Characterization of the Apolipoprotein B Polypeptide of Human Plasma Low Density Lipoprotein in Detergent and Denaturant Solutions*

John C. H. Steele, Jr. and Jacqueline A. Reynolds

From the Department of Biochemistry, Duke University Medical Center and Whitehead Medical Research Institute, Durham, North Carolina 27710

Apolipoprotein B, the polypeptide moiety of human serum low density lipoprotein, is subject to degradation (as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) both in the intact particle and after delipidation. Protease inhibitors, sodium azide, and nitrogen saturation did not influence the rate or degree of degradation. Lipid-free apolipoprotein B prepared by gel exclusion chromatography in sodium dodecyl sulfate bound a limited number of detergent molecules (up to 300) in monomeric sodium dodecyl sulfate solutions; circular dichroic spectra of this complex were similar to spectra of the intact lipoprotein. Near the critical micelle concentrations, a large cooperative increase in detergent binding occurred, accompanied by circular dichroic changes indicating increased a helicity. By sucrose density centrifugation, lysopalmitolyl phosphatidylcholine could be substituted for the anionic detergent; about 300 mol of lysolipid were bound to the polypeptide. Replacement of detergent with guanidine hydrochloride by dialysis produced a soluble polypeptide with no ordered structure at denaturant concentrations above 7 M. At lower guanidine hydrochloride concentrations, structural elements were regained in a broad, reversible transition. It appears that apolipoprotein B is an easily degraded polypeptide with regions resembling water-soluble proteins but other regions which interact with lipid (or synthetic amphiphiles) and produce an overall insolubility in aqueous solution in the absence of amphiphilic ligands.

Human plasma or serum can be separated by various means into distinct classes of lipoproteins, each of which is thought to perform different functions in the transport, distribution, and metabolism of endogenous or exogenous lipids. One of these lipoproteins, LDL, serves as the major carrier of cholesterol (primarily as cholesteryl esters) in the serum and appears to be involved in the regulation of cholesterol metabolism in extrahepatic tissues (2). Elevation of this lipoprotein has been implicated as a risk factor in the development of atherosclerosis in humans (3, 4).

LDL, as classically isolated by ultracentrifugation between solution densities of 1.019 and 1.063 g/ml, is a relatively homogeneous particle with an average diameter of 230 Å and a molecular weight of about 2.5 million (5). By weight, it is 23% protein and 78% lipid; the protein consists essentially of one polypeptide, termed apoB, although some workers have reported small amounts of other proteins present (6). While the apolipoproteins of other lipoprotein classes have been well characterized (see Ref. 7 for review), the physicochemical study of apoB has progressed slowly, due primarily to its insolubility in aqueous solutions in the absence of amphiphilic ligands. A variety of solubilization methods, employing chemical modification (8, 9), denaturants (8, 10, 11), or detergents (12, 13), have been devised in attempts to maintain apoB in solution in a state amenable to physical measurements. However, there is still controversy about many reported properties of the polypeptide with suggestions that aggregation (6) or proteolysis (14) may account for observed discrepancies.

In the present work, the method of Simons and Helenius (13, 15) utilizing gel exclusion chromatography in sodium dodecyl sulfate-containing buffer has been used to prepare lipid-free apoB (with disulfides intact). The stability, amphiphile-binding capacity, and optical properties of this protein in detergent or denaturant solutions have been measured and compared to those of the apolipoproteins of HDL. The accompanying paper (16) deals with the molecular weight and hydrodynamic properties of apoB in such solutions.

