Crystallization and Preliminary X-ray Analysis of a Low Density Lipoprotein from Human Plasma*

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Single crystals of human plasma low density lipoprotein (LDL), the major transport vehicle for cholesterol in blood, have been produced with a view to analysis of the three-dimensional structure by x-ray crystallography. Crystals with dimensions of approximately 200 × 100 × 50 μm have been reproducibly obtained from highly homogeneous LDL particle subspecies, isolated in the density ranges d = 1.0271–1.0297 g/ml and d = 1.0297–1.0327 g/ml. Electron microscopic imaging of ultrathin-sectioned preparations of the crystals confirmed the existence of a regular, quasihexagonal arrangement of spherical particles of approximately 18 nm in diameter, thereby resembling the dimensions characteristic of LDL after dehydration and fixation. X-ray diffraction with synchrotron radiation under cryogenic conditions revealed the presence of well resolved diffraction spots, to a resolution of about 29 Å. The diffraction patterns are indexed in terms of a triclinic lattice with unit cell dimensions of a = 16.1 nm, b = 38.0 nm, c = 43.9 nm; α = 96.2°; β = 92.1°; γ = 102°, and with space group P1.

The elucidation of the molecular structure of apolipoprotein B-100 (apoB-100) by x-ray crystallography is an essential prerequisite for the detailed understanding of the pathobiological function of low density lipoprotein (LDL) and for the development of rational pharmaceutical intervention in atherosclerosis. In particular, the recognition mechanisms implicated in the specific interaction of the protein moiety, apoB-100, with the cellular LDL receptor (for reviews, see Refs. 1 and 2) need to be clarified.

Small angle x-ray (3–5) and neutron (6–8) scattering investigations have shown that LDL are quasispherical particles of 20–25 nm in diameter, which contain an apolar core of cholesteryl esters and triglycerides covered by a monolayer of phospholipids and free cholesterol, into which one molecule of apoB-100 is embedded (for a recent review, see Ref. 9). Deviations from the spherical structure of LDL have also been discussed on the basis of electron microscopic observations (10, 11) and small angle x-ray scattering data (12). These studies suggested an arrangement of the apoprotein in globular domains at the surface of LDL.

ApoB-100, which constitutes approximately 20% by weight of the lipoprotein particle, is a single polypeptide chain consisting of 4536 amino acid residues, with a calculated molecular mass of 512,937 Da. The complete amino acid sequence of apoB-100 has been determined (13, 14). ApoB-100 is characterized by its high hydrophobicity (15), sensitivity to proteolytic degradation (16–18), and oxidative cleavage (19). Furthermore, the conformation of apoB-100 is an intimate feature of its interaction with the LDL lipids (20, 21), which in turn undergo a reversible, thermotropic transition between 15 and 30 °C (22, 23). All these attributes render crystallization of LDL a difficult task. They clearly indicate, on the other hand, that the structure of apoB-100 must be investigated as a component of the native LDL particle, since any attempts to solubilize the protein will almost certainly lead to structural alterations.

EXPERIMENTAL PROCEDURES

Isolation, Fractionation, and Crystallization—Discrete LDL subspecies were isolated from the plasma of normolipidemic healthy volunteers by sequential preparative ultracentrifugation followed by isopycnic density gradient fractionation, as described earlier (24). Each of the nine successive subspecies isolated within the density limits of 1.02 and 1.06 g/ml were exhaustively dialyzed in the dark under argon atmosphere against 10 mM phosphate-buffered saline (0.15 M NaCl, pH 7.2), containing 0.1% EDTA and 50 mg/liter gentamicin, at 4 °C. The solutions were stored under argon atmosphere at 4 °C until further use.

Crystallization conditions were explored by systematic variation of parameters which directly influence the solubility and nucleation process, such as pH, nature and concentration of the precipitant (ammonium sulfate, polyethylene glycol, divalent cations), protein concentration, and temperature. All reagents used were of analytical grade, if not specified otherwise, and water was double quartz-distilled. Care has been taken to inhibit any structural denaturation (bacterial growth or oxidative damage) during the crystallization procedure. The hanging- or sitting-drop vapor diffusion techniques, respectively, as well as microdrop methods were tested for their suitability to grow crystals. The best results were obtained by the hanging-drop method where the bottom well contained 10–12% (w/v) polyethylene glycol (PEG2000, Fluka, >95% purity, solutions passed through 0.45-μm filter), 0.1 M ammonium sulfate in 0.3 mM phosphate buffer, pH 6.8–7.0 at 4 °C in argon atmosphere. The drops initially contained approximately 2 mg/ml apoB-100 (corresponding to about 10 mg/ml LDL) and half the concentrations of other components in the bottom well solutions. It is noteworthy that crystals could not be obtained under any conditions at room temperature, and that crystals once formed at 4 °C disintegrated rapidly if exposed to temperatures above 10 °C. For stream-freezing in evaporating liquid nitrogen, prior to x-ray exposure, the crystals were cryopreserved by soaking in a solution containing the components used for their growth and 20% (v/v) ethylene glycol.

Preparation for Electron Microscopy—For electron microscopy, crystals were fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, for 3 h at 4 °C, stained with 6% KMnO4 and 2% uranyl acetate, collected, and mixed with 4% liquid agar at 40 °C. After cooling to 4 °C,
performed with the DENZO program system (27). Preliminary data analysis was typically collected over periods of 300 s with an oscillation angle of 0.5° about one given crystal orientation. Freezing has been described in detail elsewhere (26). The crystal-to-detector distance was varied between 400 and 600 mm. Diffraction data were poststained with 2% uranyl acetate in distilled water at 20°C for 20 min. Electron microscopy was performed with a Philips CM 12 transmission electron microscope at 30 kV using a 30 μm objective aperture.

