated by Folch (Folch et al., 1957) extraction of appropriate column fractions followed by thin-layer chromatography (silica gel H plates; 90/10/1 v/v petroleum ether–diethyl ether–acetic acid) in an effort to detect residual phospholipid, cholesterol, or cholesteryl esters associated with apoB. Quantitation of the amount of detergent present in appropriate column fractions was determined by preequilibration of the column with eluting buffer containing sodium [^{14}C]deoxycholate and by including sodium [^{14}C]deoxycholate in the incubation mixture followed by scintillation counting of aliquots from column fractions in Aquasol in a Beckman LS-250 counter.

The protein-containing fractions from the column separation were pooled and concentrated to 2 mg of protein/mL by ultrafiltration with Amicon YM-10 filters (Lexington, MA). Protein was stored at 4 °C in 10-mg aliquots. No precipitation or degradation of the protein was observed in samples stored under these conditions for up to 4 weeks.

Preparation of Mixed Micelles of DMPC and NaDC. Solid DMPC was weighed into a tared 1-dram vial to which was added the appropriate mass of solid NaDC (DMPC:NaDC, 2:1 w/w). A total of 2.0 mL of standard buffer was added to the vial, which was then capped and vigorously shaken at room temperature for 2 h. An optically clear mixed micellar solution resulted under these conditions.

Preparation of DMPC–ApoB Complexes. A total of 2.0 mL of DMPC–NaDC mixed micellar solution (ranging from 200 mg of DMPC/100 mg of NaDC to 10 mg of DMPC/5 mg of NaDC) was gently pipetted into a tube containing 10 mg of NaDC-solubilized apoB. Both solutions were at room temperature prior to mixing. The solution was gently mixed by stirring and was incubated at room temperature without agitation for 1 h.

Detergent removal was accomplished by dialysis against 3–4 L changes of standard buffer at 4 °C for 24 h followed by 6 L of standard buffer plus 10 g/L Amberlite XAD-2 for 12 h. The resulting optically clear solution was then concentrated from 8 to 2 mL by ultracentrifugation. No aggregation or precipitation was seen, and solutions remained optically clear (no turbidity) when stored for up to 2 weeks at 4 °C.

Column Chromatographic Fractionation of DMPC–ApoB Complexes. Samples of 2 mL of the concentrated DMPC–apoB incubation mixture were applied to a 2.5 × 40 cm column of Sepharose CL-4B at room temperature and eluted downward with detergent-free standard buffer. Fractions of 3 g were collected and analyzed for protein (Markwell et al., 1978) and phosphorus (McClare, 1971).

Density Gradient Fractionation of DMPC–ApoB Complexes. Following dialysis and concentration, DMPC–apoB incubation mixtures were fractionated by a modification of the density gradient lipoprotein isolation method developed by Redgrave et al. (1975). After adjustment of the density to d = 1.210 g/mL with solid KBr (0.325 g/mL), 3.0-mL aliquots of the incubation mixture were pipetted into 13.5-mL centrifuge tubes and overlayed with 2.0 mL of d = 1.120 solution, followed by 3.0 mL of d = 1.063, 2.0 mL of d = 1.060, and, finally, 3.0 mL of d = 1.057. These solutions were prepared by the addition of the appropriate amount of solid KBr to standard buffer. The samples were centrifuged for 20 h at 36 000 rpm in an SW41 rotor in a Beckman L8-70 centrifuge at 22 °C. After centrifugation, the gradients were fractionated by puncturing the bottom of the tube with a needle and collecting 0.6–0.8-g fractions.

A density calibration curve was plotted from refractive index data obtained from fractionation of tubes that contained the centrifuged salt solutions only. The refractive index of each fraction was measured with an Abbe refractometer (American Optical, Buffalo, NY) calibrated with water and potassium bromide solutions from d = 1.006 to 1.210 g/mL at 22 °C.

Preparation of DMPC–ApoB Complex with Sodium [^{14}C]Deoxycholate. A total of 10 μCi (50 μL) of [^{14}C]NaDC was added to 10 mg of NaDC-solubilized apoB in a 5-mL volume and equilibrated at room temperature for 2 h. Incubation with the DMPC–NaDC solution, dialysis, ultrafiltration, and gel filtration were accomplished as described above for unlabeled samples. Aliquots were removed from each change of dialysis buffer, from the sample after dialysis, and from protein-containing fractions from the Sepharose CL-4B column. These aliquots were monitored for radioactivity by liquid scintillation counting.

Circular Dichroism. Circular dichroic spectra of native LDL, NaDC-solubilized apoB, and DMPC–apoB complexes were recorded on a Cary 61 spectropolarimeter (courtesy of Dr. E. Simons, Department of Biochemistry, Boston University School of Medicine) calibrated with d-10-camphorsulfonic acid. A 1-cm quartz cell was used. Protein concentrations ranged from 0.03 to 0.09 mg/mL. All samples that had been prepared in standard buffer with or without 10 mM NaDC were dialyzed extensively against 0.005 M sodium tetraborate, pH 10, with or without detergent as appropriate before recording CD spectra.

All spectra reported are the average of four individual spectra on each of three to four different samples and have been corrected for base-line contributions. Following calculation of the molar ellipticity, the percentage of α-helix was calculated and the percentage of random coil and β-structure estimated according to the method of Greenfield & Fasman (1969) by utilizing the modified equation of Morrissett et al., (1973).

Electron Microscopy. DMPC–apoB complexes were negatively stained with 2% sodium phosphotungstate, pH 7.4, or 0.5% uranyl oxalate, pH 7.5, following the methods described by Melchior et al. (1980) on Formvar-coated copper grids. Electron micrographs were obtained with a Hitachi HU-11C electron microscope, calibrated with a grating replica (Pelco, Tustin, CA).