EXPERIMENTAL PROCEDURES

Materials  Sodium dodecyl sulfate ("specially pure" grade), hereafter SDS, was obtained from Gallard-Schlesinger; gas-liquid chromatography of an acid hydrolysate of this product showed no alcohols other than dodecanol to be present. Sodium dodecyl sulfate was from Amersham/Searle; a sample of the dodecanol used in the radiochemical synthesis was kindly provided by that firm and was shown to be free of alcohols of other chain lengths. Lysopalmitoyl phosphatidylcholine and [14C]lysopalmitoyl phosphatidylcholine were from Applied Science Laboratories; thin layer chromatography on Silica Gel H (Supelco) in chloroform/methanol/water (65/35/4) revealed only one spot both chemically and radiochemically. [U-14C]Phosphatidylcholine (algae) and [14C]cholesterol (from Amersham/Searle) in hydrocarbon solvents were washed extensively with distilled water to remove water-soluble impurities before use as tracers. Guanidine hydrochloride was the "ultrapure grade" from Schwarz/Mann. Chromatographic columns and Sepharose 4B were Pharmacia products. Other chemicals were reagent grade products. Deionized, glass-distilled water was used to make all solutions. All analytical glassware was washed with concentrated nitric acid and rinsed extensively with deionized water prior to use. Dialysis tubing (Union Carbide) was prepared by heating just to boiling in 1 mM EDTA, 10 mM sodium carbonate, then in distilled water.

* This investigation was supported by National Institutes of Health Grant HL 14882 (J. C. H. S.). Predoctoral Fellowship 5-TO-5-GM-01678 (J. C. H. S.). A preliminary report of portions of this work has appeared (1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* The abbreviations used are: LDL, low density lipoprotein, isolated as specified herein; HDL, high density lipoprotein; apoB, apolipoprotein B; SDS, sodium dodecyl sulfate; cmc, critical micelle concentration; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II. Especially note that apoB refers to the apolipoprotein with intact disulfide bonds except when specified otherwise.
**Analytical Procedures**—Protein was determined by the Lowry method (17) with 2% SDS included in both sample and bovine serum albumin standard solutions or, in the lysolipid experiments, by the Amido schwarz assay (18). In guanidine solutions, the protein concentration was calculated from the absorbance at 280 nm (10). Phosphorography and electron microscopy (9) were used to determine barrities, ionic strength, and sucrose concentration. The binding ratio in protein-containing fractions was calculated and found to be constant within experimental error.  

**Rate of Dialysis Measurements**—Rate of dialysis measurements. Above the critical micelle concentration (CMC) of SDS in 0.3 phosphate buffer (with azide concentrations of 0.5 or 2.5 mM SDS), appropriate fractions were collected and monitored for protein by measuring absorbance at 280 nm. Tissue containing lipid-free protein were pooled and used immediately.

**SDS Binding Measurements**—The critical micelle concentration (CMC) of SDS or 0.3 phosphate buffer was determined by 0.91 nm by rate of dialysis measures. Above the CMC, the amount of SDS bound was measured by using appropriate concentrations of radioactive SDS solutions as eluting buffer in the chromatoagrapic delipidation procedure and determining the concentration of SDS and protein in collected fractions. The binding ratio was constant (within experimental error) across the eluted protein peak and did not change by rate of dialysis measurements. Above the CMC, the amount of SDS bound was measured by using appropriate concentrations of radioactive SDS solutions as eluting buffer in the chromatographic delipidation procedure and determining the concentration of SDS and protein in collected fractions. The binding ratio was constant (within experimental error) across the eluted protein peak and did not change.

**Lysozyme Polysulfolylchloride Binding Measurements**—Lysozyme polysulfolylchloride binding was calculated and found to be constant within experimental error.  

**Guanidine Studies**—apoB in 0.1 mM SDS was dialyzed against nitrogen against concentrated solutions of guanidine hydrochloride. In some cases, the pH was adjusted to 8.6, a 10-fold molar excess of 2-mercaptoethanol was added (assuming six sulfhydryl groups/100,000 daltons (12)), and the sulfhydryl groups were blocked with iodoacetamide; completion of the reaction was verified by amino acid analysis. For the renaturation experiment, apoB in 8.6 M guanidine hydrochloride solution was dialyzed stepwise (15 to 24 h/step) against successively lower denaturant concentrations, using 1-ml Lucite cells. Then, a portion of the 2.6 M guanidine sample was successively dialyzed against increasing guanidine hydrochloride concentrations.