Synchrotron X-ray Crystallography—Single crystal x-ray diffraction was performed at the European Molecular Biology Laboratory (EMBL), Outstation Hamburg, DESY, using the wiggler beam-line BW 7B (wavelength 0.885 Å) and a MARRESEARCH imaging plate detector system (X-Ray Research, Hamburg, Germany). It was found that cryogenic conditions (boiling N2 temperature) were essential for preservation of the integrity of the crystals. The technique used in streamfreezing has been described in detail elsewhere (26). The crystal-to-detector distance was varied between 400 and 600 mm. Diffraction data were typically collected over periods of 300 s with an oscillation angle of 0.5° about one given crystal orientation. Preliminary data analysis was performed with the DENZO program system (27).

RESULTS AND DISCUSSION

LDL particles, even if isolated from a single donor, are heterogeneous with respect to particle size and lipid composition. Highly homogeneous subspecies of LDL particles have, however, been identified on the basis of their hydrated densities and physicochemical characteristics (24). These subspecies differ in their receptor binding affinity (28), susceptibility to oxidative modification (29,30), and in their atherogenic potential. In our extensive search for suitable materials and conditions for LDL crystallization, we found that only the LDL subspecies of intermediate density (d 1.0271–1.0297 g/ml) grown from 12% (w/v) PEG2000, 0.1 M ammonium sulfate at pH 7.0 in 0.3 M phosphate buffer. The bar corresponds to 500 μm.

small pieces of the solidified agar gel (about 1 mm³) containing the fixed crystals were embedded in water-miscible Nanoplast resin (Bachhuber, Ulm, Germany) polymerized by incubation first at 40°C for 48 h and then for 48 h at 60°C. Samples embedded in Nanoplast were finally transferred into Spurr resin (Merck, Darmstadt, Germany; Ref. 25) which was polymerized at 70°C for 24 h. Ultrathin sections (40–50 nm thick) were poststained with 2% uranyl acetate in distilled water at 180°C with synchrotron radiation at the beamline BW7B of the European Molecular Biology Laboratory, Hamburg, at DESY.

FIG. 1. Single crystal of LDL subspecies (d 1.0271–1.0297 g/ml) grown from 12% (w/v) PEG2000, 0.1 M ammonium sulfate at pH 7.0 in 0.3 M phosphate buffer. The bar corresponds to 500 μm.

FIG. 2. Electron micrographs of an ultrathin-sectioned preparation of a fixed and stained LDL crystal. The bars correspond to 100 nm. The inset in B shows an optical diffractogram of this image; the long axis of the lattice corresponds to a spacing of 17.5 nm.

FIG. 3. A 0.5° rotation pattern of a LDL crystal obtained at –180°C with synchrotron radiation at the beamline BW7B of the European Molecular Biology Laboratory, Hamburg, at DESY.

growth, but to date the maximum size was approximately 500 μm in the longest axis.

Electron microscopy of ultrathin-sectioned preparations of the LDL crystals showed a quasihexagonal arrangement of spheres, of approximately 17–18 nm in diameter (Fig. 2). This is convincing evidence that the crystals consist of a regular array of particles which resemble the morphology of LDL as inferred from solution scattering. The dimensions are by 10–20% smaller than those for native hydrated LDL particles as determined by x-ray and neutron small angle scattering studies (8). The smaller particle size can be rationalized by the treatment involved in fixation, staining, and thin-sectioning for electron microscopy.

Well-resolved x-ray diffraction spots were obtained from the prism-shaped crystals, under cryogenic conditions, to 29-Å resolution (Fig. 3). The intensity data are indexed corresponding to a triclinic lattice, for which the crystallographic dimensions are a = 16.1 nm, b = 39.0 nm, c = 43.9 nm; α = 96.2°, β = 92.1°, γ = 102°, space group P1. The unit cell volume is 2.8 × 10⁴ nm³. Evidence for the existence of different crystal forms, with tetragonal space group (P4₁2₁2) or P4₁2₂₂ was also obtained in some instances. The quality of the data was, however, too poor to warrant a quantitative evaluation.

An estimation of the number of LDL particles within the unit cell would require a very precise determination of the crystal densities, since the densities of the LDL subspecies are very close (within a few percent) to those of the solvent densities used in crystallization. Such precision is presently beyond experimental reach. Nevertheless, the following estimation can be made: the above values for the molecular weights and the hydrated densities lead to LDL particle volumes of about 4.5 × 10⁸ nm³. If there were four LDL per unit cell, the solvent
volume in the crystal would amount to 36\%, which is less than what would be required for a close packing of spheres. It is likely, therefore, that less than four LDL are contained in the unit cell.

This very high unit cell volume and the notion that probably only the apoprotein B-100 (i.e. less than 20\% of the total LDL mass) contributes to the long range crystallographic order, explains the very low intensity of the diffraction pattern with crystals of the given size. The lifetime of the crystals under x-ray exposure was enough to take serial exposures of about a dozen frames, with 300 s/frame. Thus, crystal size is presently the most important factor in the pursuit of higher resolution. Internal crystal disorder is certainly also a factor contributing to the limited resolution obtained so far; it seems to play a minor role, however, as even the weakest reflections are still relatively sharp.

In summary, the present results have shown that crystallization, long deemed impossible with intact human plasma lipoproteins, is a viable approach. It can be hoped that through further efforts in improving crystal size and quality, the question of protein structure in intact LDL will be resolved by x-ray crystal structure analysis, the only method presently available for a particle of this extreme size and complexity.

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