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Sulfhydryl Chemistry and Solubility Properties of Human Plasma Apolipoprotein B†

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ABSTRACT: Apolipoprotein B (apoB) was isolated from human plasma low-density lipoproteins (LDL; d = 1.02–1.05 g/mL) by delipidation with ether–ethanol, followed by solubilization of the protein with sodium decyl sulfate. After the detergent was reduced to <0.1 μg of sodium decyl sulfate/mg of protein by dialysis, apoB was next precipitated with ethanol to remove residual lipids and then solubilized in 6 M guanidine hydrochloride (Gdn-HCl). Dialysis of apoB in 6 M Gdn-HCl against 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.5, resulted in protein precipitation; however, dialysis against 6 M urea and then 10 mM Tris-HCl, pH 8.5, resulted in a water-soluble apoprotein. Water-soluble apoB gave a single precipitin line of complete identity with LDL when tested by immunodiffusion against anti-apoB. ApoB contains 13.9 ± 0.1 mol of half-cystine residue/250 000 g of polypeptide. Twelve of these residues form six intramolecular disulfide bonds in LDL while two SH groups remain free. Intermolecular disulfide bonds are formed during lipid extraction of LDL, yielding high molecular weight aggregates of apoB. The cross-linking reaction occurs by a sulfhydryl–disulfide exchange mechanism and is catalyzed by the two free SH groups originally present on each apoB molecule in LDL. Alkylation of the free SH groups with iodoacetamide blocks the exchange reaction. However, the addition of glutathione to carboxamidomethylated apoB promotes intermolecular disulfide exchange, yielding high molecular weight aggregates. The formation of intermolecular disulfide bonds occurs at the expense of intramolecular disulfide bonds and relieves secondary structural restraints. In addition, both intra- and intermolecular disulfide bonds impose modest restraints on the tertiary structure of apoB as determined by circular dichroism (CD) methods. Analysis of the far-UV CD region of apoB at various purification steps suggests that the conformation and state of association are the major factors contributing to the overall water solubility of apoB in the absence of denaturants and amphiphilic ligands.

The low-density lipoproteins (LDL)[1] of human plasma contain a high molecular weight apoprotein, apolipoprotein B (apoB), which functions in the transport of lipid to extrahepatic cells (Brown & Goldstein, 1979; Brown et al., 1978). This apoprotein has a molecular weight of 250 000 in concentrated Gdn-HCl as determined by ultracentrifugation (Steele & Reynolds, 1979b), although studies based on quantitative chemical analysis suggest the possibility of a lower unit molecular weight for apoB (Bradley et al., 1978). The structural characterization of apoB has been greatly compounded by its insolubility in aqueous buffers; detergents, denaturants (Gotto et al., 1973; Helenius & Simons, 1971; Shore & Shore, 1967; Day & Levy, 1968; Scanu et al., 1968; Pollard et al., 1969; Kane et al., 1970; 1980; Chen & Aladjem, 1978; Steele & Reynolds, 1979a; Lee, 1976), or other proteins (Shireman et al., 1977) are required for its solubilization. ApoB can also undergo extensive aggregation (Steele & Reynolds, 1979b; Socorro & Camejo, 1979), and it is highly susceptible to intact chain degradation by proteolytic

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[1] Abbreviations: VLDL, very low density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; apoB, a major protein constituent of chylomicrons, VLDL, and LDL; DPPC, dipalmitoylphosphatidylcholine; CD, circular dichroism; MCD, magnetic circular dichroism; PMSF, phenylmethanesulfonyl fluoride; EDTA, disodium salt of ethylenediaminetetraacetic acid; CAM-apoB, carboxamidomethylated apoB; RCAM-apoB, reduced and carboxamidomethylated apoB; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, reduced form of glutathione; BHT, butylated hydroxytoluene; CAM-LDL, carboxamidomethylated LDL; Gdn-HCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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(Krishnaiah & Weigandt, 1974; Steele, 1979) and oxidative bond cleavage reactions (Schuh et al., 1978). The present study describes a method for obtaining the intact chain of apoB from human plasma LDL as a lipid-free, water-soluble apoprotein. In addition we show that certain procedures used to inhibit oxidative chain degradation promote covalent aggregation. Alternative methods are presented to inhibit this aggregation, and a critical evaluation of factors that influence the solubility of apoB in aqueous solutions is presented.

Materials and Methods

Materials. Sequenal grade Gdn-HCl and urea were obtained from Pierce Chemical Co. Phenylmethanesulfonyl fluoride (PMSF), butylated hydroxytoluene (BHT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione (GSH), and iodoacetamide were obtained from Sigma Chemical Co. Diethyl ether (anhydrous) was purchased from J. T. Baker Chemical Co. and used without further purification. [^{14}C]-Urea (5.35 Ci/mol), [1-^{14}C]iodoacetamide (23 Ci/mol), and di[1-^{14}C]palmitoylphosphatidylcholine (100 Ci/mol) were obtained from New England Nuclear; radiolabeled DPPC was greater than 99% pure by thin-layer chromatography on silica gel in either $CHCl_3$–CH_3OH–H_2O (65:25:4 v/v) or in benzene–hexane (1:1 v/v).

Prior to use, fresh solutions of 8 M urea, in the presence and absence of [^{14}C]urea, were passed through an ion-exchange column of Rexyn I-300 (Fisher Scientific) to remove free cyanates and then used immediately. All buffer solutions were prepared from deionized–distilled water. Unless stated otherwise, standard buffer is 10 mM Tris-HCl, pH 8.5, containing 1 mM PMSF, 1 mM EDTA, and 0.01% NaN_3.

Isolation of LDL. Human plasma was obtained by plasmapheresis from normal fasting donors or subjects with familial homozygous type II hyperlipoproteinemia. Sodium azide, PMSF, and EDTA were added immediately to the plasma to final concentrations of 0.01%, 0.5 mM, and 1 mM, respectively. In some experiments glutathione was also added to the plasma to a final concentration of 0.05%.

LDL were isolated between d = 1.02 and d = 1.05 g/mL by ultracentrifugation in salt solutions of KBr. All centrifugations were carried out in a Beckman L8 ultracentrifuge operating at 48 000 rpm for 20 h at 4 °C with a Beckman Type 50.2 rotor. Plasma was adjusted to d = 1.063 g/mL, and after ultracentrifugation the lipoproteins of d < 1.063 g/mL were removed by aspiration. The lipoproteins were dialyzed against a buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.9% NaCl, 0.01% NaN_3, 0.5 mM PMSF, and plus or minus 0.05% glutathione, as indicated under Results, and subjected to ultracentrifugation at d = 1.02 g/mL. The infranatant fraction was then removed and subjected to ultracentrifugation at d = 1.05 g/mL. LDL were then dialyzed against 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.9% NaCl, 0.01% NaN_3, 0.5 mM PMSF, and plus or minus 0.05% glutathione, as indicated, and stored at 4 °C. LDL were used within 1 month of preparation and filtered through a 0.45-μm filter (Millipore Corp.) prior to use in order to remove small amounts of aggregated lipoprotein.