Gel Electrophoresis. Sodium dodecyl sulfate (NaDodSO$_4$)–polyacrylamide gel electrophoresis was performed according to the method of Weber & Osborn (1969) on 3.0 and 5.0% gels. The 3.0% gels were routinely used to analyze column fractions during the solubilization of apoB and recombination of apoB with DMPC, both for assessment of apoB degradation and for estimation of apoB molecular weight.

Quantitative Amino-Terminal Analysis. Quantitative amino-terminal analysis of LDL, NaDC-solubilized apoB, and DMPC–apoB complexes was performed according to the method reviewed by Levy (Fraenkel-Conrat et al., 1955). All dinitrophenyl-labeled amino acids were chromatographed on silica gel G plates to 70/30/5 v/v chloroform–methanol–acetic acid (Keller & Pataki, 1963).

Results

Solubilization and Delipidation of ApoB by NaDC. Alkylation of the two free sulfhydryl groups on apoB of LDL was performed with iodoacetamide in order to prevent the possible formation of intermolecular disulfide bonds (Steele & Reynolds, 1979a,b; Watt & Reynolds, 1981) and, hence, avoid formation of high molecular weight aggregrates. Recently, Cardin et al. (1982) have shown that high molecular

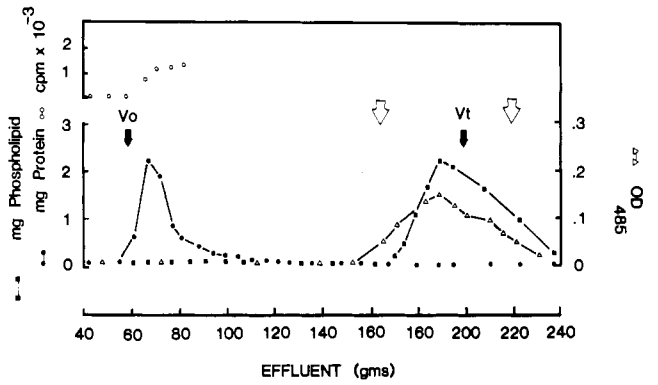

FIGURE 1: Gel-filtration chromatography of $1.025 < d < 1.050$ g/mL LDL solubilized with NaDC. LDL at 20 mg of LDL-protein in 2 mL of standard buffer was incubated with 55 mg of NaDC/mg of protein and applied to a Sepharose CL-4B column of dimensions 2.5 × 40 cm and eluted downward with standard buffer plus 10 mM NaDC at room temperature. Solid arrows mark V_0 (Blue Dextran 2000) and V_t (tryptophan) of the column. Open arrows mark elution position of [^{14}C]NaDC micelles. (○) cpm ([[^{14}C]NaDC]); (●) protein (mg/fraction); (■) phospholipid (mg/fraction); (△) OD_{485}.

weight aggregates of apoB are formed during lipid extraction of LDL by a sulfhydryl–disulfide exchange reaction that results in the formation of intermolecular disulfide bonds.

Complete solubilization and delipidation of apoB were obtained at a weight ratio of 55 mg of solid NaDC/mg of protein (total protein concentration of 5–10 mg/mL) (Helenius & Simons, 1971). As shown in Figure 1, on gel chromatography the protein elutes as a single peak over the range 60–120 g of effluent. Protein recovery was 71% (14.4 ± 1.0 mg—average of 20 experiments). A small, but variable amount of protein was detected in the void volume. Depending on the donor, the exact elution position of the solubilized apoB may vary slightly over the range 60–140 g. However, multiple samples from the same donor were reproducible. Rechromatography of the protein fractions that elute immediately after the void volume gave identical elution behavior of the protein-containing fractions. Carotenoids, phospholipid, cholesterol, triglyceride, and detergent elute as a broad fraction close to the total volume of the column, well separated from the NaDC-solubilized apoB.

Column fractions containing the solubilized apoB, for example, in Figure 1 from 65 to 100 g of effluent, were pooled and concentrated. The pooled and concentrated fractions containing 10 mg of NaDC-solubilized apoB in a volume of 2 mL were extracted by the method of Folch et al. (1957) after acidification to pH 2 (Helenius & Simons, 1971). No residual cholesterol, other neutral lipids, or phospholipids were detectable.

When sodium [^{14}C]deoxycholate was included in the solubilization mixture, radioactivity eluted in two peaks (see Figure 1): the first peak coeluting with the protein and the second (sodium deoxycholate micelles) coeluting with phospholipid and carotenoids. The NaDC-solubilized apoB from appropriate column fractions, e.g., 65–100 g in Figure 1, was used for subsequent experiments.

Gel-Filtration Chromatography of DMPC–ApoB Incubation Mixtures. Figure 2 illustrates elution profiles of a series of DMPC–apoB incubation mixtures on a Sepharose CL-4B column equilibrated and eluted with standard buffer. These profiles were reproducibly obtained from incubation mixtures ranging from 10:1 to 1:1 w/w DMPC:apoB.

