multiple enhancements observed for the longer wavelength bands. This parallelism supports the assignment of the absorption bands to the same pair of π–π^* transitions, shifted to lower energy for the semiquinone.

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Solubilization and Characterization of Apolipoprotein B from Human Serum Low-Density Lipoprotein in *n*-Dodecyl Octaethylene Glycol Monoether†

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ABSTRACT: Apolipoprotein B, the core polypeptide of human serum low-density lipoprotein, retains its native association state (500 000 g/complex), as well as its native conformation as judged by circular dichroism, when all lipid has been replaced by a nonionic detergent. Protein solubilized in this detergent should therefore be well suited for lipid binding studies. The native association state is also preserved when lipid is replaced by ionic detergents, but in this case the protein undergoes a conformational change, which can be reversed if the ionic detergent is replaced by nonionic detergent. The constancy of the state of association of the B polypeptide in a variety of amphiphilic environments contrasts with what has been observed with polypeptides from high-density lipoproteins which exist in different states of association under different conditions.

Low density lipoproteins (LDL),[1] the main cholesteryl ester transport vehicles in human serum, arise in the plasma as catabolic products of the triglyceride-rich very low density lipoproteins (VLDL). This metabolic processing gives rise to two distinct subclasses of LDL termed LDL_1 and LDL_2 with density ranges of 1.006–1.019 g/cm^3 and 1.02–1.063 g/cm^3, respectively. LDL_2 is composed of 78% lipid and 22% protein, the latter consisting exclusively of a single polypeptide, apolipoprotein B (apo-B). In all classes of LDL and VLDL that

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[1] Abbreviations used: LDL, human low-density serum lipoprotein; VLDL, human very low density serum lipoprotein; $NaDodSO_4$, sodium dodecyl sulfate; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether.

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have been carefully examined, there is an invariant mass of 510000 g of apo-B per particle, suggesting that this is a constant structural feature of these lipoprotein families (Schumaker, 1973; Fisher et al., 1975; Tanford & Reynolds, 1979).

Numerous structural studies of holo-LDL have shown it to be a roughly spherical particle with a diameter of 230 Å and a molecular weight of (2–3) × 10^6 (Laggner, 1976). Oncley et al. (1955) demonstrated that although LDL is 80% lipid by weight, the particle exhibits solubility properties similar to those of typical water-soluble proteins, a property attributable to the presence of most of the titratable amino acid residues on the surface. More recent NMR investigations indicate that all of the phospholipid head groups are also present at the surface but ~20% of them interact directly with the protein component (Yeagle et al., 1977, 1978).

Studies of the delipidated protein from LDL_2, apo-B, have been difficult since it is insoluble in aqueous solution in the absence of bound ligands or a strong denaturant such as guanidinium chloride and it is particularly susceptible to proteolytic cleavage (Banaszak & McDonald, 1962; Margolis & Langdon, 1966; Triplett & Fisher, 1978; Steele, 1979). In concentrated solutions of guanidinium chloride, apo-B is a random coil and has a polypeptide molecular weight of 255000 (Smith et al., 1972; Steele & Reynolds, 1979b). This molecular weight contains the contribution from bound carbohydrate which is ~7% by weight. Delipidation of apo-B with sodium dodecyl sulfate leads to a thermodynamically stable dimer of the protein. At low levels of this detergent (400–600 mol/500000 g of protein), the circular dichroic spectrum of apo-B is nearly identical with that observed in the holoparticle. However, at saturation levels a large conformational change occurs without consequent dissociation to a monomeric state (Steele & Reynolds, 1979a).