Preparation of Di[1-^{14}C]palmitoylphosphatidylcholine-Labeled LDL. Human plasma LDL were labeled with di[1-^{14}C]palmitoylphosphatidylcholine by using bovine liver phosphatidylcholine exchange protein as described previously (Jackson et al., 1980a); radiolabeled LDL contained 172 cpm/μg of phospholipid.

Preparation of Water-Soluble ApoB under Standard (Nonreducing) Conditions. For preparation of water-soluble apoB under standard conditions, LDL (4 mg of protein/mL) were dialyzed 2 h against 1 mM EDTA, pH 8.0. The LDL (5 mL) were then lyophilized and subjected to three extractions with 50 mL of ether–ethanol (3:1 v/v). At each step of the extraction, the protein–solvent mixture was incubated at −20 °C for 1 h and pelleted by low-speed centrifugation, and the solvent was removed by aspiration. After the final ether–ethanol extraction, the protein (apoB) was washed once with anhydrous ether and pelleted, and the solvent was removed by aspiration while being careful to leave the protein moist. ApoB (20 mg) was suspended in 15 mL of standard buffer containing 100 mM sodium decyl sulfate. Complete solubilization of apoB was achieved by gentle vortexing, followed by gentle stirring at 23 °C. ApoB was then dialyzed at 4 °C against consecutive 6-L changes of standard buffer to remove the sodium decyl sulfate. The detergent concentration was monitored by the methylene blue assay for quantitating protein-bound ionic detergent (Hayashi, 1975). After dialysis, apoB contained <0.1 μg of sodium decyl sulfate/mg of protein; 2–6 mg of protein was used in the assay.

ApoB was next delipidated with ethanol to remove any residual lipid. To 5.0 mL of apoB (1 mg/mL) in standard buffer was added 45 mL of absolute ethanol at room temperature; 5.0 mL of 1 M Tris-HCl, containing 0.01 M EDTA, pH 8.5, was then added to precipitate the protein. The precipitate was washed with water, dissolved in standard buffer containing 6 M Gdn-HCl, and then reprecipitated by dialysis against standard buffer. The protein pellet was redissolved in 4 mL of standard buffer containing 6 M Gdn-HCl and then dialyzed consecutively against three 1-L changes of standard buffer containing 6 M urea, followed by 1 M urea and finally standard buffer. The apoprotein prepared in this way did not pellet following centrifugation for 30 min at 48000g. For determination of whether carbamylation occurred during the preparation of apoB, 25 μCi of [^{14}C]urea was added to apoB in 6 M urea (step 9, Chart I). The sample was then dialyzed against three 2-L changes of standard buffer containing 1 M urea and then against three 2-L changes of standard buffer; 3.0 mg of protein was taken for determination of radioactivity.

Preparation of Water-Soluble ApoB under Reducing Conditions. Water-soluble apoB prepared under reducing conditions was achieved by the inclusion of 0.02% BHT in all organic solvents during delipidation of LDL, the inclusion of 0.05% glutathione (GSH) in all aqueous buffers, and the presaturation of these aqueous buffers with nitrogen. Dialyses were performed with gentle stirring with a continuous nitrogen purge by use of a gas dispersion tube (Fisher Scientific) immersed into the dialysis tank.

Quantitation of Sulfhydryls. The number of free sulfhydryls in LDL was determined by the method of Habeeb (1972) with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and by the reaction of LDL with [1-^{14}C]iodoacetamide (2.14×10^6 dpm/mg). Both reactions were conducted in the presence and absence of 2% sodium dodecyl sulfate. To 3 mL of LDL (2 mg of protein/mL) in 0.1 M $NaHCO_3$, pH 8.6, containing 0.5 mg/mL EDTA was added 0.1 mL of a 4 mg/mL DTNB solution. After 15 min the apparent absorbance was corrected by a reagent blank to yield the net absorbance at 412 nm. An ϵ_{412nm} of 13 600 M^{-1} cm^{-1} was used to calculate the concentration of free sulfhydryl. For reaction with iodoacetamide, 5 mL of LDL (4 mg of protein/mL) in 0.5 M Tris-HCl, pH 8.6, and 30 mM EDTA was reacted with 5 mg of [1-^{14}C]-iodoacetamide for 1 h in the dark at 24 °C. The carboxamidomethylated LDL (CAM-LDL) were then dialyzed against standard buffer to remove excess reagent. The extent of label incorporation was then calculated from the specific

radioactivity of [1-^{14}C]iodoacetamide.

The total sulfhydryl content of lipid-free apoB was determined by reaction with [1-^{14}C]iodoacetamide as described by Haeberli et al. (1975). Reduction of disulfide bonds was performed at room temperature for 4 h at a protein concentration of 2 mg/mL in 6 M Gdn-HCl, 0.5 M Tris-HCl, and 30 mM EDTA, pH 8.6. A 100-fold molar excess of 2-mercaptoethanol per protein disulfide bond was employed. Alkylation was carried out in the dark at 24 °C for 30 min with a molar ratio of [1-^{14}C]iodoacetamide to 2-mercaptoethanol of 2.5. The fully reduced and ^{14}C-labeled carboxamidomethylated apoB (RCAM-apoB) was then precipitated by dialysis against three 2-L changes of standard buffer. After low-speed centrifugation, no radioactivity was found in the supernatant. The pellet was then dissolved in standard buffer containing 6 M Gdn-HCl. The sample was next dialyzed against two 1-L changes of standard buffer containing 6 M urea, followed by standard buffer containing 1 M urea and finally standard buffer, to yield water-soluble RCAM-apoB. The concentration of RCAM-apoB in standard buffer was typically 1.9–2.5 mg/mL. The number of modified sulfhydryl groups was determined from the specific radioactivity of [1-^{14}C]iodoacetamide.

