Journal of Structural Biology 180 (2012) 488–496

Contents lists available at SciVerse ScienceDirect

Journal of Structural Biology

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

Unraveling the structure of membrane proteins *in situ* by transfer function corrected cryo-electron tomography

Matthias Eibauer [1], Christian Hoffmann [2], Jürgen M. Plitzko, Wolfgang Baumeister, Stephan Nickell [3], Harald Engelhardt *

Max-Planck-Institut für Biochemie, Martinsried, Germany

ARTICLE INFO

Article history:
Received 6 July 2012
Received in revised form 30 August 2012
Accepted 3 September 2012
Available online 18 September 2012

Keywords:
Cryo-electron microscopy
3D electron microscopy
Contrast transfer function (CTF)
Modulation transfer function (MTF)
Subtomogram averaging
Porin
Outer membrane protein
Mycobacterium smegmatis

ABSTRACT

Cryo-electron tomography in combination with subtomogram averaging allows to investigate the structure of protein assemblies in their natural environment in a close to live state. To make full use of the structural information contained in tomograms it is necessary to analyze the contrast transfer function (CTF) of projections and to restore the phases of higher spatial frequencies. CTF correction is however hampered by the difficulty of determining the actual defocus values from tilt series data, which is due to the low signal-to-noise ratio of electron micrographs. In this study, an extended acquisition scheme is introduced that enables an independent CTF determination. Two high-dose images are recorded along the tilt axis on both sides of each projection, which allow an accurate determination of the defocus values of these images. These values are used to calculate the CTF for each image of the tilt series. We applied this scheme to the mycobacterial outer membrane protein MspA reconstituted in lipid vesicles and tested several variants of CTF estimation in combination with subtomogram averaging and correction of the modulation transfer function (MTF). The 3D electron density map of MspA was compared with a structure previously determined by X-ray crystallography. We were able to demonstrate that structural information up to a resolution of 16.8 Å can be recovered using our CTF correction approach, whereas the uncorrected 3D map had a resolution of only 26.2 Å.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Cryo-electron tomography (CET) is a versatile technique for investigating the structure and interactions of macromolecules in their natural environment (Lucic et al., 2005). This applies to soluble protein complexes and assemblies (Brandt et al., 2010; Ortiz et al., 2010) as well as to membrane proteins (Bartesaghi and Subramaniam, 2009). CET in combination with subtomogram averaging routinely provides structural information of membrane proteins in the range of 2 to 3 nm as shown for viral proteins (Förster et al., 2005; Liu et al., 2008; Zanetti et al., 2006) and of 3 to 6 nm for complexes that are located in cellular membranes or were solubilized and then reconstituted (Khursigara et al., 2008; Dudkina et al., 2010; Trépout et al., 2010). The reconstruction of complexes at a molecular level (<2 nm), however, is hampered by the substantial defocus (between −5 and −12 μm) required for imaging vitrified specimens of a few hundred nanometers in thickness, which limits the resolution. While correction of the contrast transfer function (CTF) in single particle approaches is well established and straightforward (van Heel et al., 2000; Frank, 2006; Grigorieff, 2007), allowing to generate 3D reconstructions in the subnanometer range (e.g. Booth et al., 2004; Bohn et al., 2010; Frauenfeld et al., 2011), due to practical reasons CTF correction is currently not routinely applied in cryo-electron tomography. Tilt series images show a focus gradient in the direction perpendicular to the tilt axis and thus a varying CTF and have a very low signal-to-noise ratio. If the defocus of an image reference point is known the focus gradient can be calculated from geometric considerations and corrected for by one of the approaches described previously (Winkler and Taylor, 2003; Fernández et al., 2006; Zanetti et al., 2009; Mariani et al., 2011; Voortman et al., 2011). The challenge is to reliably determine the actual defocus of projections in a tilt series. Fernández et al. (2006) introduced the method of strip-based periodogram averaging that uses the information of all images recorded to determine a common defocus value. Xiong et al. (2009) modified this approach such that the defoci of subsets of a tilt series can be extracted. There is currently no experimental technique available that allows an accurate CTF determination of an individual cryo-electron tomography projection.