Nonionic detergents such as poly(oxyethylenes) do not bind to most water-soluble proteins (Makino et al., 1973; Helenius & Simons, 1975; Tanford & Reynolds, 1976). They interact with hydrophobic portions of intrinsic membrane proteins, often successfully replacing the in vivo lipid and leaving native function intact. We have employed octaethylene glycol dodecyl ether ($C_{12}E_8$) as a delipidating and solubilizing agent for apo-B in order to obtain a soluble protein–detergent complex which retains its native structure. This detergent is particularly convenient for ultracentrifugal analysis since it has a partial specific volume of 0.973 cm^3/g. Additionally, it is a homogeneous species with a sufficiently high critical micelle concentration (9 × 10^{-5} M) that exchange can be carried out for other amphiphilic ligands. Since apo-B undergoes essentially irreversible conformational changes in the absence of bound amphiphiles and is consequently rendered insoluble, it is particularly important that detergents used for delipidation and solubilization be readily exchangeable. Ultimately, one wishes to investigate the binding properties of apo-B with respect to varying hydrophobic and hydrophilic groups on amphiphilic ligands as a first step in understanding the remarkable lipid solubilizing ability of this protein.

Experimental Procedures

Materials

n-Dodecyl octaethylene glycol monoether ($C_{12}E_8$) was obtained as a homogeneous product from Nikko Chemicals Co., Tokyo, Japan. [The relevant properties of this detergent may be found in Tanford et al. (1977).] Sodium dodecyl sulfate was BDH Chemical Corp. specially pure grade purchased from Gallard-Schlesinger. Sepharose 4B, Sepharose CL-4B, and chromatographic columns were products of Pharmacia Fine Chemicals. Regenerated cellulose membranes were from Schleicher & Schuell. All other materials were standard reagent grade.

Methods

Preparation of holo-LDL_2 was carried out as described by Steele & Reynolds (1979a) using blood from fasting, normal human volunteers. After isolation, the two free sulfhydryls per 250000 g of protein were alkylated with iodoacetamide, and the reaction was terminated by dialysis against 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) buffer, pH 7.4, ionic strength 0.3 (hereafter referred to as standard Tes buffer). Holo-LDL_2 was then passed through sterile 0.22-μm Millipore filters into sterile test tubes and stored at 4 °C.

Analytical Procedures. The purity of all preparations was assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis using either 3.3 or 5% gels according to the method of Weber & Osborn (1969). Protein concentrations were determined by using the procedure of Lowry et al. (1951) with 2% $NaDodSO_4$ included in both the sample and bovine serum albumin standards. Lipid phosphorus was assayed according to the micromethod of Bartlett (1959). $C_{12}E_8$ concentrations were measured by the protocol of Garewal (1973) except that the absorbance was read at 319 nm.

Preparation of Delipidated Apo-B. Either $C_{12}E_8$ (at 30 mg/mg of LDL protein) or $NaDodSO_4$ (at 30 mg/mg of LDL protein) was added to a solution of holo-LDL_2 at 2–3 mg of LDL protein in a total volume of 1.5 mL. After 3 h of mild stirring in the dark at room temperature, the solution was applied to a 1.5 × 90 cm column of Sepharose 4B (or CL-4B) equilibrated with an eluant buffer containing either 1 mM $C_{12}E_8$ or 2.5 mM $NaDodSO_4$. Fractions were collected and the weight, optical density at 280 nm, protein concentration, phospholipid content, and amount of detergent of appropriate fractions were measured. Generally the buffer utilized was standard Tes buffer, pH 7.4. However, for studies with $C_{12}E_8$ delipidated apo-B at pH greater than 7.4, other buffer systems were employed and will be described under Results where relevant. Routinely, 5% acrylamide gels were used to analyze column fractions.

Circular dichroic spectra of holo-LDL_2 and apo-B in detergents were recorded on a Jobin-Yvon Dichrographe Mark III calibrated with a 0.1% aqueous solution of *d*-10-camphorsulfonic acid. One-millimeter cells were used, and a mean residue weight of 112.5 was utilized to calculate molar ellipticity (Steele & Reynolds, 1979a).