Pore–Gradient Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Socorro & Camejo (1979). Electrophoresis was performed on a gel (3 mm thick) in a vertical slab cell unit (Bio-Rad Model 220) at a constant current of 10 mA/gel. The ratio of acrylamide to bis(acrylamide) was 30:1; polymerization was induced by the addition of 1.5 μL of *N,N,N′,N′*-tetramethylethylenediamine and 2.5 mg of ammonium persulfate per 10 mL of gel. A 0–20% (w/v) sucrose gradient was superimposed over the acrylamide gradient (3–20% w/v) to improve gradient linearity. The gel contained 0.25 M Tris-HCl, 0.1% sodium dodecyl sulfate, and 6 M urea, pH 9.0. The electrophoresis buffer contained 25 mM Tris-HCl, 0.2 M glycine, and 0.1% sodium dodecyl sulfate, pH 8.4. The samples were adjusted to 0.5 mg/mL in a buffer containing 10 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 20% sucrose, 1 mM EDTA, 6 M urea, and 0.03% bromophenol blue and then heated at 60 °C for 15 min either in the presence or in the absence of 1% 2-mercaptoethanol. Following electrophoresis, gels were fixed overnight in 2-propanol–acetic acid–water (25:10:65 v/v), stained for 6 h in 0.2% Coomasie brilliant blue R (Pierce Chemicals) dissolved in methanol–acetic acid–water (50:10:40 v/v), and destained in 2-propanol–acetic acid–water (10:10:80 v/v).

Immunological Studies. A single adult male goat was used for the preparation of anti-apoB. ApoB (5 mg) in 1 mL of 0.15 M NaCl was mixed with 1 mL of complete Freunds adjuvant (Difco) and the emulsion injected at 50 different sites on the back of the goat. After 6 and 8 weeks, injections of 2 mg of apoB in complete Freunds adjuvant were given intramuscularly (im). Two weeks later, 10 mg of antigen was given im, and after 10 days, the goat was exanguinated by cardiac puncture. The cells were removed from the blood, and PMSF and ϵ-aminocaproic acid were added to final concentrations of 0.5 mM and 10 mM, respectively, to inhibit proteolysis (Bjerrum et al., 1975; Steele, 1979). The resulting antiserum was then utilized for the immunologic studies.

Immunodiffusion was carried out in premade Ouchterlony plates (ICN Pharmaceuticals). The plates were developed overnight at room temperature and then at 4 °C for 48–72 h. The gels were extensively washed with 0.9% NaCl and then stained with a solution of 0.02% Coomassie Blue containing 2-propanol–acetic acid–water (10:10:80 v/v); the gels were destained in a solution of 2-propanol–acetic acid–water (10:10:80 v/v).

CD and MCD Measurements. CD measurements were made on a Cary 61 spectropolarimeter at 25 °C. All measurements were made at optical density readings that maintained the dynode voltage < 0.8. The ellipticity [θ] in units of degrees centimeters squared per decimole is given as

$$[\theta] = (\theta^\circ/10)[M/(lc)]$$

where θ° is the observed ellipticity in degrees, l is the cell path length in centimeters, and c is the protein concentration in grams per milliliter. In the far-UV region, M was taken as 112 (mean residue weight) whereas in the near-UV region, M was equal to 250 000.

MCD spectra were recorded on a Cary 61 spectropolarimeter equipped with a Varian horizontal bore superconducting magnet. All MCD spectra were corrected for natural CD contributions by recording the CD spectra prior to energizing the solenoid. The superconducting magnet was energized to a current reading of 29.3 A, which corresponds to a field of 50 000 G. The Verdet constant of water was defined as negative to maintain the proper sign convention as suggested by McCaffery et al. (1967). The units of molecular magnetic ellipticities [θ_M] were expressed as degrees centimeters squared per decimole per kilogauss and were calculated by using a molecular weight of 25 000.

Far-UV CD Spectral Analyses. The far-UV CD data were analyzed with the HELIX computer program modified to accomodate experimental error as described by Thompson et al. (1976). The program is a curve-fitting procedure over the wavelength interval between 190 and 245 nm. The algorithm consists of adjusting estimates of α-helical and β-pleated sheet contents such that the model best describes the data according to the least-squares criterion. Computer-fitted curves were generated from ellipticity values computed from fractional conformations and standard ellipticity values obtained from proteins of known secondary structural content (Chen et al., 1974).

Other Methods. Amino acid analyses were performed on a Durrum amino acid analyzer. Samples were hydrolyzed for 24 h at 110 °C in 6 M HCl containing 0.1% phenol. The concentration of apoB in detergent and standard buffer was determined both by amino acid analysis and by the method of Lowry et al. (1951) with bovine serum albumin as standard. Protein concentrations in Gdn-HCl solution were determined spectrophotometrically at 280 nm with an $\epsilon_{280nm}^{0.1\%}$ of 0.77 (Smith et al., 1972). All absorption measurements in the UV range were made on a Hitachi 340 UV–vis recording spectrophotometer.

Results

Preparation of Water-Soluble ApoB. Chart I shows the preparation of water-soluble apoB; at certain steps of the procedure the percent recoveries of protein are indicated. A number of preliminary studies were required to maximize these recoveries. The optimal amount of LDL protein in the delipidation procedure (step 2) was 20 mg/50 mL of organic solvent. Higher concentrations of protein resulted in the formation of a protein–gel that had a markedly reduced solubility in 0.1 M sodium decyl sulfate. The solubility of LDL protein in 0.1 M sodium decyl sulfate was maximal at pH values >8.0; at pH 8.5, the recovery of solubilized LDL protein was 100%. At pH values <8.0, a precipitate developed that remained insoluble even when the pH was readjusted to pH 8.0–9.5. Sodium decyl sulfate was removed (step 4) from the

Chart I: Preparation of Water-Soluble ApoB[a]

step	procedure	recovery of protein (%)
1	20 mg of LDL, d = 1.02–1.05 g/mL; lyophilize	100
2	3 times ether–ethanol extraction (3:1 v/v); 1 ether extraction; −20 °C for 1 h	N.D.[b]
3	solubilize in 0.1 M sodium decyl sulfate	100
4	extensive dialysis to remove detergent	75
5	ethanol precipitation	N.D.
6	solubilize in standard buffer containing 6 M Gdn-HCl	65
7	dialysis vs. standard buffer	N.D.
8	resolubilize apoB in standard buffer containing 6 M Gdn-HCl	65
9	dialysis vs. standard buffer containing 6 M urea	N.D.
10	dialysis vs. standard buffer containing 1 M urea	N.D.
11	dialysis vs. standard buffer	55

[a] Standard buffer is 10 mM Tris-HCl, 1 mM PMSF, 1 mM EDTA, and 0.01% NaN_3, pH 8.5. [b] N.D., not determined.