Figure 2A shows a representative elution profile from a DMPC:apoB 10:1 w/w incubation mixture. All the protein applied to the column elutes as a fairly symmetrical peak from

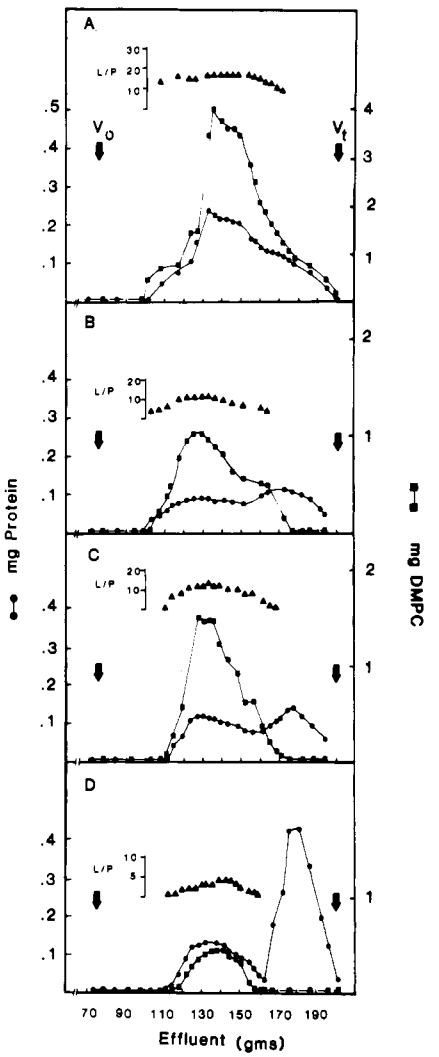

FIGURE 2: Gel-filtration chromatography of DMPC–apoB incubation mixtures on a Sepharose CL-4B column of dimensions 2.5 × 40 cm equilibrated with standard buffer as stated under Experimental Procedures. The following weight ratios (DMPC:apoB) were used: (A) 10:1; (B) 5:1; (C) 2.5:1; (D) 1:1. Arrows mark V_0 and V_t. (●) mg of protein/fraction; (■) mg of DMPC/fraction; (▲) L:P (mg of DMPC to mg of protein) per fraction.

100 to 190 g of effluent, with a suggestion of a slight shoulder at 170 g. The phospholipid coelutes with the major portion of the protein. Over the region of 130–150 g, where protein and phospholipid coelute, the weight ratio of lipid to protein is relatively constant and approximately 16:1 w/w as shown in Figure 2A.

Figure 2B shows an elution profile from a DMPC:apo B 5:1 w/w incubation mixture. At this weight ratio, protein elutes as two partially resolved peaks centered at 130 g (range 100–150 g) and 175 g (range 150–200 g). The major portion of the phospholipid elutes as a peak centered at 130 g (range of 100–150 g). However, a small phospholipid peak can also be seen at 165 g. Over the region from 118 to 138 g, where protein and phospholipid coelute, a relatively constant DMPC:apoB ratio is observed. However, in this case, the ratio corresponds to a lipid to protein ratio of 12:1 w/w.

An elution profile from a DMPC:apoB 2.5:1 w/w incubation mixture is illustrated in Figure 2C. At this weight ratio, protein elutes as two more distinctly resolved peaks centered at 130 (range 110–115 g) and 180 g (range 155–190 g). Phospholipid elutes as one peak and coelutes with the first protein peak at 130 g. In the central region of the coeluting phospholipid and protein peaks (125–145 g), a fairly constant

DMPC:apoB ratio is found once again. In this case the weight ratio of lipid to protein is 10.5:1.

The elution profile from a DMPC:apoB 1:1 w/w incubation mixture in Figure 2D shows that as observed for DMPC:apoB 5:1 and 2.5:1 w/w ratios, two protein peaks are observed. However, at this ratio, they are very distinct and appear to be eluting as totally separate species. A single phospholipid peak is observed that coelutes with the first protein peak at 135 g (range 110–160 g). Again, in the central region of the coeluting protein and phospholipid peaks (130–150 g), a constant lipid to protein ratio is observed, in this case 4:1 w/w.

At all phospholipid to protein ratios neither phospholipid nor protein elutes in the void volume. Quantitative thin-layer chromatography of Folch (Folch et al., 1957) extracts of phospholipid-containing fractions across the elution profiles show less than 1% degradation of DMPC to lyso-PC over the time course of preparation, dialysis, and gel filtration. The coelution of protein and phospholipid at constant ratios suggests the presence of a stable phospholipid–apoB complex with a lipid to protein ratio that varies with the incubation ratio.

When [^{14}C]NaDC was included in the incubation mixture for DMPC–apoB complexes, 99.8% of the detergent was found in the dialysis buffer after 36 h. After chromatography on Sepharose CL-4B, radioactivity was not detectable in association with protein, indicating that a negligible amount of detergent remains bound to the protein.

Gel-Filtration Chromatography of Detergent-Free ApoB. As shown in elution profiles of DMPC–apoB incubation mixtures (Figure 2), protein may elute as a two fractions. The first peak is centered at 130 g and coelutes with phospholipid, whereas the second peak elutes in the region 160–200 g, a region where little to no phospholipid elutes. The amount of protein eluting in this latter region is dependent on the initial incubation ratio of lipid and protein. Gel-filtration chromatography of apoB from which the detergent has been removed by extensive dialysis in the absence of phospholipid showed the protein to elute as one fairly symmetrical peak from 150 to 200 g of effluent, corresponding to the second protein peak present in chromatographic separations of DMPC–apoB mixtures. No protein elutes in the void volume.

Density Gradient Ultracentrifugation of DMPC–ApoB Incubation Mixtures. Figure 3 illustrates results obtained from density gradient ultracentrifugation and fractionation of a series of DMPC–apoB incubation mixtures ranging from 10:1 to 1:1 w/w.

Figure 3A shows a fractionation pattern of a 10:1 w/w incubation mixture. The major fraction of protein bands at the center of the tube between d = 1.105 and 1.063 g/mL. No protein is found in the top fractions; however, some protein is observed in the bottom of the tube. Phospholipid is located between d = 1.105 and 1.055 g/mL with the peak maximum at d = 1.084 g/mL. The higher density region of the phospholipid band overlaps the protein band centered at d = 1.086 g/mL. The lipid to protein ratio shows a relatively constant value of 4:1 w/w over the initial region where protein and phospholipid overlap. This ratio markedly increases toward the top of the gradient. Protein found in the higher density fractions is not associated with lipid.