Sedimentation velocity and equilibrium measurements were performed on a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. The concentration of protein as a function of radial distance from the center of rotation was determined by scanning the cell at 280 nm. For sedimentation velocity experiments 300-μL samples (obtained immediately after elution from a gel filtration column) were employed. Centrifugation was carried out at or above 20 °C. Molecular weights were obtained from sedimentation equilibrium according to Reynolds & Tanford (1976). The slope of ln OD vs. R^2 gives $M_r(1 - \phi'\rho)$ directly. This latter term is expanded to $M_p[1 - \bar{v}_p\rho + \delta_d(1 - \bar{v}_d\rho)]$ where M_p is the molecular weight of the protein, $\bar{v}_p$ and $\bar{v}_d$ are the partial specific volumes of the protein and detergent, respectively, δ_d is grams of detergent bound per gram of protein, and ρ is the density of the solution. $\bar{v}_p$ = 0.725 cm^3/g for apo-B including carbohydrate (Steele & Reynolds, 1979b) and $\bar{v}_D$ = 0.973 cm^3/g for $C_{12}E_8$ (Tanford et al., 1977).

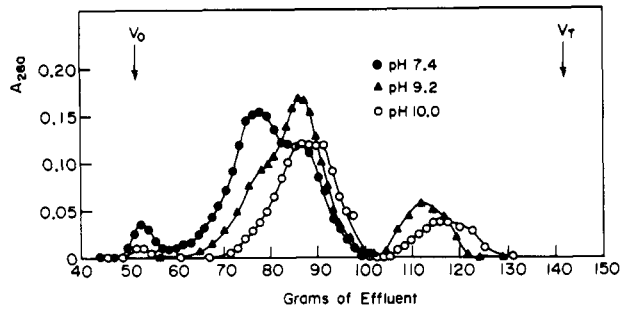

FIGURE 1: Gel filtration chromatography of LDL_2 in $C_{12}E_8$. LDL_2 at 2–3 mg/mL protein was incubated with 30 mg of detergent per mg of protein and applied to the column in three different buffer systems. The eluant for each of the three conditions had the following composition: pH 7.4, 1 mM $C_{12}E_8$, 20 mM Tes, and NaCl to a final ionic strength of 0.3; pH 9.2, 1 mM $C_{12}E_8$, 20 mM Tris-HCl, and NaCl to a final ionic strength of 0.3; pH 10.0, 1 mM $C_{12}E_8$, 20 mM sodium carbonate–bicarbonate, and NaCl to a final ionic strength of 0.3. Arrows mark the void (V_0) and total (V_t) volumes of the column. Sepharose 4B or Sepharose CL-4B was the support material in a Pharmacia column of dimensions 1.5 × 90 cm. T = 22 °C.

Detergent Exchange. Lucite cells containing two 1-mL compartments separated by a Schleicher & Schuell regenerated cellulose membrane were used for detergent exchange studies. Typically, a 25-mm RC-54 membrane with a pore size of 0.020–0.035 μm was employed in order to allow free passage of the detergent micelles but to retain the protein–detergent complex. In the experiments reported here, Na-$DodSO_4$ delipidated apo-B (Steele & Reynolds, 1979a) was concentrated to ~1 mg/mL on an Amicon XM-100A membrane, diluted 1:10 with a buffered solution containing 1 mM $C_{12}E_8$, and dialyzed against repeated changes of solution containing 1 mM $C_{12}E_8$. Total removal of residual $NaDodSO_4$ is readily monitored by using sodium dodecyl [^{35}S]sulfate.

Results

Solubilization and Delipidation of Apo-B in $C_{12}E_8$. Complete solubilization and delipidation of apo-B are obtained at a weight ratio of 30 mg of $C_{12}E_8$ per mg of protein (total protein concentration 2–3 mg/mL). Lower weight ratios lead to incomplete removal of the cholesteryl esters and the formation of large detergent–cholesteryl ester micelles which coelute with the protein–detergent complex. Gel filtration chromatography of the solubilized particle on Sepharose 4B equilibrated with 1 mM $C_{12}E_8$ is shown in Figure 1. Three separate conditions of pH are given. At the void volume of the column (irrespective of pH) is a small amount of aggregated material, followed by a large included peak corresponding to 90% or more of the applied protein. Mixed micelles of lipid (phospholipid, triglycerides, cholesterol, and cholesteryl esters) and $C_{12}E_8$ eluted later as a defined fraction between 100 and 130 g of effluent. This latter fraction is monitored conveniently at 485 nm since carotenoids are also associated with this complex.