sample by extensive dialysis against standard buffer. ApoB remained in solution at concentrations of 1–3 mg/mL after dialysis and contained <0.1 μg of sodium decyl sulfate/mg of apoB. For removal of any residual lipid or detergent not detected by the respective assays, apoB was delipidated with ethanol as previously described for glycophorin (van Zoelen et al., 1978). The precipitated apoB (step 5) was completely solubilized in standard buffer containing 6 M Gdn-HCl with an overall 65% recovery of protein. Removal of the Gdn-HCl by dialysis against standard buffer resulted in precipitation of the protein. At step 8, the protein was redissolved in standard buffer containing 6 M Gdn-HCl and then dialyzed consecutively against standard buffer containing 6 M urea, 1 M urea, and finally standard buffer. At step 11, the concentration of water-soluble apoB was 3 mg/mL. The overall recovery of apoB protein from LDL ranged from 48 to 62% on five different preparations. The recoveries of apoB were the same whether LDL from normal controls or subjects with familial homozygous type II hypercholesterolemia were used. No precipitate was observed at any of the solubilization steps (steps 3, 4, 6, and 8–11). The progressive decrease in recoverable protein was attributed to the loss of water-soluble apoB that adhered to the dialysis bags (steps 4 and 11) or to the manipulation of insoluble protein at the ethanol-precipitation step. When 7 mg of water-soluble apoB was subjected to scintillation counting, no di[^{14}C]palmitoylphosphatidylcholine originally incorporated into LDL was detected; moreover, apoB at step 11 did not contain any detectable radioactivity derived from [^{14}C]urea.

Polyacrylamide Gel Electrophoresis. Figure 1 shows a polyacrylamide slab gel of two preparations of apoB at various stages of purification. The gel pattern of apoB at steps 4, 7, and 11 prepared under standard conditions is shown in lanes a, c, and e, respectively (Figure 1). Lanes b, d, and f show the gel pattern of apoB prepared under reducing conditions at steps 4, 7, and 11, respectively. Lane g shows the protein standards. The gel patterns in Figure 1 represent apoB from a subject with familial homozygous type II hyperlipoproteinemia. Identical gel patterns were obtained for three normal subjects and two other subjects with type II hyperlipoproteinemia (not shown). Figure 1 shows that in the *presence* of 2-mercaptoethanol identical gel patterns are obtained when either standard or reducing conditions are employed in the delipidation of LDL and solubilization of apoB in aqueous

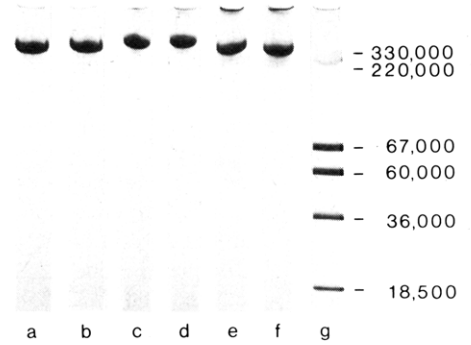

FIGURE 1: Polyacrylamide gel electrophoresis of apoB prepared from LDL under either standard conditions or reducing conditions. The gel represents a 3–20% pore–gradient gel containing 0.1% sodium dodecyl sulfate and 6 M urea (see Materials and Methods for details). Lanes a–f received 45 μg of protein each; electrophoresis was performed in the presence of 1% 2-mercaptoethanol. Lanes a, c, and e represent apoB at steps 4, 7, and 11, respectively; these samples were prepared under standard conditions for delipidation of LDL and solubilization of apoB (see Materials and Methods). Lanes b, d, and f represent apoB at steps 4, 7, and 11, respectively; these samples were prepared under reducing conditions (see Materials and Methods) for delipidation of LDL and solubilization of apoB. Lane g represents the protein standards (20 μg each) electrophoresed in the presence of 1% 2-mercaptoethanol; the following molecular weight standards (from top to bottom) were used: thyroglobulin (330 000), ferritin half unit (220 000), bovine serum albumin (67 000), catalase (60 000), lactate dehydrogenase (36 000), and ferritin monomer (18 500).

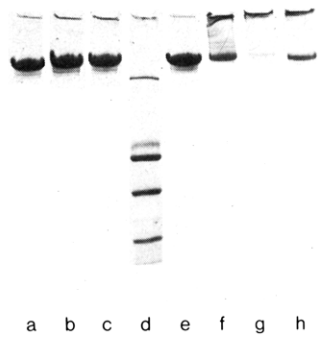

FIGURE 2: Effect of 2-mercaptoethanol on electrophoretic mobility of apoB at various steps of purification procedure. The gel was prepared as described in Figure 1. Samples of apoB at steps 4, 7, and 11 in 10 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 6 M urea, 1 mM EDTA, 20% sucrose, and 0.03% bromophenol blue were divided into two equal portions. One portion received 2-mercaptoethanol to a final concentration of 1%, while the remaining portion received an equal volume of H_2O. Each sample was then heated at 60 °C for 15 min and subjected to electrophoresis as described under Materials and Methods. Lanes a–c represent apoB (45 μg each) at steps 4, 7, and 11, respectively, to which 1% 2-mercaptoethanol was added. Lane d represents the following molecular weight standards (from top to bottom): thyroglobulin (330 000), ferritin half unit (220 000), bovine serum albumin (67 000), catalase (60 000), lactate dehydrogenase (36 000), and ferritin monomer (18 500). Lane e represents 45 μg of RCAM-apoB dissolved in dissociation buffer to which 2-mercaptoethanol was omitted. Lanes f–h represent apoB (45 μg each) at steps 4, 7, and 11, respectively, in the absence of 2-mercaptoethanol.

buffer. At each step of the purification, apoB shows a single band with an apparent M_r of approximately 500 000. Only slight variations in electrophoretic mobility were observed in this gel system when 5 and 45 μg of protein were loaded per well; only on overloaded gels (>45 μg of protein) were additional faint bands of slightly lower molecular weight observed.

Figure 2 compares the gel patterns of a preparation of apoB electrophoresed in the *presence* and *absence* of 2-mercapto-

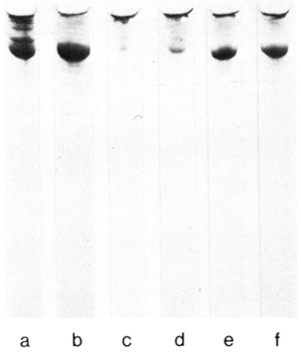

FIGURE 3: Polyacrylamide gel electrophoresis of apoB prepared from carboxamidomethylated LDL. CAM-apoB containing ~2 mol of [^{14}C]acetamide/250 000 g of protein was prepared as described under Materials and Methods. Standard conditions were employed during the purification of CAM-apoB. The gel was prepared as described in Figure 1. Each lane (a–f) received 30 μg of protein. (Lane a) CAM-apoB electrophoresed in the absence of 2-mercaptoethanol. (Lane b) CAM-apoB electrophoresed in the presence of 1% 2-mercaptoethanol. (Lane c) CAM-apoB (0.37 mg/mL) in standard buffer was adjusted to 4×10^{-5} M in 2-mercaptoethanol. The reducing agent was then removed by extensive dialysis against standard buffer. CAM-apoB was then electrophoresed in the absence of 2-mercaptoethanol. (Lane d) CAM-apoB (0.37 mg/mL) in standard buffer was adjusted to 0.05% in GSH. The GSH was then removed by extensive dialysis against standard buffer, and the sample was electrophoresed in the absence of 2-mercaptoethanol. (Lanes e and f) These lanes represent the same samples as those in lanes c and d, respectively; electrophoresis was performed in the presence of 1% 2-mercaptoethanol.