**RESULTS**

**Stability of LDL**—Most preparations of LDL showed only one band on SDS-polyacrylamide gel electrophoresis (Fig. 1A), although one or two very minor bands with slightly faster mobility were noted at times on heavily overloaded gels (Fig. 1B). Identical gel patterns were obtained when 2-mercaptoethanol was excluded from the sample and when the sample was heated at 100°C for 0 to 10 min. On heating at 100°C for more than 1 h or at 50°C overnight, marked degradation was observed with disappearance of the major band and the appearance of numerous bands in the 100,000- to 200,000-dalton range (Fig. 1D). Inclusion of phenylmethanesulfonyl fluoride or of 1,10-phenanthroline (23) failed to prevent the degradation. LDL stored at room temperature for more than 4 days also gave gel patterns, indicating degradation had occurred to a variable extent; delipidated apoB in micellar SDS solutions appeared to be more stable, although breakdown began to occur gradually after 4 to 6 days at room temperature. Finally, intact LDL stored at 4°C in azide or EDTA, or both, was stable for only 3 to 4 weeks; gels run after that time showed several bands of lower molecular weight (Fig. 1C). The use of nitrogen-saturated solution throughout the isolation and storage procedures did not retard these changes.

**Delipidation of LDL and Solubility of ApoB (with Disulfides Intact)**—LDL delipidated by gel exclusion chromatography in SDS solution gave a pattern similar to that observed using slightly different conditions by Helenius and Simons (13), with a small peak of aggregated protein plus lipid in the void volume, a well included symmetrical protein peak which was stable for only 3 to 4 weeks; gels run after that time showed several bands of lower molecular weight (Fig. 1C). The use of nitrogen-saturated solution throughout the isolation and storage procedures did not retard these changes.

**Fig. 1.** SDS-gel electrophoresis of holo-LDL. A, freshly prepared LDL, 10 µg of protein; B, freshly prepared LDL, 50 µg of protein; C, stored LDL, 50 µg of protein; D, freshly prepared LDL heated for 4 h at 100°C, 50 µg of protein. Gels are 3.3% acrylamide; origin is at top. Dotted lines represent bands faintly stained with Coomassie blue; solid lines indicate more intensely stained bands. Appropriate weights determined using standard proteins are indicated.
eluted in an identical position when rechromatographed in the presence or absence of 2-mercaptoethanol, and two lipid peaks, the latter near the column’s internal volume. Protein analysis showed a small amount to be present in the void volume, with the remainder in the large protein peak and none in subsequent fractions. On columns run using 1.0 mM SDS in N-[(tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid, pH 7.4 (ionic strength, 0.3), buffer rather than in 0.3 phosphate buffer, phosphorus analysis of protein peak tubes indicated less than 1 mol of phosphorus/mol of protein, i.e. per 250,000 g (10, 16). Using radioactively labeled diacylphosphatidylcholine, cholesterol, cholesterol oleate, or triolein in tracer amounts, less than 1 mol of phospholipid, cholesterol ester, or triolein per mol of apoB was found using 1.0 or 2.5 mM SDS buffer. However, 50 to 60 mol of free cholesterol remained associated with each mol of protein at 1.0 mM SDS, while after delipidation using 2.5 mM SDS, 3 to 4 mol of cholesterol were still present.

Delipidated apoB maintained in micellar SDS solutions appeared to be completely soluble indefinitely. However, reducing the SDS concentration below its cmc led to gradual precipitation of protein after 4 to 6 days. When dialyzed from SDS into concentrated guanidine solutions, apoB remained soluble indefinitely, unless the guanidine concentration was subsequently decreased to less than 2 M. Attempts to dialyze apoB from SDS solutions directly into guanidine solutions of concentration less than 3 M were unsuccessful, as the protein precipitated rapidly and could not be resolubilized.