Acrylamide gel electrophoresis of column fractions reveals that the entire region between 60 and 100 g of effluent in Figure 1 contains apo-B in intact form (i.e., no degradation has occurred). However, the striking feature apparent in this figure is that as the pH is increased from 7.4 to 10.0 the elution profile changes dramatically. The protein peak at 75 g of effluent observed at pH 7.4 is further included in the column and a more symmetrical elution pattern is observed as the pH is increased.

A complete description of the results obtained at pH 10.0 is provided in Figure 2. No detectable phospholipid is found associated with the protein, and thin-layer chromatography of these fractions revealed no neutral lipids (i.e., less than 5 mol of neutral lipid per 250000 g of apo-B was present). The binding of $C_{12}E_8$ appears to be slightly lower on the leading edge of the protein peak, but, in view of the insensitivity of the assay and the low protein concentrations, caution must be exercised in quantitative interpretation of the variations in binding ratios seen across the peak.

Characterization of the $C_{12}E_8$–Apo-B Complex. The state of association of the complex was determined by analytical ultracentrifugation. Sedimentation velocity studies were carried out with samples immediately after elution from the gel filtration column at pH 7.4, pH 9.2, and pH 10.0. In all cases a rapidly moving, diffuse boundary was observed which decreased in magnitude with increasing pH. Approximately one-third of the total optical density was present as this heterogeneous aggregate at pH 9.2, while from 9 to 20% was observed in different preparations at pH 10.0. The majority of the protein–detergent complex moved with an s value of

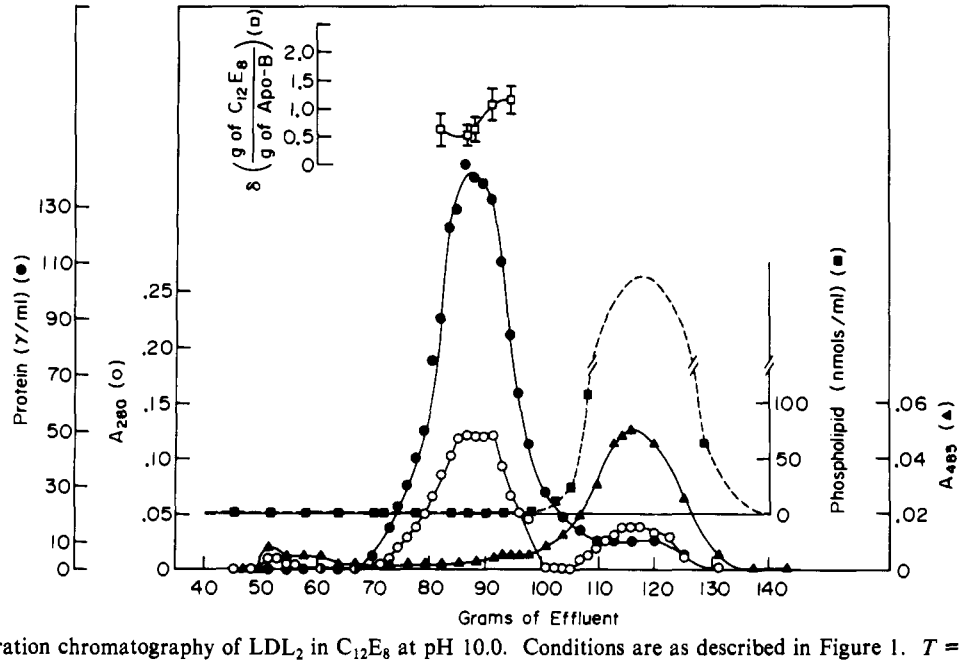

FIGURE 2: Gel filtration chromatography of LDL_2 in $C_{12}E_8$ at pH 10.0. Conditions are as described in Figure 1. T = 22 °C.