ethanol. This sample was prepared under standard conditions during the purification. Lanes a, b, and c show apoB at steps 4, 7, and 11, respectively. Lane d shows the protein standards. These samples were electrophoresed in the presence of 2-mercaptoethanol and show gel patterns similar to those of Figure 1. Lane e represents RCAM-apoB (14 mol of acetamide/250 000 g of apoB). Lanes f, g, and h represent apoB at steps 4, 7, and 11, respectively, electrophoresed in the *absence* of 2-mercaptoethanol. Only a fraction of the apoB at steps 4, 7, and 11 entered the gel when 2-mercaptoethanol was omitted from the sample buffer; the percent of the protein that entered the gel at steps 4, 7, and 11 (lanes f, g, and h) was 43, 12, and 30%, respectively. These values, determined by densitometry in the linear range of absorbance at 750 nm vs. protein concentration, were normalized relative to the amounts of apoB that entered the gel at steps 4, 7, and 11 in the presence of 2-mercaptoethanol. The RCAM-apoB (lane e) completely entered the gel. When water-soluble apoB (step 11) was prepared under reducing conditions (GSH and BHT) during the purification procedure, less than 5% of the sample entered the gel when electrophoresis was conducted in the absence of 2-mercaptoethanol compared to the amount that entered the gel in the presence of 2-mercaptoethanol. The data shown in Figures 1 and 2 suggest that sulfhydryl groups mediate the covalent aggregation of apoB. Moreover, this aggregation was more extensive when water-soluble apoB was purified under reducing conditions. LDL have 1.65 ± 0.05 (n = 4) and 1.82 ± 0.06 (n = 4) free sulfhydryl residues/250 000 g of polypeptide as determined by reaction with DTNB and [1-^{14}C]iodoacetamide, respectively. Moreover, water-soluble apoB (step 11) has 13.9 ± 0.1 (n = 2) mol of half-cystine/250 000 g of protein. These data suggest that each apoB polypeptide chain in LDL has 2 mol of free SH and six intramolecular disulfide bonds. Reaction of CAM-apoB (2 mol of acetamide/250 000 g of apoB) with fluorescein mercuric acetate (Karush et al., 1964) yielded 5.8 ± 0.53 (n = 2) disulfide bonds/250 000 g of protein.

Table I: Amino Acid Analysis[a]

AA	present study ± SEM[b]	Margolis & Langdon (1966a)	Kane et al. (1980)	Lee & Alaupovic (1970)
Asp	27.6 ± 0.7	20.5	22.2	21.6
Thr	14.0 ± 0.5	12.3	13.8	13.6
Ser	24.0 ± 1.0	15.7	18.0	16.8
Glu	27.9 ± 0.9	23.9	24.2	25.5
Pro	8.3 ± 0.6	7.3	8.0	7.3
Gly	12.0 ± 0.8	9.3	9.9	9.1
Ala	15.2 ± 1.0	11.8	12.6	13.0
Cys	1.4[c]	1.3	0.93	1.1
Val	7.8 ± 0.6	11.8	11.6	9.2
Met	2.8 ± 1.3	3.5	3.3	3.0
Ile	7.9 ± 0.6	11.8	12.6	10.0
Leu	23.2 ± 1.2	22.8	24.7	22.7
Tyr	6.2 ± 0.2	5.7	7.0	6.2
Phe	11.6 ± 0.2	10.2	10.5	9.1
His	5.7 ± 0.2	4.9	5.4	4.4
Lys	13.7 ± 0.8	13.0	16.6	12.3
Arg	6.0 ± 0.7	6.2	7.0	5.5
Trp	1.3[d]	1.1	N.D.[e]	1.3

[a] Expressed in moles per 25 000 g of polypeptide, excluding weight contributions due to carbohydrate (5–7%). [b] These data represent a total of nine determinations of 24-h hydrolysates obtained from three separate donors for the apoB preparation obtained at step 11 of Chart I. [c] Based on the chemical reactivity of apoB with [1-^{14}C]iodoacetamide. [d] Determined by MCD analysis. [e] N.D., not determined.

Figure 3 shows the polyacrylamide gel patterns of water-soluble carboxamidomethylated apoB (CAM-apoB) at step 11 electrophoresed in the absence (lane a) and presence (lane b) of 2-mercaptoethanol. CAM-apoB was prepared from LDL that was first modified with iodoacetamide in order to block the free SH groups on apoB, and then the CAM-apoB was purified under standard conditions. As shown by the gel patterns, nearly all of the CAM-apoB entered the gel in the absence of 2-mercaptoethanol. Thus, blocking the half-cysteine residues in LDL prior to lipid extraction inhibits the formation of high molecular weight covalent aggregates. Lane a also shows several minor bands representing oligomers >500 000. These aggregates, which are absent in lane b, either are formed from apoB molecules in LDL that were not modified by iodoacetamide or represent apoB that has undergone reduction of its disulfide bonds during purification to yield free sulfhydryls. For determination of the relationship between free sulfhydryl groups and the aggregation state of apoB, 2-mercaptoethanol or glutathione was first added to CAM-apoB in standard buffer, and the exogenous sulfhydryl compounds were removed by dialysis (Figure 3). As is shown in lanes c and d in which the CAM-apoB (0.37 mg/mL) was exposed to either 4×10^{-5} M 2-mercaptoethanol or 0.05% glutathione, respectively, only a limited amount of apoB entered the gel, suggesting that extensive cross-linking had occurred. In the latter experiment, the electrophoresis was performed in the *absence* of 2-mercaptoethanol. However, when electrophoresis was performed in the *presence* of 2-mercaptoethanol, most of the protein entered the gel (lanes e and f).