Figure 3B shows a fractionation pattern of a 5:1 w/w incubation mixture. Protein once again is found in the bottom of the tube. However, the major portion of the protein bands between d = 1.112 and 1.074 g/mL. Phospholipid is found from d = 1.105 to 1.056 g/mL, with the peak maximum at d = 1.072 g/mL. The high-density region of the phospholipid band overlaps the protein band. Again, in the region of the peak protein band centered at d = 1.090 g/mL, the lipid to protein ratio is 4:1 w/w and increases at greater distances up the tube.

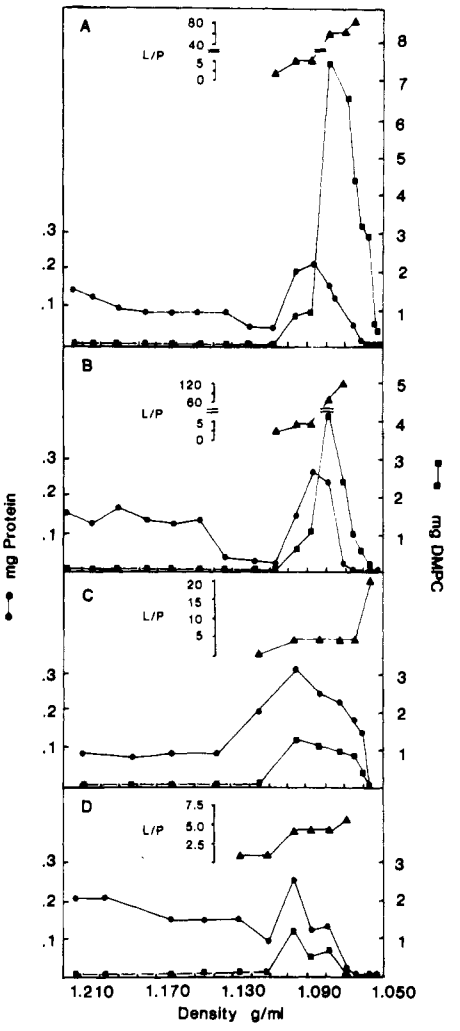

FIGURE 3: Density gradient centrifugation of DMPC–apoB incubation mixtures on a gradient of potassium bromide [see Methods and Redgrave et al. (1975)]. (A–D) DMPC–apoB incubation mixtures. The bottom of the tube is at the left, and the top of the tube is at the right. The following weight ratios (DMPC:apoB) were used: (A) 10:1; (B) 5:1; (C) 2.5:1; (D) 1:1. (●) mg of protein/fraction; (■) mg of DMPC/fraction; (▲) L:P (mg of DMPC to mg of protein) per fraction.

Figure 3C shows a fractionation pattern of a 2.5:1 w/w incubation mixture. The major portion of protein bands between d = 1.115 and 1.058 g/mL, virtually identical with the phospholipid. The lipid to protein ratio is constant at 4:1 w/w over the range d = 1.105–1.064 g/mL. At lower densities, the lipid to protein ratio increases.

Figure 3D shows a fractionation pattern of a 1:1 w/w incubation mixture. Protein migrates at the bottom of the tube. At this incubation ratio, both protein and lipid form a well-defined band on the density gradient between d = 1.110 and 1.070 g/mL. However, a significant amount of protein bands in the higher density regions of the gradient. The lipid to protein ratio over the majority of the protein and phospholipid band is again 4:1 w/w.

Density gradient ultracentrifugation of detergent-free apoB shows that all protein is recovered between d = 1.210 and 1.162 g/mL, with no protein found in the central region (data not shown). In contrast to the gel-filtration chromatography data, density gradient fractionation suggests a single stable DMPC–apoB complex of 4:1 w/w that coexists with excess