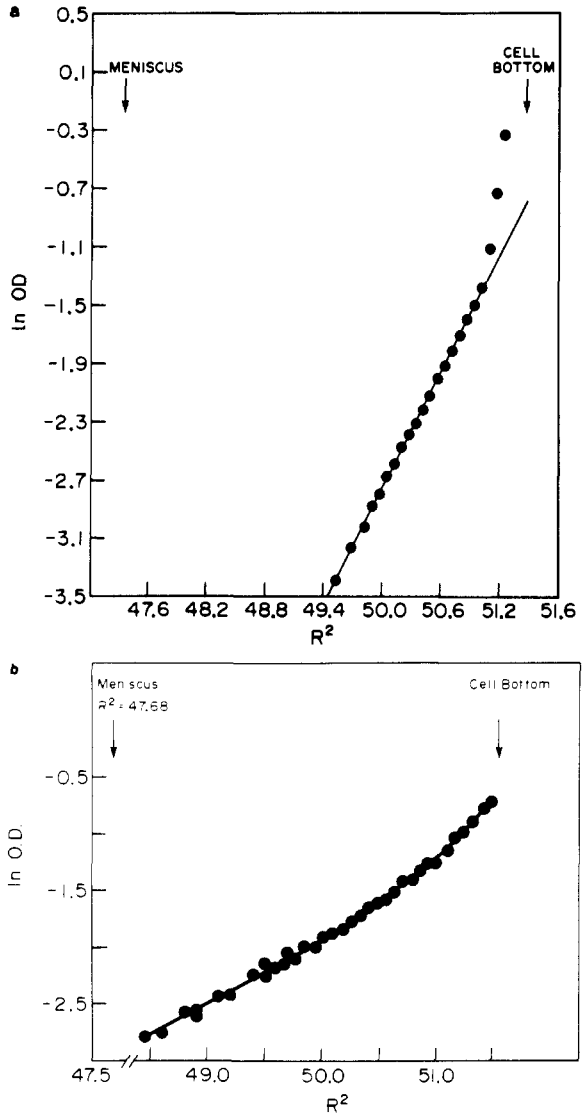

FIGURE 3: Sedimentation equilibrium of the apo-B–$C_{12}E_8$ complex at pH 10.0 in the solvent system described in Figure 1. Initial protein concentration was 0.13 mg/mL. The sample eluted from the gel filtration column shown in Figure 2 at 87 and 91 g. (a) 6000 rpm; (b) 4000 rpm.

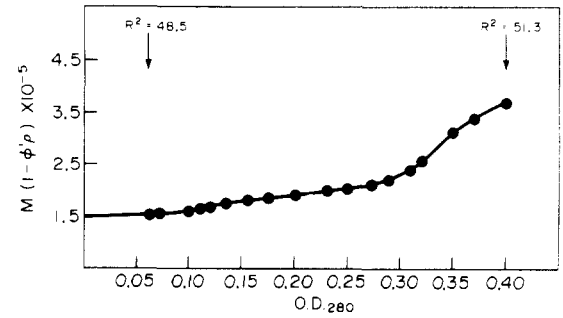

FIGURE 4: $M_r(1 - \phi'\rho)$ vs. OD_{280} for the data shown in Figure 3b.

