Journal of Structural Biology 164 (2008) 190–198

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Streptavidin crystals as nanostructured supports and image-calibration references for cryo-EM data collection

Liguo Wang, Puey Ounjai [1], Fred J. Sigworth *

Department of Cellular and Molecular Physiology, Yale University, 333 Cedar Street, New Haven, CT 06520, USA

ARTICLE INFO

Article history:
Received 12 May 2008
Received in revised form 14 July 2008
Accepted 18 July 2008
Available online 5 August 2008

Keywords:
2D crystal
Nano-support
Cryo-EM
Liposome
Tethering

ABSTRACT

For cryo-EM structural studies, we seek to image membrane proteins as single particles embedded in proteoliposomes. One technical difficulty has been the low density of liposomes that can be trapped in the ~100 nm ice layer that spans holes in the perforated carbon support film of EM grids. Inspired by the use of two-dimensional (2D) streptavidin crystals as an affinity surface for biotinylated DNA (Crucifix et al., 2004), we propose to use the crystals to tether liposomes doped with biotinylated lipids. The 2D crystal image also serves as a calibration of the image formation process, providing an absolute conversion from electrostatic potentials in the specimen to the EM image intensity, and serving as a quality control of acquired cryo-EM images. We were able to grow streptavidin crystals covering more than 90% of the holes in an EM grid, and which remained stable even under negative stain. The liposome density in the resulting cryo-EM sample was uniform and high due to the high-affinity binding of biotin to streptavidin. Using computational methods, the 2D crystal background can be removed from images without noticeable effect on image properties.

1. Introduction

Membrane proteins are involved in many essential biological processes, such as ion and solute transport, energy conversion, and cell signaling. Despite their pivotal roles, only about 170 unique membrane protein structures have been identified (Raman et al., 2006), as compared with over 46,000 known structures for soluble proteins, due to the difficulty of forming crystals for X-ray or electron crystallography. Recently, single-particle electron cryomicroscopy (cryo-EM) has been used to study structures of membrane proteins solubilized in detergents. With the use of detergents, however, there always arises the question of whether the native conformation is maintained.

A natural way to preserve the native conformation of membrane proteins for structural study is to reconstitute them into liposomes (lipid vesicles). The resulting proteoliposomes are applied to perforated carbon films, fast-frozen and imaged using cryo-EM, and we expect that the three-dimensional structure can be reconstructed from the cryo-EM images (Jiang et al., 2001).

During our attempts to image proteoliposomes, we found that there were two technical difficulties. First, the density of proteoliposomes varies with ice thickness and adsorptive forces, so that they are found predominantly near the edges of holes in the perforated carbon film where the quality of acquired images is poor. Second, when only microgram quantities of the membrane protein are available, the proteoliposome concentration is low and few images are obtained. Inspired by the immobilization of biotinylated DNA on two-dimensional (2D) streptavidin crystals (Crucifix et al., 2004), we sought to use these crystals to tether proteoliposomes decorated with biotinylated lipids, which will be valuable to increase the proteoliposome density in acquired cryo-EM images.

Streptavidin, synthesized by *Streptomyces avidinii*, is a 15 kDa protein of 159 residues (Sano et al., 1995). It is proteolyzed naturally at both ends, and the most stable and well-studied form of this protein (called core-streptavidin) contains 125–127 residues. The atomic structure was first determined independently by Weber et al. (1989) and Hendrickson et al. (1989) using X-ray diffraction. Other forms (full length, biotin-bound complex, and mutants) have been studied extensively by X-ray diffraction (Freitag et al., 1997; Freitag et al., 1999; Izrailev et al., 1997) and by electron crystallography (Avila-Sakar and Chiu, 1996; Darst et al., 1991; Kubalek et al., 1991; Le Trong et al., 2006; Wang et al., 1999). The crystal structures show that the protein is a homotetramer of identical subunits with a D2 symmetry. Each subunit contains a β-barrel with the biotin-binding site located at one end. The 2D crystal has a unit cell with $a = b = 8.23$ nm, and $\gamma = 90°$ (Avila-Sakar and Chiu, 1996). Due to the mirror symmetry in the projection map, the reflection spots with indices $h + k = 2n + 1$ are absent; thus in projection the crystal appears to have a square lattice with $a = b = 5.82$ nm and $\gamma = 90°$. We use these parameters in analyzing our projection images.