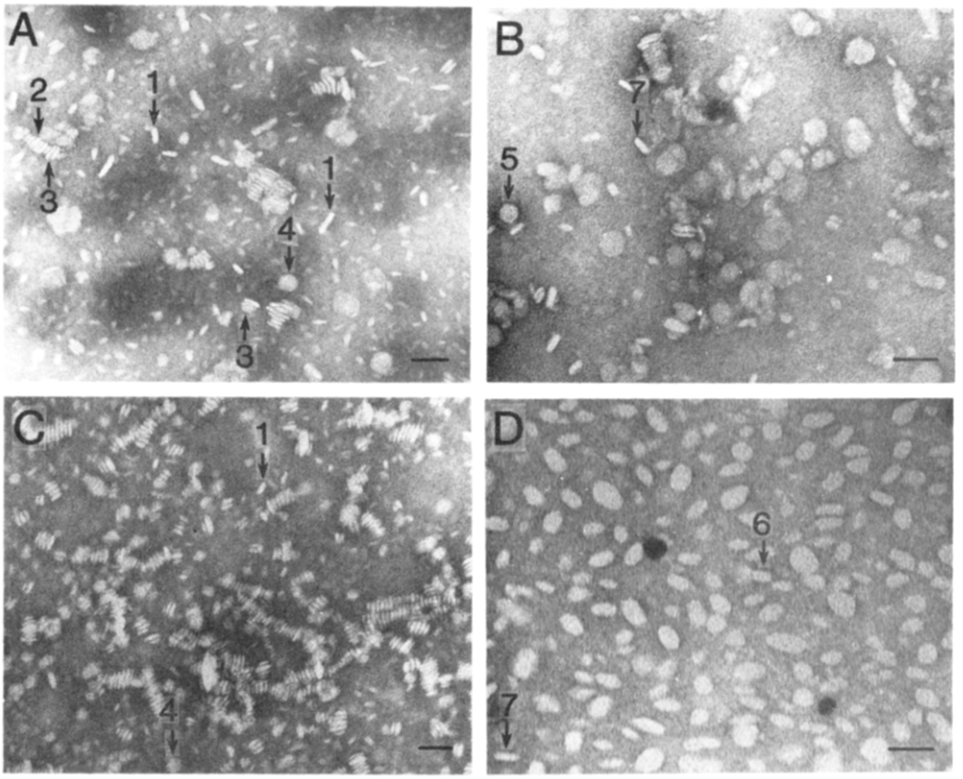

FIGURE 4: DMPC–apoB 4:1 w/w complexes isolated by density gradient ultracentrifugation, negatively stained with sodium phosphotungstate (A and C) or uranyl oxalate (B and D). (A and B) Isolated from 2.5:1 w/w incubation ratio at d = 1.074 g/mL. (C and D) Isolated from 1:1 w/w incubation ratio at d = 1.098 g/mL. (Arrows) (1) Elongated structures of 100 Å thickness; (2) elongated structures of 50 Å thickness; (3) U-shaped particles; (4) free-standing circular particles; (5) circular particle; (6) flattened elongated particle; (7) completely flattened particle. Bar = 500 Å.

protein or phospholipid, depending on the initial incubation ratio.

Electron Microscopy of DMPC–ApoB Complexes. Electron micrographs of fractions with a constant lipid to protein ratio of 4:1 w/w isolated from the density gradient are illustrated in Figure 4. When stained with NaPTA, the fractions at d = 1.074 and 1.097 g/mL from the fractionation of a 2.5:1 and 1:1 incubation mixture, respectively, showed similar appearance on electron microscopy (Figure 4A,C). This appearance is typified by a heterogeneous population of elongated structures of thickness ranging between 100 (arrow 1) and 50 Å (arrow 2). The 50 Å thick particles are found exclusively in stacked structures. In these micrographs, U-type structures (arrow 3) can also be observed. Occassionally a free-standing particle with circular morphology (arrow 4) (diameter 210 ● 20 Å) is also observed. This appearance described extensively by Melchior et al. (1980) is typical of a population of phospholipid single-bilayer vesicular structures where the vesicle has ruptured and flattened during the staining procedures. The 50 Å thick elongated appearance correlates with a single phospholipid bilayer, and the 100 Å thick elongated structure correlates with a double phospholipid bilayer.

Identical samples stained with uranyl oxalate (Figure 4B,D) also showed a similar appearance. However, in this case, the appearance is typified by particles with a circular morphology (diameter 210 ± 20 Å; arrow 5) together with particles that appear flattened and elongated (arrow 6). The particles that appear completely flattened (arrow 7) have a thickness of approximately 100 Å, corresponding to the thickness of two phospholipid bilayers. This appearance, also extensively discussed by Melchior et al. (1980), is again typical of a phospholipid vesicular particle. The uranyl oxalate stain appears to preserve the appearance of the vesicular particle more precisely than NaPTA, which causes vesicle rupture and flattening on the electron microscope grid.

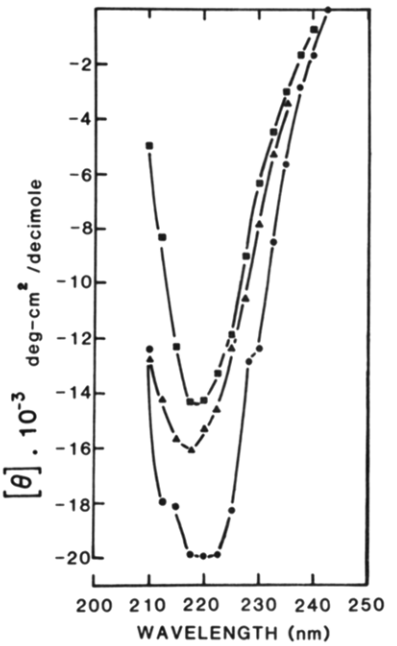

FIGURE 5: Circular dichroic spectra: (▲) intact LDL; (■) NaDC-solubilized apoB; (●) DMPC:apoB 4:1. Spectra were recorded at room temperature in a 1-cm quartz cell at protein concentrations of 0.03–0.09 mg/mL. All samples were dialyzed extensively against 0.005 M sodium tetraborate, pH 10 (±10 mM NaDC where appropriate).

Circular Dichroism. The far-ultraviolet circular dichroic spectra of LDL, NaDC-solubilized apoB, and DMPC–apoB from the 4:1 w/w region isolated from 10:1 and 1:1 w/w incubation mixtures by density gradient ultracentrifugation were recorded at 20 °C. As shown in Figure 5, within ex-

FIGURE 6: 3.0% $NaDodSO_4$–polyacrylamide gels. (Left to right) High molecular weight standard [Bio-Rad high molecular weight protein standards (top to bottom): myosin (200 000), β-galactosidase (116 250), phosphorylase B (92 500), bovine serum albumin (66 200), and ovalbumin (45 000)]; LDL; NaDC-solubilized apoB; DMPC:apoB 4:1 w/w isolated by density gradient ultracentrifugation and prepared at a ratio of 1:1 w/w.

perimental error, there is little difference between spectra of apoB in intact LDL and apoB that has been solubilized with NaDC. By use of the molar ellipticities at 217 and 222 nm, an apparent protein conformation of 45.0 ± 0.3% ($\bar{x}$ ± 1 SD) α-helix, 15 ± 5% β-sheet, and 40 ± 5% random coil is calculated for LDL (Greenfield & Fasman, 1969, Morrissett et al., 1973). For NaDC-solubilized apoB, 42.1 ± 0.7% α-helix, 17 ± 5% β-sheet, and 40 ± 5% random coil is calculated, which is in close agreement with values obtained by other investigators for apoB solubilized with other detergents (Steele & Reynolds, 1979a,b; Watt & Reynolds, 1980; Cardin & Jackson, 1981). For apoB in association with DMPC at a 4:1 w/w ratio isolated from density gradient fractions, the pronounced minimum at 222 nm indicates the presence of more α-helix. Quantitated estimates of secondary structure give 58.7 ± 0.1% α-helix, approximately 1% β-sheet, and 40 ± 5% random coil.