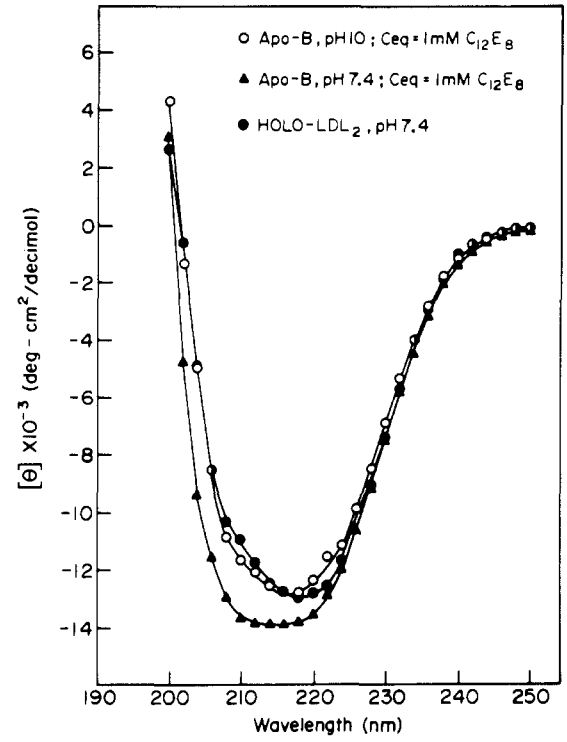

FIGURE 5: Circular dichroism of LDL_2 and the $C_{12}E_8$–apo-B complex at pH 7.4 and pH 10.0. The solvent system was as described in Figure 1. Spectra were obtained in 1-mm cells at a protein concentration of 10^{-1} mg/mL. T = 22 °C.

9–10 S and displayed a symmetrical boundary at pH 10.0. Sedimentation equilibrium of the highly aggregated, heterogeneous mixture formed at pH 7.4 demonstrated that over a period of 2 or 3 days time further aggregation occurred as the concentration in the bottom of the cell was increased. Addition of excess $C_{12}E_8$ or alteration of the ionic strength from 0.02 to 2.5 did not prevent this phenomenon.

At pH 9.2 and pH 10.0 stable equilibrium protein concentration gradients were obtained in the ultracentrifuge. Sample data at pH 10.0 are shown in Figure 3a,b. These samples were obtained directly from the gel filtration column and eluted at 91 g of effluent (Figure 2). At 6000 rpm the small amount of aggregated complex observed in sedimentation velocity experiments and apparent in the curvature at the bottom of the cell does not contribute significantly to the optical density in the remainder of the cell. The straight line in Figure 3a is the calculated distribution for a 550 000 molecular weight protein containing the experimentally determined 1.16 ± 0.28 g of $C_{12}E_8$ per g of protein by using an initial protein concentration determined from sedimentation velocity as 80% of the total initial concentration. At 4000 rpm (Figure 3b) the aggregated species makes a larger contribution to the optical density equilibrium distribution. A plot of $M_r(1 - \phi'\rho)$ vs. optical density is presented in Figure 4 where the limiting value is equivalent to M_p = 500 000. At no point was there any evidence of degradation of apo-B as assayed by $NaDodSO_4$ gel electrophoresis after centrifugation, nor was any species smaller than a dimer observed.

It is difficult to assess the retention of native structure of apo-B since one of the in vivo functions is interaction with cell surface receptors and cannot be assayed in the presence of detergents. We have demonstrated that in $C_{12}E_8$ the state of association is the same as that found in vivo. Another criterion for structural integrity is a comparison of circular dichroic spectra. Figure 5 shows the far-ultraviolet CD spectra of apo-B in holo-LDL and in $C_{12}E_8$ at two different pH values. Within experimental error there is no difference between the spectra of the native particle and apo-B–$C_{12}E_8$ at pH 10.0. At pH 7.4 the small alterations observed in the detergent–protein complex are probably attributable to the time-dependent aggregation observed in the ultracentrifuge. By use of the molar ellipticity at 217 and 208 nm, an apparent protein conformation of 25% α helix, 40% β structure, and 35% random coil is calculated (Greenfield & Fasman, 1969). These values agree closely with those estimated by Gotto et al. (1973) for