* Corresponding author. Fax: +1 203 785 4951.
E-mail address: fred.sigworth@yale.edu (F.J. Sigworth).
[1] Present address: Department of Cell Biology, UT Southwestern Medical Center, Dallas, TX 75390, USA.

1047-8477/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2008.07.008

The 2D streptavidin crystal has been shown to be readily picked up by an EM grid coated with a holey carbon film (Avila-Sakar and Chiu, 1996; Crucifix et al., 2004; Kubalek et al., 1991), and crystal sizes up to a few μm^2 have been observed (Crucifix et al., 2004; Kubalek et al., 1991). Crystals fast-frozen in vitreous ice show electron diffraction to atomic resolution (Avila-Sakar and Chiu, 1996), and the 2D crystals can also serve as tethering surfaces, for example for tethering of biotinylated DNA molecules (Crucifix et al., 2004). These authors demonstrated that the crystal information can be removed computationally, allowing 3D models to be obtained by single-particle reconstruction to a resolution of 3 nm.

In the experiments described here, we use this 2D crystal as a nano-support and a tethering template for liposomes, and as a built-in reference for the calibration of the image formation process.

2. Methods

2.1. Growth of 2D streptavidin crystals

Streptavidin was purchased from Sigma–Aldrich (St. Louis, MO). Biotinylated dipalmitoyl-phosphatidyethanolamine (biotin–DPPE) and dioleyl-phosphatidlycholine (DOPC) (Avanti, Alabaster, AL) were used as received. Two-dimensional streptavidin crystals were grown at room temperature using a procedure similar to that described by Kubalek et al.(1991). Sixty-seven microliters of Tris buffer (150 mM NaCl, 50 mM Tris, pH 7.0) containing 0.05–0.40 mg/ml of streptavidin was deposited in a micro-well formed by a polypropylene PCR tube cap. Then 0.5–1.5 μl of a 0.5 mg/ml chloroform/hexane (1:1) solution of biotin–DPPE and DOPC, at a mass ratio of 1:4, was spread on the surface of the protein solution. The PCR caps were placed in a humid chamber and kept at room temperature for 2–20 h. A standard 400-mesh EM grid coated with a perforated carbon film made by the stamping method (Chester et al., 2007), was washed with hexane and dried in air for an hour, and then was placed onto the surface of a well for one minute. It was withdrawn, washed with three droplets (25 μl) of Tris buffer, and was either negatively stained or frozen for examination by electron microscopy.

2.2. Preparation and tethering of liposomes

Diphytanoyl phosphatidylcholine (DPhPC) (Avanti, Alabaster, AL) was used as received. DPhPC and biotin–DPPE (3600:1 mol/mol) were mixed in chloroform, dried under nitrogen, and re-hydrated in Hepes-buffered KCl solution (135 mM KCl, 5 mM NaCl, 1 mM EDTA, 10 mM HEPES, pH = 7.4) to a concentration of 2.9 mg/ml. The lipid suspension was frozen and thawed 10 times, and extruded through an 80-nm polycarbonate membrane filter (Whatman) using a Lipex™ extruder (Northern Lipids Inc.) (Mayer et al., 1986). We expect there to be about 16 biotin–DPPE molecules in each 80 nm liposome. To obtain highly spherical liposomes, we swelled them by repeated osmotic shocks, adding water to the liposome suspension (11%, 14%, 18%, 24%, and 33% of the original volume) at 1 h intervals at room temperature.