Gel Electrophoresis and End-Group Determination. Electrophoresis on 3.0% $NaDodSO_4$–polyacrylamide gels performed on preparations of LDL, NaDC-solubilized apoB, and apoB in association with DMPC shows one band that corresponds to an apparent M_r of 366 000 ± 10 000 (Figure 6). No additional bands are observed at lower molecular weights on similar gels after prolonged sample storage (up to 6 weeks at 4 °C). Over the time course of detergent removal and chromatography, no degradation of apoB has occurred.

Quantitative end-group determination performed on samples of intact LDL and NaDC-solubilized apoB showed one dinitrophenyl-labeled amino acid, which is (dinitrophenyl)-glutamic acid. Thus, the minimum number of peptide chains per apoB molecule is one. By this method alone, it is not possible to estimate the maximum number of polypeptide chains; however, if there are multiple chains, all must have glutamic acid at the N terminus.

Discussion

Utilizing the method of LDL solubilization reported by Helenius & Simons (1971) and incorporating the sulfhydryl-blocking reaction utilized by Steele & Reynolds (1979a,b) and Watt & Reynolds (1980), we have developed a method by which LDL apoB can be completely delipidated and purified by gel-filtration chromatography and recovered in high yield.

Comparison of the circular dichroic spectrum of sodium deoxycholate solubilized apoB obtained by these methods with that of native LDL indicates that the protein retains a similar secondary conformation to that in LDL. This is in agreement with observations by other investigators using other detergents (Steele & Reynolds, 1979a,b; Watt & Reynolds, 1980; Cardin & Jackson, 1981).

The solubilized apoB was incubated with NaDC-solubilized DMPC, and the incubation mixture was fractionated by gel-filtration chromatography and density gradient ultracentrifugation. On gel-filtration chromatography, the coelution of phospholipid and apoB at a well-defined elution volume on the column with a fairly constant lipid to protein ratio suggests the formation of a stable homogeneous population of DMPC–apoB complexes with a lipid to protein ratio that varies with the incubation ratio. Apparent lipid to protein ratios of 16:1, 12:1, 10.5:1, and 4:1 w/w are suggested from 10:1, 5:1, 2.5:1, and 1:1 w/w incubation ratios, respectively. However, density gradient fractionation of DMPC–apoB incubation mixtures suggests the presence of a stable DMPC–apoB complex with a lipid to protein ratio of 4:1 at all incubation ratios examined. On the density gradient, the complex is separated from either uncomplexed protein or excess lipid depending on the incubation ratio. The 4:1 w/w lipid to protein ratio obtained by density gradient ultracentrifugation agrees with the constant ratio of lipid to protein obtained by gel-filtration chromatography of a 1:1 w/w incubation mixture.

At 10:1 w/w incubation ratio of lipid to protein, although lipid is in excess of protein, a large amount of protein is found in the high-density region (from d = 1.210 to 1.162 g/mL), the region where detergent-free apoB alone bands. Due to the extensive manipulation of the protein during the preparative methods, the physical state of some of the apoB molecules may have been changed, and they may no longer be in the appropriate form for incorporation into vesicles, and thus, the uncomplexed apoB would band in the high-density region of the gradient.

In general, the 4:1 w/w relatively constant lipid to protein ratio, for the incubation ratios studied, is obtained at 1.074 $\leq d \leq$ 1.115 g/mL by density fractionation. On the basis of the partial specific volumes of 0.956 (Small, 1967) and 0.725 mL/g (Steele & Reynolds, 1976b) for DMPC and apoB, respectively, a complex with a 4:1 lipid to protein ratio would have a density of 1.098 g/mL, which is in excellent agreement with the median density of 1.095 g/mL for the observed density range.

In gel-filtration chromatography elution profiles of DMPC:apoB 10:1, 5:1, and 2.5:1 w/w mixtures, a population of DMPC:apoB 4:1 w/w particles may well be coeluting with a population of protein-free phospholipid vesicles. An increase in the mass of DMPC per fraction would occur, therefore decreasing the apparent ratio of protein to phospholipid.

The *apparent* molecular weight of LDL–apoB and NaDC–apoB as determined by electrophoresis on 3.0% gels is 366 000 ± 10 000. In agreement with other recent investigations (Steele & Reynolds, 1979a,b; Morrissett et al., 1975; Smith et al., 1972; Elovson, 1981), apoB migrates as a single high molecular weight species and with a single amino-terminal residue. The apoB of the 4:1 w/w DMPC–apoB complex exhibits identical behavior on 3.0% gels. Thus, the mild conditions under which apoB is isolated, as well as the experimental protocols used to produce and isolate the complexes, preserve the integrity of the large apoB molecule. However,

circular dichroic spectra of the 4:1 w/w DMPC:apoB shows an increased molar ellipticity at 222 nm and an increased percentage of α-helix, suggesting a conformational change of apoB when phospholipid is reintroduced.

Electron microscopy shows the structural organization of the 4:1 w/w DMPC–apoB complex to be similar to a phospholipid single-bilayer vesicle. An estimate of the vesicle diameter is difficult to obtain because of the distortions of the particle morphology that occur during negative staining. However, an estimate from the micrographs obtained with uranyl oxalate stain and measurement of only free-standing particles with approximately circular morphology gives 210 ± 20 Å for the vesicle diameter.