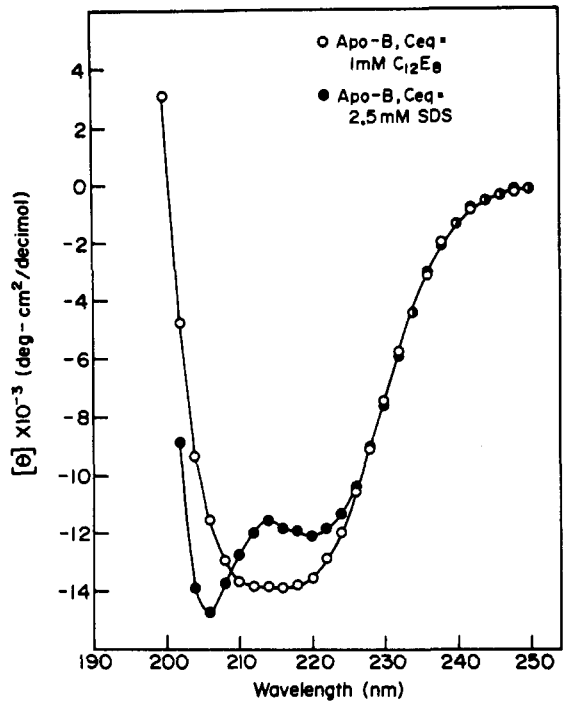

FIGURE 6: Reversibility of denaturation of apo-B by sodium dodecyl sulfate. Pooled fractions of the apo-B–$NaDodSO_4$ complex from a Sepharose 4B column equilibrated in 2.5 mM $NaDodSO_4$ in standard Tes buffer, pH 7.4, were concentrated and dialyzed against either 2.5 mM $NaDodSO_4$ or 1 mM $C_{12}E_8$ as described under Experimental Procedures. T = 22 °C.

holo-LDL and by Steele & Reynolds (1979a,b) for apo-B in low levels of $NaDodSO_4$.

Reversibility of $NaDodSO_4$ Denaturation of Apo-B. At the high binding levels of $NaDodSO_4$ required to totally delipidate apo-B, a major conformational change occurs as shown in Figure 6 and by Steele & Reynolds (1979). However, these alterations are readily reversible by exchanging $NaDodSO_4$ for $C_{12}E_8$ as described under Methods. The circular dichroic spectrum in the latter detergent after prior delipidation with $NaDodSO_4$ is identical with that obtained when delipidation is carried out in $C_{12}E_8$ (lowest curve in Figure 5).

Discussion

Apo-B can be completely delipidated and solubilized with $C_{12}E_8$[2] at pH values greater than 7.4 with retention of the native dimeric state and no detectable alteration in the CD spectrum. Further, this form of the protein reacts with antisera directed against native LDL_2 (data not shown). The protein–detergent complex is highly asymmetric in that estimates of f/f_{min} from sedimentation velocity (not extrapolated to zero concentration) and sedimentation equilibrium measurements give values of 2.0–2.3.

The maximal binding of $C_{12}E_8$ to apo-B is ~930 mol/500 000 g of protein, a value far higher than one micelle per dimer (average aggregation number = 120; Tanford et al., 1977). In general, the binding of detergents to hydrophobic segments of intrinsic membrane proteins does not significantly perturb the size distribution of the pure detergent micelle, and it is unlikely that 930 mol of $C_{12}E_8$ is associating with a single hydrophobic region of apo-B. This information taken together with the large asymmetry of the particle suggests the presence of several hydrophobic regions along the length of the apo-B molecule.

The preferred thermodynamic state of apo-B appears to be dimeric in the presence of bound amphiphilic ligands. In the nonionic detergents $C_{12}E_8$ (this work) and Triton X-100 (Ikai & Hasegawa, 1978), no species of apo-B smaller than a dimer has been observed. In the present paper we show that small percentages of higher aggregates are formed. However, Ikai and Hasegawa imply their preparation was homogeneous. Even in the denaturing detergent, $NaDodSO_4$, apo-B retains a molecular weight of 500 000.

The pH-dependent aggregation of the detergent–protein complex studied here is particularly interesting in that this phenomenon was not observed when an ionic detergent at neutral pH was used. We suggest that this aggregation is occurring through interactions of the water-soluble portion of apo-B and is prevented by titration to higher pH, thus increasing the total charge on the particle (or, alternatively, by binding a charged ligand). It is noteworthy that holo-LDL_2 also aggregates when it is highly concentrated during the process of purification from serum.