**SDS Binding to ApoB (with Disulfides Intact)**—The binding of SDS to delipidated apoB was studied over the concentration range 10 μM to 10 mM by equilibrium dialysis (below the cmc) or by gel exclusion chromatography (above the cmc). The results are shown in Fig. 2 as grams of SDS bound per g of protein plotted versus the logarithm of the unbound SDS concentration; reversibility shown by the filled circles indicates that equilibrium was attained during the measurements. The isotherm shows that little detergent is bound at concentrations below 0.1 mM; as the cmc of 0.91 mM is approached, a limited amount of 0.2 to 0.4 g of SDS/g of protein is bound. Near the cmc, a large, cooperative increase in binding is observed which appears to plateau at 1.66 + 0.05 g/g, or 1440 ± 50 mol of detergent/mol of protein. Results were not significantly different using LDL delipidated at 1.0 or at 2.5 mM SDS, indicating that the free cholesterol molecules still associated with the protein do not alter the detergent-binding capacity of the molecule.

**Lysopalmitoyl Phosphatidylcholine Binding**—As similar experiments with radioactive lysolecithin are extremely expensive, a different method of measuring bound amphiphile was used for preliminary studies. ApoB in low levels of detergent (0.1 mM SDS) was incubated briefly in 1.0 mM radiolabeled lysopalmitoyl phosphatidylcholine solution, then the mixture was centrifuged in a 5/40/50% step sucrose gradient to equilibrium. As shown in Fig. 3, the lysolecithin-protein complex banded at a density of about 1.17 g/ml with excess unbound lipid found near density 1.09 g/ml. When buffer was added instead of protein, the lysolecithin band near densities 1.09 and 1.04 g/ml. From the estimation of lipid concentrations by scintillation counting and of protein concentrations by Amido Schwarz assay, it can be calculated that some 300 ± 50 mol of lysolecithin are associated with each mol of protein. Parallel centrifugations using [35S]SDS and unlabeled lipid indicated that essentially all the detergent remained at the top of the gradient, at densities less than 1.08 g/ml; no more than 3 mol of SDS sedimented with the protein-lysolecithin complex.

**Circular Dichroic Studies**—The far ultraviolet circular dichroic spectra of apoB (with disulfides intact) in SDS solutions of 0.1 and 2.5 mM in 0.3 phosphate buffer are shown in Fig. 4; the spectrum at 1.0 mM SDS was identical within experimental error with that at 2.5 mM SDS. At low binding levels, the wide negative trough at 212 to 220 nm indicates a significant amount of β structure, although the shoulder seen near 209 nm suggests that some α helix is also present. Analysis by the method of Greenfield and Fasman (24) confirms this qualitative assignment and also reveals that there is a strong contribution to the spectrum from nonstructured elements; to the nearest 5%, the spectrum indicates 25% helix, 40% β structure, and 35% disordered regions. With maximal SDS binding to apoB (1.66 g/g), the CD spectrum changes markedly; most of the structured regions now appear to be helical, but the relatively low molar ellipticity values at 208 and 220 nm reflect a large amount of "random coil." More quantitative analysis
polypeptide. Within experimental error, the same pattern is observed in fully reduced and carboxyamidomethylated apoB, alkylated) transferred from SDS into concentrated guanidine solutions is also given in Fig. 4; the generally featureless spectrum indicates the loss of all ordered structure in the polypeptide. Within experimental error, the same pattern is observed in fully reduced and carboxyamidomethylated apoB, indicating that intact disulfide bonds do not prevent the loss of all ordered structure. The dependence upon guanidine concentration of the 220 nm molar ellipticity of the fully reduced and carboxyamidomethylated polypeptide, shown in Fig. 5, reveals a very broad reversible renaturation transition which continues down to the solubility limit of apoB in guanidine solution.