Values for the molecular weight of apoB quoted in the literature range from 250 000 for the apoB monomer (Smith et al., 1972; Steele & Reynolds, 1979a) through an apparent molecular weight of 360 000 (Elovson et al., 1981; this work) to 500 000 or more for an apoB dimer (Smith et al., 1972; Steele & Reynolds, 1979a; Kane et al., 1980). On the basis of the chemical composition of the DMPC–apoB 4:1 w/w complex and these values for the molecular weight of apoB, the complex contains either 1 molecule of apoB of M_r 250 000 (Smith et al., 1972, Steele & Reynolds, 1979a) and 1440 molecules of DMPC, 1 molecule of apoB of M_r 366 000 and 2100 molecules of DMPC, or 1 dimer of apoB of M_r 500 000 (Smith et al., 1972; Steele & Reynolds, 1979a,b) and 2900 molecules of DMPC.

Given the surface area occupied by a DMPC molecule (70 Å^2) and its partial specific volume (Small, 1967), the partial specific volume of apoB (Steele and Reynolds, 1979b), and the above chemical compositions of the DMPC–apoB complexes, vesicle diameters of 156, 180, and 204 Å may be calculated, respectively. The calculated values of 180 and 204 Å are in close agreement with our estimate of particle diameter of 210 ± 20 Å obtained from electron microscopy. However, without a more definitive value for the molecular weight of apoB no firm conclusion may be drawn concerning the precise molecular composition of the complex.

Studies on the incorporation of intrinsic membrane proteins into single-bilayer phospholipid vesicles have identified a potential problem arising from the trapping of protein molecules in the enclosed aqueous compartment of the vesicle rather than true incorporation into the phospholipid bilayer (Mimms et al., 1981). The DMPC–apoB 4:1 w/w complex may be precipitated by polyanions (e.g., dextran–magnesium sulfate). These methods are used to precipitate native apoB-containing lipoproteins (Waugh & Small, 1982), suggesting that apoB of the DMPC–apoB complex is accessible from the outer surface of the vesicular particle, rather than encapsulated in the aqueous space of the vesicle (data not shown).

Recently, Watt & Reynolds (1981) reported the preparation of a soluble complex of apoB with egg yolk phosphatidylcholine in which apoB binds a small number of phospholipid molecules. Cardin & Jackson (1981) report interaction of apoB with DMPC to form stacked disk structures. The type of detergent utilized in solubilization and delipidation and the method and rate of detergent removal after reintroduction of lipid may govern the structure and composition of the final complex that is formed between lipid and protein. Since the detergents and technical methods in the above studies (Watt & Reynolds, 1981; Cardin & Jackson, 1981) differ from those presented in this paper, it is perhaps not surprising that different apoB–phospholipid complexes may be formed.

Bile salts have been used to solubilize, delipidate, and isolate various membrane proteins (Helenius et al., 1979; Razin, 1972; Furth, 1980; Racker, 1979; Hwang & Stoeckenius, 1977). The preparation of lipid–protein particles of a reproducible composition requires detergent removal in a controlled, reproducible manner. In spite of all the possible problems, we have succeeded in preparing and isolating reproducibly a DMPC–apoB 4:1 w/w complex, using extensive dialysis to remove detergent.

By this series of protocols, we have produced a DMPC-apoB complex at a 4:1 w/w ratio that is a vesicular particle. An understanding of the interaction of apoB with lipid may aid in the determination of the structural organization of the protein in the intact LDL particle as well as the functional aspects of the lipoprotein.

Acknowledgments

We are grateful to Dr. Donald M. Small, Geoffrey S. Ginsburg, and Margaret M. Prack for helpful discussions. We also thank Eileen Fonferko and Ronald P. Corey for excellent technical assistance and Anne M. Gibbons for her expertise in preparing the manuscript.

Registry No. NaDC, 302-95-4; DMPC, 13699-48-4.

References

Basu, S. K., Anderson, R. G. W., Goldstein, J. L., & Brown, M. S. (1977) *J. Cell Biol. 74*, 119–135.

Brown, M. S., & Goldstein, J. L. (1976) *Science (Washington, D.C.) 191*, 150–154.

Cardin, A. D., & Jackson, R. L. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1636.

Cardin, A. D., Witt, K. R., Barnhart, C. L., & Jackson, R. L. (1982) *Biochemistry 21*, 4503–4511.

Dearborn, D. G., & Wetlaufer, D. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 179–185.

Elovson, J., Huang, Y. O., Baker, N., & Kannan, R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 157–161.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Forte, T. M., & Nichols, A. V. (1972) *Adv. Lipid Res. 10*, 1–41.

Fraenkel-Conrat, H., Harris, J. S., & Levy, A. L. (1955) *Methods Biochem. Anal. 2*, 359–425.

Furth, A. J. (1980) *Anal. Biochem. 109*, 207–215.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.

Gurd, F. R. N. (1967) *Methods Enzymol. 11*, 532–541.

Havel, R. J., Eder, J. A., & Bragdon, J. H. (1955) *J. Clin. Invest. 34*, 1345–1353.

Helenius, A., & Simons, K. (1971) *Biochemistry 10*, 2541–2547.

Helenius, A., McCaslin, D. R., Fries, E., & Tanford, C. (1979) *Methods Enzymol. 56*, 734–749.

Hirs, C. H. W. (1967) *Methods Enzymol. 22*, 199–203.

Hwang, S.-B., & Stoeckenius, W. (1977) *J. Membr. Biol. 33*, 325–350.

Kane, J. P., Hardman, D. A., & Paulus, H. F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2465–2469.