Amino Acid Composition. Table I shows the amino acid composition of water-soluble apoB obtained at step 11 of the purification procedure. This composition agrees favorably with those reported from other laboratories (Margolis & Langdon, 1966a; Triplett & Fisher, 1978; Kane et al., 1980; Lee & Alaupovic, 1970). ApoB contains ~14 mol of half cystine/250 000 g of apoB of which two residues exist as free sulfhydryls in LDL. The tryptophan content of water-soluble apoB was determined by MCD. This method relates the magnitude of the magnetically induced CD to the number of

Table II: Secondary Structural Composition of LDL and of ApoB at Various Purification Steps[a]

purification step[b]	sample	buffer conditions	% α helix	% β-pleated sheet	% remainder
	LDL	S.B.[c]	44	16	40
4	apoB	S.B.	42	20	38
8	apoB	S.B. + 6 M Gdn-HCl	6	15	79
9	apoB	S.B. + 6 M urea	18	18	64
11	apoB	S.B.	28	15	57
11	CAM-apoB	S.B.	30	28	42
11	RCAM-apoB	S.B.	27	13	60

[a] Data were analyzed by the HELIX program as described previously (Chen et al., 1974; Thompson et al., 1976). [b] Refer to Chart I. [c] S.B., standard buffer.

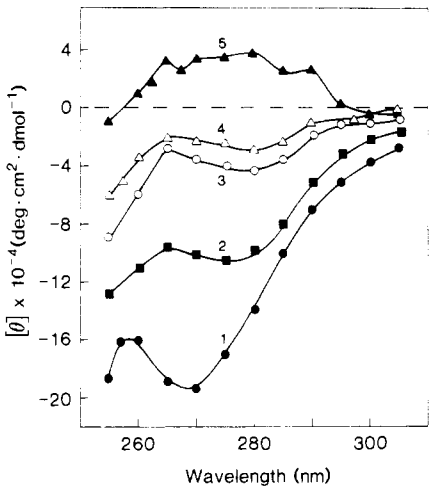

FIGURE 4: Near-UV CD spectra of various water-soluble apoB preparations: (curve 1) apoB purified from LDL under reducing conditions; (curve 2) apoB purified from LDL under standard conditions; (curve 3) the sample of curve 1 adjusted to 10 mM in 2-mercaptoethanol; (curve 4) fully reduced and carboxamidomethylated apoB (RCAM-apoB); (curve 5) carboxamidomethylated apoB (CAM-apoB).

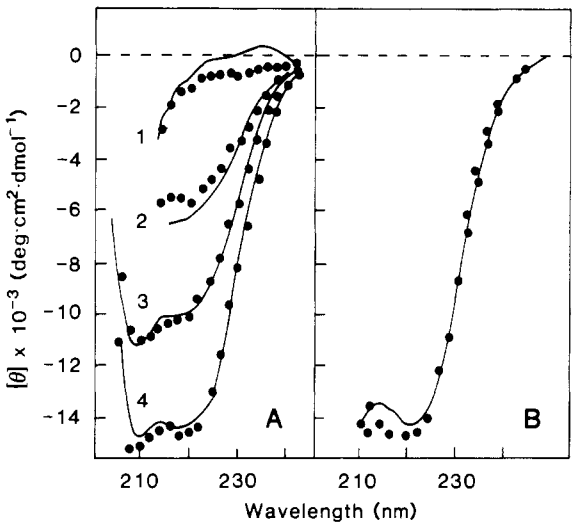

FIGURE 5: Far-UV CD spectra of apoB at various steps of purification procedure of Chart I (panel A) and of human plasma LDL (panel B): (curve 1) apoB in standard buffer containing 6 M Gdn-HCl (step 8); (curve 2) apoB in standard buffer containing 6 M urea (step 9); (curve 3) apoB in standard buffer (step 11); (curve 4) apoB in standard buffer (step 4). The concentration of apoB and of LDL in each experiment was 300 μg/mL. Data were analyzed by the HELIX program (Chen et al., 1974; Thompson et al., 1976). The dots show the experimental points, and the solid lines show the computer-fitted curves. Spectra were recorded at 23 °C in a cell of 0.2-cm path length.

tryptophan residues per polypeptide chain and is independent of the nature of the chemical environment in which the tryptophan resides (Barth et al., 1972; McFarland & Coleman, 1972; Holmquist & Vallee, 1973). On the basis of MCD measurements, water-soluble apoB contains 13 mol of tryptophan/250 000 g of polypeptide. When subjected to in lanes Edman degradation, apoB was found to have a blocked N terminus.

Physical Properties. Figure 4 shows the near-UV CD spectra of several water-soluble apoB preparations. Curve 1 is the spectrum for a preparation of apoB (step 11) that was purified from LDL under reducing conditions and then dialyzed against standard buffer to remove the glutathione; buffer alone or buffer containing 0.05% glutathione had a negligible CD contribution. This sample has a broad negative trough between 255 and 310 nm and exhibits a band minimum at ~270 nm; $[\theta]^{min}_{270nm} = -198\,000$ deg·cm²·dmol⁻¹. The addition of 2-mercaptoethanol (curve 3) causes a diminution in the magnitude of the negative ellipticity at all wavelengths and a 10-nm red shift in the band minimum to ~280 nm ($[\theta]^{min}_{280nm} = -43\,370$ deg·cm²·dmol⁻¹); furthermore, removal of the 2-mercaptoethanol results in the regeneration of curve 1. By itself, 2-mercaptoethanol in standard buffer showed a negligible CD contribution. The spectrum for apoB in the presence of 2-mercaptoethanol (curve 3) closely resembles that for RCAM-apoB (curve 4), which has a $[\theta]^{min}_{280nm} = -38\,870$ deg·cm²·dmol⁻¹. Water-soluble apoB isolated from LDL in the absence of glutathione (curve 2) exhibits a CD spectrum much less intense ($[\theta]^{min}_{275nm} = -107\,000$ deg·cm²·dmol⁻¹) than that of apoB purified from LDL in the presence of glutathione ($[\theta]^{min}_{270nm} = -198\,000$ deg·cm²·dmol⁻¹). CAM-apoB (curve 5) exhibits a broad positive spectrum with a $[\theta]^{max}_{280nm} = +40\,560$ deg·cm²·dmol⁻¹. The addition of 2-mercaptoethanol to CAM-apoB results in a spectrum (not shown) identical with that of RCAM-apoB (curve 4). The above findings suggest that both intramolecular and intermolecular disulfide bonds make major contributions to the near-UV CD spectrum of water-soluble apoB preparations.