The different classes of serum lipoproteins contain different core polypeptides (Tanford & Reynolds, 1979) which appear to be designed to transport specific lipid classes and display correspondingly different modes of interaction with amphiphiles. AI and AII from HDL have been shown to "package" specific hydrophobic volumes of normal alkyl chains. The maximal amount bound is not related to the normal size of a micelle formed in the absence of protein and is much less than a single micelle for those amphiphiles with large aggregation numbers (Reynolds et al., 1977). Apo-B, on the other hand, binds more than the equivalent of one micelle per mol of protein, probably at several hydrophobic domains. Nevertheless, apo-B differs also from intrinsic membrane proteins in its ability to direct the formation of a lipid–protein complex which is water soluble and cannot resemble a phospholipid bilayer since cholesteryl esters and triglycerides are virtually insoluble in that bilayer structure (Loomis et al., 1974).

AI and AII are water soluble in the absence of ligands and self-associate only weakly in that state. In contrast, apo-B is dimeric under all conditions thus far investigated including in vivo particles containing this polypeptide as a core protein. Direct protein–protein interaction (not dependent upon the presence of ligands) thus plays a role in the structure of LDL but not necessarily of HDL.

Acknowledgments

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[2] We have noted in this work as well as in collaborative studies with Dr. Pierre Wong and D. McCaslin that $C_{12}E_8$ and $C_{12}E_9$ solutions become turbid at temperatures less than 10 °C. We have no explanation for this phenomenon since these detergents should show no anomalous temperature-dependent behavior below their cloud points. However, these observations suggest caution in working with protein–detergent complexes of this type below room temperature.

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Fluorescent Probe Study of Temperature-Induced Conformational Changes in Cytochrome Oxidase in Lecithin Vesicle and Solubilized Systems†

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ABSTRACT: A protein-bound label, *N*-(1-anilinonaphthyl-4)-maleimide (ANM), was used to investigate conformational changes in bovine heart cytochrome oxidase. The fluidity of cytochrome oxidase vesicles was monitored by a lipophilic probe, 1,6-diphenyl-1,3,5-hexatriene. The fluorescence intensity and emission anisotropy of these probes were examined between 4 and 60 °C in enzyme–dipalmitoyllecithin vesicles, in enzyme–dimyristoyllecithin vesicles, in enzyme–dioleoyllecithin vesicles, and in the soluble enzyme. The temperature-dependent changes in these quantities indicated that there were two types of conformational changes in oxidized cytochrome oxidase: one was attributed to an intrinsic enzyme conformational change which occurred around 20 °C, and the other was attributed to a conformational change induced by the lipid phase transition. Although ANM-reactive subunits of cytochrome oxidase in these four lecithin vesicle and solubilized systems were different from each other, subunit I always reacted with ANM in preference to other subunits.

Cytochrome oxidase is the terminal enzyme of the respiratory chain in the mitochondrial inner membrane (Boyer et al., 1977). Three-dimensional analysis of the structure of cytochrome oxidase in vesicle crystals (Henderson et al., 1977) has shown that the enzyme molecule sticks out on both side surfaces of the vesicle.

An interesting question regarding membrane proteins is how their functions and conformations are influenced by the physical states of membrane lipids (Vanderkooi, 1974; Yu et al., 1975; Carroll & Racker, 1977; Jost et al., 1977; Longmuir et al., 1977). Sharp breaks have been observed in the temperature dependences of the activities of several membrane enzymes (Inesi et al., 1973; Houslay et al., 1975; Lee et al., 1974; Hesketh et al., 1976). These breaks were often attributed to lipid phase transitions or lateral lipid phase separations.

Raison et al. (1971) and Erecińska & Chance (1972) observed a break at around 20 °C in the Arrhenius plot of cytochrome oxidase activity in rat liver and pigeon heart mitochondria, respectively. They attributed the break to the lipid phase transition. However, the mitochondrial membranes of rat liver, pigeon heart, and bovine heart have been shown to be in the liquid-crystalline state above 10 °C (Blazyk & Steim, 1972; Vanderkooi, 1973; Cannon et al., 1975; Shinitzky & Inbar, 1976). In solubilized and dioleoyllecithin vesicle systems, we (Yoshida et al., 1979) have shown that the break occurs at 21 and 24 °C, respectively, in the Arrhenius plot of

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