Keller, M., & Pataki, G. (1963) *Helv. Chim. Acta 46*, 1687–1691.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mahley, R. W., & Innerarity, T. L. (1978) in *Sixth International Symposium on Drugs Affecting Lipid Metabolism* (Kritchevsky, D., Paoletti, R., & Holmes, W. L., Eds.) pp 99–127, Plenum Press, New York.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.
McClare, C. W. F. (1971) *Anal. Biochem. 39*, 527–530.
Melchior, V., Collingshead, C. J., & Cahoon, M. E. (1980) *J. Cell Biol. 86*, 881–884.
Mimms, L. F., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. A. (1981) *Biochemistry 20*, 883–840.
Morrissett, J. D., David, J. S. K., Pownall, H., & Gotto, A. M. (1973) *Biochemistry 12*, 1290–1299.
Morrissett, J. D., Jackson, R. L., & Gotto, A. M. (1975) *Annu. Rev. Biochem. 44*, 183–207.
Nelson, G. J. (1979) in *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism*, p 300, Robert E. Krieger Publishing Co., New York.
Noble, R. P. (1968) *J. Lipid Res. 9*, 693–700.
Racker, E. (1979) *Methods Enzymol. 55*, 699–711.
Razin, S. (1972) *Biochim. Biophys. Acta 265*, 241–296.
Redgrave, T. G., Roberts, D. C. K., & West, C. E. (1975) *Anal. Biochem. 65*, 42–49.
Rudel, L. L., & Morris, M. D. (1973) *J. Lipid Res. 14*, 364–366.
Small, D. M. (1967) *J. Lipid Res. 8*, 551–560.
Smith, R., Dawson, J. R., & Tanford, C. (1972) *J. Biol. Chem. 247*, 3376–3381.
Steele, J. C. H., & Reynolds, J. A. (1979a) *J. Biol. Chem. 254*, 1633–1638.
Steele, J. C. H., & Reynolds, J. A. (1979b) *J. Biol. Chem. 254*, 1639–1643.
Watt, R. M., & Reynolds, J. A. (1980) *Biochemistry 19*, 1593–1598.
Watt, R. M., & Reynolds, J. A. (1981) *Biochemistry 20*, 3897–3901.
Waugh, D. A., & Small, D. M. (1982) *J. Lipid Res. 23*, 201–204.
Weber, K., & Osborn, M. J. (1969) *J. Biol. Chem. 244*, 4406–4412.
Yeagle, P. L., Langdon, R. G., & Martin, R. B. (1977) *Biochemistry 16*, 3487–3491.

Lipid and Subunit III Depleted Cytochrome *c* Oxidase Purified by Horse Cytochrome *c* Affinity Chromatography in Lauryl Maltoside†

D. A. Thompson and S. Ferguson-Miller*

ABSTRACT: Cytochrome oxidase is purified from rat liver and beef heart by affinity chromatography on a matrix of horse cytochrome *c*–Sepharose 4B. The success of this procedure, which employs a matrix previously found ineffective with beef or yeast oxidase, is attributed to thorough dispersion of the enzyme with nonionic detergent and a low density of cross-linking between the lysine residues of cytochrome *c* and the cyanogen bromide activated Sepharose. Beef heart oxidase is purified in one step from mitochondrial membranes solubilized with lauryl maltoside, yielding an enzyme of purity comparable to that obtained on a yeast cytochrome *c* matrix [Azzi, A., Bill, K., & Broger, C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2447–2450]. Rat liver oxidase is prepared by hydroxyapatite and horse cytochrome *c* affinity chromatography in lauryl maltoside, yielding enzyme of high purity (12.5–13.5 nmol of heme *a*/mg of protein), high activity (TN = 270–400 s^{-1}), and very low lipid content (1 mol of DPG and 1 mol of PI per mol of aa_3). The activity of the enzyme is characterized by two kinetic phases, and electron transfer can be stimulated to maximal rates as high as 650 s^{-1} when supplemented with asolectin vesicles. The rat liver oxidase purified by this method does not contain the polypeptide designated as subunit III. Comparisons of the kinetic behavior of the enzyme in intact membranes, solubilized membranes, and the purified delipidated form reveal complex changes in kinetic parameters accompanying the changes in state and assay conditions, but do not support previous suggestions that subunit III is a critical factor in the binding of cytochrome *c* at the high-affinity site on oxidase or that cardiolipin is essential for the low-affinity interaction of cytochrome *c*. The purified rat liver oxidase retains the ability to exhibit respiratory control when reconstituted into phospholipid vesicles, providing definitive evidence that subunit III is not solely responsible for the ability of cytochrome oxidase to produce or respond to a membrane potential or proton gradient.

Cytochrome *c* oxidase (EC 1.9.3.1) is a complex, multisubunited enzyme, which contains four metal centers and spans the inner mitochondrial membrane. Electrons liberated during metabolism are transferred from cytochrome *c* through cytochrome oxidase to molecular oxygen, resulting in formation of water and conservation of energy by mechanisms not fully understood. Elucidation of the structure–function correlations in this elaborate protein has been difficult. Much controversy still exists concerning the number of subunits required for the native functions of the enzyme, and their operational roles, as well as the significance of the biphasic steady-state kinetic pattern observed for the interaction of cytochrome *c* with cytochrome oxidase (Nicholls, 1964, 1965; Ferguson-Miller et al., 1976; Errede & Kamen, 1978; Antalis & Palmer, 1982). Studies on the activity of cytochrome oxidase have been complicated by its functional dependence on the hydrophobic environment. An absolute requirement for specific phospholipids has been postulated (Awasthi et al., 1971; Robinson et al., 1980; Fry & Green, 1980; Vik et al., 1981; Robinson, 1982), and oxidase activity has been proposed to be further influenced by interactions with the membrane, with other membrane proteins, and with itself (by formation of a dimer consisting of two complete sets of subunits) (Robinson & Capaldi, 1977; Bisson et al., 1980; Wikstrom, 1981; Ferguson-Miller et al., 1982).

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. *Received December 30, 1982.* This research was supported by National Institutes of Health Grant GM 26916 to S.F.-M.

0006-2960/83/0422-3178$01.50/0