**DISCUSSION**

The protein moiety of LDL appears to be a relatively unstable molecule (as judged by the gradual appearance of lower molecular weight bands in SDS-gel electrophoretic patterns) when the lipoprotein is isolated. The course of the observed changes is dependent on the temperature at which the LDL is maintained, occurring much faster and progressing to a much greater extent at elevated temperatures. Further, contributing destabilizing factors may be present in holo-LDL, as chromatographically delipidated apoB degrades much more slowly than intact LDL at the same temperature and in the same buffer. However, a role for a co-purifying protease in the phenomenon, as suggested by Krishnaiah and Wiegandt (14), is unlikely, as inhibitors of serine, sulfhydryl, and cation-dependent proteases failed to retard the changes. A slow, sequential disaggregation of the protein into smaller identical subunits is probably not involved, as the large number of irregularly spaced bands seen on gel electrophoresis of samples heated at 100°C for more than 2 h are not consistent with a specific subunit molecular weight and since the monomeric molecular weight appears to be 250,000 (10, 16). Recently, it has been suggested (27) that a free radical process dependent on molecular oxygen may be responsible; the observations reported here could be the result of such a process, but this would necessitate a high degree of specificity in the reaction, as identical degradation patterns have been seen in several LDL preparations from a large number of individuals; further, nitrogen saturation of all solutions did not prevent the changes in gel patterns. This reproducible, precise pattern may be the result of scission at relatively unstable regions of the polypeptide chain; these could be covalent cross-links (but not disulfide bonds, as 2-mercaptoethanol has no effect on the gel electrophoretic or chromatographic patterns) or amide bonds in certain inherently less stable amino acid sequences.

Measurements of amphiphile binding capacity of a variety of proteins have been made in the past and have led to the identification of several modes of binding. A limited number of proteins have discrete binding sites for monomeric amphiphile; other proteins bind many amphiphile molecules in a cooperative fashion, either as a result of mixed micelles forming at a pre-existing hydrophobic area on the polypeptide, or of a conformation change which exposes a large number of new binding sites to individual amphiphile molecules (28). Studies in this laboratory (29–32) of the two major polypeptides of high density lipoprotein have shown that both have three to four discrete, noninteracting binding sites for monomeric amphiphiles, but that at amphiphile concentrations sufficient to saturate these sites a conformation change occurs which produces a cooperative increase in the binding of amphiphiles. CD spectra of the resulting complexes showed that both polypeptides are in conformations very similar to their native structures in the presence of bound lipid.

Similar studies of amphiphile binding to apoB from LDL have been hampered by the insolubility of the lipid-free polypeptide in aqueous solutions not containing detergents, lipids, or denaturants. Simons and Helenius (15, 33) have measured the binding of SDS (2.6 g/g), sodium dodecyl sulfate (0.64 g/g) and Triton X-100 (0.52 g/g) at a single concentration above the cmc of each detergent; their value for SDS has been

**Fig. 4.** Circular dichroism of apolipoprotein B in sodium dodecyl sulfate and guanidine hydrochloride solutions. The computed CD spectra for apolipoprotein B in 0.1 mM SDS (---), in 2.5 mM SDS (----), and in 7.6 mM guanidine hydrochloride (-----) solutions are shown. Only the free sulfhydryl groups of the polypeptide have been carboxyamidomethylated.

**Fig. 5.** Conformational stability of apolipoprotein B in guanidine hydrochloride [G&HCI] solution. Shown is the molar ellipticity at 222 nm of reduced and carboxyamidomethylated apolipoprotein B in varying concentrations of guanidine hydrochloride. Measurements made at increasing (●) or decreasing (○) denaturant concentrations are indicated.
disputed by Smith et al. (10), who found 1.50 g/g bound at SDS concentrations below the cmc in low ionic strength buffer using fully reduced apoB. Clarke (34) has used sucrose density gradient centrifugation to determine Triton X-100 binding to delipidated LDL; his estimate of 0.92 g/g at the same free detergent concentration used by Simons and Helenius (33) is nearly twice their value. This may result from residual protein-associated lipid in Clarke’s (34) preparation (no criteria for completeness of delipidation were given) or from possible perturbing effects of sucrose on amphiphile binding, state of association, and preferential hydration (35, 36). Consequently, the Triton X-100 binding isotherm given by Clarke (34) is of questionable validity, although the qualitative conclusion that detergent monomers can bind to apoB is presumably justified.