Figure 5 shows the far-UV CD spectra of apoB at various stages of purification (panel A) and of LDL (panel B). Secondary structural compositions are listed in Table II. LDL exhibit 44% α helix and 16% β pleated sheet. Water-soluble apoB obtained at step 4 (curve 4) has 42% α helix and 20% β-pleated sheet. In the presence of Gdn-HCl (step 8, curve 1), apoB exhibits a large decrease in α helix to 6% with a concomitant increase in nonstructured elements; the β-pleated sheet is slightly reduced to ~15%. After apoB is dialyzed against 6 M urea (step 9, curve 2), there is a large increase in ordered structure to 18% α helix, whereas the β-pleated sheet remains fairly constant, i.e., ~18%. Following dialysis against standard buffer (step 11) to remove the urea, the α-helical content increases to 28% (curve 3), whereas the β structure does not significantly change. RCAM-apoB exhibits 27% α helix and 13% β-pleated sheet, while CAM-apoB ex-

hibits a slightly increased ordered structure, 30% α helix and 28% β-pleated sheet.

Discussion

Preparation of Water-Soluble ApoB. The primary aim of this study was to develop a method to obtain apoB as a lipid-free, water-soluble apoprotein. The initial steps in the preparation of water-soluble apoB are similar to those described by Gotto et al. (1973). As is shown in Chart I, LDL were lyophilized to dryness and delipidated with organic solvents. ApoLDL were then dissolved in sodium decyl sulfate, and the detergent was removed by extensive dialysis; sodium decyl sulfate is easily removed by dialysis due to its high critical micelle concentration (Mukerjec & Mysels, 1971). Although others have reported that decreasing the concentration of sodium dodecyl sulfate by dialysis causes apoB to precipitate (Steele & Reynolds, 1979a; Simons & Helenius, 1970), we find that after the removal of sodium decyl sulfate, apoB remains water soluble. For removal of tightly bound lipids or possible traces of detergent, apoB was further delipidated by ethanol precipitation as described previously for glycophorin (van Zoelen et al., 1978) and (Na^+ and K^+)-ATPase (Jackson et al., 1980b); this procedure removes tightly associated residual lipids, and especially acidic phospholipids, from these proteins. After ethanol precipitation, no residual radiolabeled phosphatidylcholine was detected, and no inorganic phosphate or cholesterol was detected by standard mass measurements. Although the protein was completely soluble in 6 M Gdn-HCl, dialysis against standard buffer caused apoB to precipitate. However, if apoB in 6 M Gdn-HCl were dialyzed first against 6 M urea, then 1 M urea, and finally standard buffer, apoB was totally water soluble. The concentration of apoB in standard buffer at steps 4 and 11 was typically 2–3 mg/mL. ApoB showed a major band of ~500 000 at each step of purification when electrophoresis was conducted in 0.1% sodium dodecyl sulfate and 6 M urea. This value of the molecular weight, however, may be fortuitous as apoB is known to exhibit an anomalous electrophoretic migration (Lee et al., 1981). Moreover, goat antisera prepared against apoB (step 4) gave a single precipitin line of complete identity with LDL, water-soluble apoB at steps 4 and 11, CAM-apoB, and RCAM-apoB. Rabbit antisera prepared against human apoE, C-I, C-II, C-III, and A-I did not give an observable reaction when tested over a range of apoB concentrations.

Factors Influencing Solubility of ApoB. So that the importance of reducing conditions in the solubilization of apoB could be tested, LDL were purified and delipidated in the absence and presence of GSH, BHT, and nitrogen; no quantitative differences in protein recoveries were obtained at step 11 in four different preparations of apoB. Thus, the inclusion of antioxidants is not a requirement for obtaining nondegraded water-soluble apoB preparations.

The results in Table II suggest that the conformation and state of noncovalent association of apoB play an important role in determining its solubility properties. LDL, apoB (steps 4 and 11), RCAM-apoB, and CAM-apoB all exhibit highly ordered structures in aqueous buffer. ApoB, RCAM-apoB, and CAM-apoB in 6 M Gdn-HCl have <6% α helix, and these proteins precipitate when dialyzed against standard buffer. Steele & Reynolds (1979a) reported a random-coil structure for RCAM-apoB in 7.6 M Gdn-HCl. Moreover, lowering the Gdn-HCl concentration to <2 M resulted in precipitation of the apoprotein (Steele & Reynolds, 1979a). In concentrated Gdn-HCl, RCAM-apoB exists in a monomeric state of M_r 250 000 (Steele & Reynolds, 1979b). The precipitation may be due to the inability of the apoprotein to reassociate and to renature properly to achieve an aqueous soluble state. As is shown in Figure 5 and Table II, dialysis of apoB in 6 M Gdn-HCl against 6 M urea (step 9) increases the α-helical content to 18%. The increase in α helix may be explained by an effective decrease in the denaturant concentration experienced by the apoprotein when it is transferred from 6 M Gdn-HCl to 6 M urea; i.e., on a molar basis, urea is a less effective denaturant than Gdn-HCl (Greene & Pace, 1974). The data of Table II suggest that the α-helical regions of apoB are conformationally more labile than the β-pleated sheet regions. Preliminary experiments suggest that the increase in ordered structure and water solubility properties reflect the state of noncovalent aggregation or association that occurs as the denaturant concentration is decreased. We postulate that the sequestration of hydrophobic domains away from the aqueous media and the exposing of hydrophilic domains account for the overall solubility of apoB in aqueous solutions in the absence of denaturants and amphiphilic ligands. Covalent aggregation due to intermolecular disulfide bond formation does not explain the aqueous solubility of apoB. The addition and removal of 2-mercaptoethanol to water-soluble apoB results in the reduction and reoxidation, respectively, of the disulfide bonds without altering the solubility properties. Moreover, RCAM-apoB, which has no disulfide bonds, and CAM-apoB, which only has intramolecular disulfides, are both highly water soluble. The aqueous solubility of apoB cannot be explained by carbamylation, the presence of residual denaturants, the selective enrichment of a soluble subunit, the degradation of the apoB polypeptide chain by oxidation (Schuh et al., 1978; Lee, 1980), proteolysis (Steele, 1979; Coetzee et al., 1980; Krishnaiah & Weigandt, 1974; Triplett & Fisher, 1978), or the presence of other serum proteins (Shireman et al., 1977).