* Corresponding author. Address: Max-Planck-Institut für Biochemie, Am Klopferspitz 18, D-82152 Martinsried, Germany. Fax: +49 89 8578 2641.
E-mail address: engelhar@biochem.mpg.de (H. Engelhardt).
[1] Present address: Biochemisches Institut, Universität Zürich, Switzerland.
[2] Present address: Sandoz GmbH, Langkampfen, Austria.
[3] Present address: Carl Zeiss Microscopy GmbH, BioSciences Deivision, Oberkochen, Germany.

1047-8477/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jsb.2012.09.008

In this study, we describe a procedure employing an extended acquisition scheme for cryo-electron tomography. Additional high-dose images were used to precisely determine the actual defocus and so the CTF condition for each image of the tilt series. We applied our approach to the 160 kDa outer membrane protein MspA from *Mycobacterium smegmatis* reconstituted in lipid vesicles. In addition, we improved the resolution of MspA by subtomogram averaging of the CTF and modulation transfer function corrected data sets and compared our results with the structure of MspA, determined by X-ray crystallography (Faller et al., 2004).

2. Materials and methods

2.1. Purification of MspA

MspA was expressed and purified as described by Heinz and Niederweis (2000). Briefly, *M. smegmatis* ML10 cells harboring the MspA-encoding plasmid pMN016 (Stephan et al., 2004) were grown in Middlebrook 7H9 medium (Difco Laboratories Inc., Franklin Lakes, New Jersey) supplemented with 0.2% glycerol, 0.05% Tween80, and hygromycin at a final concentration of 50 μg/ml. After incubation for 48 h at 37 °C the cells were harvested and resuspended in extraction buffer (100 mM NaH_2PO_4/Na_2HPO_4 pH 6.5, 0.1 mM EDTA, 150 mM NaCl, 0.5% *n*-octyl polyoxyethylene). The suspension was boiled in a water bath for 30 min and cooled on ice for 10 min. After centrifugation for 30 min at 4,000*g*, the supernatant was carefully mixed with the same volume of ice cold acetone and incubated for 1 h at −20 °C. After centrifugation for 30 min at 8,000*g* and 4 °C, the pellet was dissolved in low salt buffer (25 mM HEPES/NaOH pH 7.5, 10 mM NaCl, 0.5% *n*-octyl polyoxyethylene) and filtered (Sartorius, 5 μm pore size) to remove insoluble material. Chromatographic purification was done following the protocol from Heinz and Niederweis (2000). The filtered solution was loaded on an anion-exchange column (POROS 20HQ; PerSeptive Biosystems, Framingham, MA) and bound protein was eluted with a gradient from 10 mM to 2 M NaCl over five column volumes. The collected fractions were analyzed using SDS–PAGE. Fractions containing pure MspA were combined and, after a second acetone precipitation, the pellet was dissolved in 1–2 ml storage buffer (25 mM NaH_2PO_4/Na_2HPO_4 pH 7.5, 0.5% *n*-octyl polyoxyethylene), filtered, and separated by gel filtration (column G3000SW_{XL}; TosoHaas, Stuttgart, Germany) at a flow rate of 0.5 ml/min. Fractions were analyzed by SDS–PAGE and the protein concentration was determined by the bicinchoninic acid assay.

2.2. Preparation of proteoliposomes

Proteoliposomes were prepared by dissolving 1,2-diphytanoyl-sn-glycero-phosphocholine (DiphPC; Avanti Polar Lipids, Alabaster, AL) in chlorophorm. The mixture was dried to a thin film by evaporation under a stream of nitrogen for at least 2 h. The lipid film was hydrated in liposome buffer (25 mM NaH_2PO_4/Na_2HPO_4 pH 7.5) to a final DiphPC concentration of 5 mg/ml, and subjected to five cycles of freeze–thawing (dry-ice in ethanol), followed by 11 to 15 cycles of extrusion through a Nuclepore® track-etched membrane (pore size 100 nm) using a mini-extruder (Avanti Polar Lipids). During vesicle formation, the temperature was kept at 37 °C to ensure the lipid being in the liquid phase. Five hundred microlitre vesicle preparation was mixed with 25 μg MspA in solution containing 0.5% *n*-octyl polyoxyethylene. To remove the detergent from the protein and to force reconstitution 50 mg BioBeads® SM-2 (Bio-Rad, Munich, Germany) were added, and the mixture was incubated for at least 12 h at 37 °C with gentle rotation. Samples from the liquid phase that was free of BioBeads were used for electron microscopy.