The studies reported here show that apoB binds a limited amount of SDS monomers below the cmc; at or near the cmc, a large cooperative binding increase occurs which saturates at 1.66 g/g or about 1400 mol/mol. It is not certain whether this represents the formation of mixed micelles around hydrophobic regions of the polypeptide, as has been found with some integral membrane proteins (28), or a cooperative binding of monomers triggered by a conformational change occurring when the free monomer concentration nears 0.9 mM. The circular dichroic spectra also support the latter interpretation as they show that a conformational change has occurred in this cooperative binding region; further, the spectral changes are consistent with the observed effects of SDS on a variety of water-soluble proteins, i.e. an increase in helical content to 30 to 40% in a protein which initially has a smaller percentage of helix (25, 26). The significance of the binding below the cmc is also unclear at this time; apparently, a limited number of monomers (up to about 300) bind to the polypeptide with a higher affinity than the 1000 or so molecules involved in the cooperative binding, but the experimental scatter of the results (presumably due to the limited solubility of the polypeptide at low levels of detergent binding) make a more quantitative interpretation difficult. The conformation of the polypeptide appears to be similar by CD criteria to the structure seen in low levels of sodium decyl sulfate (37) and in the intact LDL particle (37, 38); it should be noted that this is after exposure of the polypeptide to high concentrations of SDS (in the delipidation procedure), during which it has assumed a more helical conformation.

The binding measurements using lysopalmitoyl phosphatidylcholine, while subject to some uncertainty due to the use of sucrose gradients as discussed above, indicate that apoB does interact with this naturally occurring lipid, as does the apo-B1 polypeptide of high density lipoprotein (52), to form a particle of defined stoichiometry. Quantitatively, apoB appears to bind about 3 times as much lysolecithin as does apoA-I at saturation. Furthermore, these experiments show that lysolceithin can readily displace essentially all of the bound SDS on the polypeptide and maintain apoB in a soluble state. More detailed investigation of this interaction is underway.

Circular dichroism measurements of fully reduced and carboxamidomethylated apoB transferred from SDS to concentrated guanidine solutions confirm the ORD results of Smith et al. (10) which showed loss of all ordered structure. The fact that the polypeptide is in a random coil configuration is illustrated by the thermal stability studies of that the polypeptide is in a random coil configuration is illustrated by the thermal stability studies of Ikai (40) has observed spectral and ORD changes in intact porcine LDL consistent with denaturation of solvent-exposed polypeptide over a guanidine hydrochloride concentration range of 2 to 4 M. A similar situation may exist here, with portions of the polypeptide (resembling water-soluble protein) being denatured in this guanidine concentration range and with additional regions (those protected by lipid in Ikai’s work) undergoing denaturation at higher denaturant concentrations, analogous to (but not as stable as) the hydrophobic sequences of integral membrane proteins, several of which retain structural features at guanidine concentrations above 8 M (28). The denaturation behavior of apoB differs markedly from that of the high density lipoprotein polypeptides apoA-I and apoA-II, both of which are conformationally very flexible and are completely denatured at very low levels of guanidine hydrochloride (41).

The results presented here provide necessary information for the interpretation of hydrodynamic studies we report in the accompanying paper (16). However, they are also of interest because they reveal that apoB behaves quite differently than integral membrane proteins or the two major polypeptides of high density lipoprotein in terms of amphiphile binding mode and capacity in the nonreduced state, conformational stability in the fully reduced state, and resistance to degradation. This suggests that models (such as the amphipathic helix (42)) proposed to explain the interaction of membrane proteins or of the high density lipoprotein polypeptides with amphiphilic compounds will not be similarly applicable in attempts to understand the structure of the LDL particle.

Acknowledgments—Our appreciation is extended to Dr. C. Tanford for many helpful discussions and to Dr. H. Steinman for advice regarding the possible instability of certain amino acid sequences.

REFERENCES

Apolipoprotein B in Detergent and Denaturant Solutions

Characterization of the apolipoprotein B polypeptide of human plasma low density lipoprotein in detergent and denaturation solutions.

J C Steele, Jr and J A Reynolds


Access the most updated version of this article at http://www.jbc.org/content/254/5/1633

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/5/1633.full.html#ref-list-1