After the 2D streptavidin crystal was transferred to the perforated carbon film, the crystal was incubated with liposome suspensions for 0.5–16 min, depending on the desired liposome density on the grid. Then the grid was washed with two droplets (25 μl) of 2 times diluted Hepes-buffered KCl solution and one droplet (25 μl) of 3 times diluted Hepes-buffered KCl solution, after which either a negative stain or cryo-EM sample was prepared. For negative stain, 2% uranyl acetate was applied to the sample and blotted away after 1 min. For a cryo-EM sample, the grid was side-blotted briefly to remove excess KCl solution, and then 6 μl of 5 times diluted HEPES-buffered KCl solution was applied to the grid, and blotted away for 2–5 s using a slip of filter paper (Whatman) at room temperature. The specimen was rapidly frozen by plunging into liquid ethane and stored in liquid nitrogen.

2.3. EM imaging

Images of liposomes within the holes in the carbon film were taken at Brandeis University using a Tecnai F30 microscope at 300 keV with a 30 μm objective aperture and a Gatan Imaging Filter (GIF). The electron dose for each exposure was 1000–3000 e/nm^2. Images were taken at 50,000 magnification and 0.2–4.5 μm defocus, and recorded on the GIF 2K × 2K UltraScan 1000 FT (Frame Transfer) camera with an effective pixel size of 0.253 nm. Some data were taken at Yale University using a Tecnai F20 electron microscope at 200 keV using a 20 or 30 μm objective aperture. The dose for each exposure was about 2000 e/nm^2. Images were taken at 25,000 or 50,000 magnification and 2.0–3.3 μm defocus and recorded on Kodak SO-163 film. Negatives were scanned with a Zeiss SCAI film scanner to an effective pixel size of 0.28 nm.

2.4. MD simulations of the solvated streptavidin crystal

MD simulations of the streptavidin crystal were performed on the Bulldog-I computer cluster at Yale University using NAMD2.6 (Kale et al., 1999) with version 27 of the CHARMM force field. The structure of a streptavidin monomer was obtained from the atomic coordinates (PDB code: 1STP). However, because the quaternary structure of the tetramer in 2D crystals differs from that in 3D crystals, we constructed a tetramer (Fig. 1A) by docking monomers to match the high-resolution projection map of the 2D crystal (Avila-Sakar and Chiu, 1996). Water molecules (TIP3) were added around the tetramer to a thickness of about 2 nm in all directions: in total 23,260 water molecules were added, resulting in a system of 76,765 atoms in a cubic box with periodic boundaries. After energy minimization with a conjugate gradient for 10,000 steps, the system was subjected to 0.5 ns of constant pressure and temperature (NPT) simulations (1 fs per integration step) at 1 bar and 300 K using the Nosé–Hoover method. The cutoff distance for van der Waals interactions was 1.2 nm, with the pair list distance extended to 1.35 nm. The long-range full electrostatic interactions were evaluated every time step using the particle mesh Ewald method. Harmonic constraints were applied to maintain the tetramer structure, while the water molecules were allowed to move freely. The mean number-density of each atom type was obtained by averaging the trajectories of the last 0.2 ns of the simulation.

From the projection $\rho_i(x,y)$ of the atom density along the z direction we computed the neutral-atom phase shift ϕ_n (units of mrad) of electrons passing through the specimen according to

$$\phi_n(x,y) = \sigma_e \sum_i V_i \rho_i(x,y). \quad (1)$$

Here V_i is the spatially integrated, shielded coulomb potential for an isolated, neutral-atom (V_i = 0.025, 0.130, 0.108, 0.097, and 0.267 V nm^3 for hydrogen, carbon, nitrogen, oxygen, and phosphorous, respectively, calculated from published parameters (Kirkland, 1998)); and the interaction parameter σ_e describes the first-order dependence of the electron phase on the projected electrostatic potential (7.3 and 6.5 mrad/V nm for 200 and 300 keV electrons, respectively) (Kirkland, 1998). The resulting projection maps (Fig. 1B and C) are used as a model for one unit cell of the crystal. The average internal potential is 5.6 and 7.1 V, which for 300 keV electrons results in an electron phase shift of 36.5 and 46.2 mrad/nm of path length for the water and protein regions, respectively. In total, there is about 48 mrad more electron phase shift for an electron