2.3. Cryo-electron tomography

C-Flat copper grids (Plano, Wetzlar, Germany) were loaded with 3.5 μl of a suspension containing colloidal gold clusters (10 nm) followed by 5 μl of the proteoliposome preparation. After blotting samples were vitrified in liquid ethane by plunge freezing. Tilt series were recorded on a Tecnai Polara transmission electron microscope (FEI Company Inc., Hillsboro, USA) equipped with a GIF2002 energy filter and a 2 × 2 k Multiscan CCD camera (Gatan Inc., Pleasanton, USA). The microscope was operated at 300 keV in zero-loss mode with a slit width of the energy filter of 20 eV. Micrographs were taken at a final magnification of 61,000*x*, resulting in a pixel size of 2.1 Å on the specimen level; the intended underfocus was set to −5 μm. The tilt series comprised of projections from −60° to 60° with an angular increment of 3°; the cumulative electron dose ranged from 27 to 64 $e^-/Å^2$. The acquisition of two high-dose electron micrographs (*focus images*) for each tilt angle was spatially separated from the sample position (exposure site) as exemplified in the Section 3. The *Xplore3D* software (FEI Company Inc., Hillsboro, USA) was used throughout.

2.4. Defocus measurement

The defocus values of individual images from focus sites (*focus images*) were determined by means of periodogram averaging (Fernández et al., 1997). In brief, each *focus image* was subdivided into tiles of 512 × 512 pixels with an 256 pixels overlap, the power spectra of the tiles were calculated and averaged. The background of periodograms was removed by bandpass filtering (van Heel et al., 2000). Defocus values were determined by cross correlating the periodograms with a set of theoretical CTF spectra (van Heel et al., 2000; Mindell and Grigorieff, 2003) for an interval of ±2 μm around the nominal defocus z_0 in 100 nm steps. For reasons of simplicity, only the phase contrast term was used for CTF calculations whereas the small (but unknown) contribution of amplitude contrast was set to zero. The defocus values were determined by finding the highest correlation coefficient.

2.5. Defocus correction

Defocus correction was performed following the approach of Zanetti et al. (2009), which does not require to align the tilt axis with the direction of a coordinate axis. In brief, the *exposure images* were subdivided into tiles of 16 × 16 pixels (3.4 × 3.4 nm) and these extracted as central areas of image subframes 256 × 256 pixels in size. The defocus value for each tile was calculated from the defocus value of the respective exposure image adding the difference in z between the centers of the image and the actual tile according to the tilt angle ϑ_i (angle of the *ith* projection of the tilt series). The 256 × 256 pixel sized subframes centered at the tiles were Fourier-transformed and the inverted phases corrected (phase flipping; Frank, 2006) according to the theoretical CTF based on the defocus value as calculated before. Following inverse Fourier transformation, the 16 × 16 pixel tiles were extracted and successively assembled to the final CTF-corrected *exposure image*.

2.6. Image alignment and tomographic reconstruction

Projection alignment and tomographic reconstruction were done using procedures of the *TOM Toolbox* (Nickell et al., 2005) and all adaptations were implemented in MATLAB (Mathworks, Natick, USA) for later integration. For alignment, coordinates of eight or more gold markers in *exposure images* and four gold markers in *focus images* (used for test reconstructions only) were selected interactively. Initial alignments were calculated and refined by the approach of Lawrence (1992).

Download English Version:

https://daneshyari.com/en/article/5914403

Download Persian Version:

https://daneshyari.com/article/5914403

Daneshyari.com