Mechanisms of Covalent Aggregation of ApoB. ApoB forms covalent aggregates following the delipidation of LDL (Figure 2). In sodium dodecyl sulfate and urea aggregation is reversed by 2-mercaptoethanol, suggesting that sulfhydryls participate in the cross-linking reaction. LDL contain 14 mol of half-cystine/250 000 g of apoB polypeptide. Since two of these residues are free sulfhydryls in LDL, there are approximately six disulfide bonds in LDL. These findings are consistent with those of Margolis & Langdon (1966a,b), who reported ~13 mol of half-cystine/250 000 g of protein; two of these residues were detected as free sulfhydryls in LDL. However, it has not been demonstrated previously that apoB forms covalent aggregates if these sulfhydryl residues are not first blocked. We observed that carboxamidomethylation of LDL (CAM-LDL) inhibited the covalent aggregation of apoB when the purification was conducted in the absence of GSH but not in its presence (Figure 3). These data strongly suggest that a free sulfhydryl is required to initiate the cross-linking reaction. Addition of GSH and other mercaptides to disulfide-containing proteins is known to catalyze cross-linking reactions (Jocelyn, 1972; Huggins et al., 1951). Cross-linking reactions also occur between heterologous proteins such as serum albumin, which contains one free sulfhydryl group, and γ-globulins, which only contain disulfide bonds (Huggins et al., 1951). This type of reaction may explain the reported aqueous solubilization of apoB by bovine serum albumin (Shireman et al., 1977). The ability of copper to induce the solubilization of apoB as reported by Huang & Lee (1979) might be related to the fact that copper is a strong mercaptide-forming agent (Huggins et al., 1951; Jocelyn, 1972). The present findings with apoB are compatible with a sulfhydryl–disulfide exchange reaction mechanism (Huggins et al.,

1951; Jocelyn, 1972). The result is the formation of an intermolecular disulfide bond and a new free SH group at the expense of the intramolecular disulfide bond. This process is then reiterated by the newly generated SH group and ultimately results in polymerization.

Effect of Disulfide on ApoB Structure. The data of Table II suggest that intermolecular disulfide bond formation relieves internal secondary structural restraints, possibly β structure. Consistent with this hypothesis, Steele & Reynolds (1979b) showed that CAM-apoB (intramolecular disulfide bonds intact) has a Stokes radius of 137 Å, whereas RCAM-apoB (disulfides fully reduced and alkylated) has a radius of 147 Å. Triplett & Fisher (1978) reported an increase in intrinsic viscosity following reductive alkylation of apoB consistent with internal relaxation of the polypeptide structure. Disulfide bonds also appear to influence the tertiary structure of apoB. As shown in Figure 4, CAM-apoB (curve 5) has a positive aromatic CD spectrum whereas RCAM-apoB (curve 4) and highly cross-linked water-soluble apoB (curves 1 and 2) have slightly negative and intensely negative CD bands, respectively. These bands are assigned to the presence of intramolecular and intermolecular disulfide bonds in CAM-apoB and water-soluble apoB, respectively. These transitions arise directly from disulfide bonds (Sears & Beychok, 1973; Jocelyn, 1972) or indirectly by disulfide bond induced conformational restraints that influence aromatic transitions. Moreover, the magnitude of these bands varies as a function of the purification step and is directly proportional to the degree of polymerization as assessed by polyacrylamide gel electrophoresis. Thus, both solubility and aggregation are modulated by experimental procedure.

Degradation of ApoB. Nitrogen saturation and the inclusion of antioxidants such as GSH, BHT, and EDTA in buffers have been shown to minimize lipid peroxidation of LDL and possibly polypeptide chain degradation whereas azide may promote such reactions (Schuh et al., 1978; Lee, 1980; Lee et al., 1981). Currently, the exact mechanism by which polypeptide degradation occurs is not known nor at which sites such reactions are initiated. In addition to lysines that may form condensation products with malondialdehyde, the sulfhydryl chemistry of apoB may influence its stability in solution. Protein thiols serve as preferred free-radical traps (Henricksen, 1961) and may themselves form free radicals (Jocelyn, 1972). The possible radiomimetic potentiating effects of azide (Schuh et al., 1978) may promote thiol-free radical formation. Biological reactions that possibly involve protein thiol free-radicals include autoxidation reactions catalyzed by trace metals and reactions with fatty acid peroxides (Jocelyn, 1972). Thus, the fully reduced and alkylated apoprotein should be used as a precautionary measure to inhibit intermolecular disulfide exchange reactions and potential thiol-mediated degradation reactions.

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Molecular Properties of Calcium-Pumping ATPase from Human Erythrocytes†

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ABSTRACT: The Ca^{2+}-pumping ATPase from human erythrocyte membranes, purified by the method previously reported [Niggli, V., Penniston, J. T., & Carafoli, E. (1979) *J. Biol. Chem. 254*, 9955–9958], was freed of minor impurities by extensive washing while bound to the calmodulin–Sepharose column. The pure enzyme showed a single band of M_r 138 000, which contained no stainable carbohydrate. The enzyme retained calmodulin-stimulable ATPase activity; with appropriate assay conditions, an activity of 21.2 μmol/(mg·min) was obtained. Amino acid analysis showed that the ATPase had a larger proportion of polar amino acids than do other integral membrane proteins. Despite this, the ATPase showed a tendency to form dimers and higher aggregates even in the presence of sodium dodecyl sulfate and urea. The enzyme required Mg^{2+} but showed little activity unless a second ion was added. With regard to this second ion, the enzyme responded to alkaline earth metal ions in the order $Ca^{2+} > Sr^{2+} \gg Ba^{2+}$. It was highly specific for ATP and was stimulated by Na^+ or K^+; in all of these properties it resembled the enzyme in unfractionated membranes. Limited proteolysis using trypsin yielded, at short times, many fragments of various molecular weights; continued proteolysis resulted in two trypsin-resistant fragments of M_r 81 000 and 33 500. Analysis of the time course of proteolysis indicated that the ATPase existed in two or more conformations that had differing susceptibilities to proteolysis. It is suggested that these correspond to active and inactive conformers of the enzyme.

Several laboratories have established that Ca^{2+}–ATPase is responsible for active Ca^{2+} extrusion across the plasma membrane of human erythrocytes (Schatzmann, 1973; Schatzmann & Vincenzi, 1969; Roufogalis, 1979; Carafoli et al., 1980). Its enzymatic and physiological properties have been widely studied with red blood cell membranes, but only recently has it been purified nearly to homogeneity (Niggli et al., 1979). Because of the low concentration of this enzyme in the membrane, it has been difficult to free it of minor impurities; in the original preparation (Niggli et al., 1979) about 6% of the stain appeared in a position corresponding to band 3 (90 000 daltons).

In this paper we report a method for removing minor impurities from the enzyme. We also report chemical and catalytic properties of the purified enzyme, the absence of stainable carbohydrate in the enzyme, and the probable existence of two or more conformations of the enzyme, based on an analysis of the time course of proteolysis.

Materials and Methods

L-α-Phosphatidylcholine (type IX-E from egg yolk) was purchased from Sigma Chemical Co. Cyanogen bromide activated Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden. [γ-^{32}P]ATP[1] (2–10 Ci/mmol) was purchased from New England Nuclear. Bovine pancreatic trypsin was purchased from Calbiochem. Bovine brain calmodulin

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[1] Abbreviations: TEA, triethanolamine; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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