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The ultrastructure of *Chlorobaculum tepidum* revealed by cryo-electron tomography

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ABSTRACT

Chlorobaculum (*Cba*) *tepidum* is a green sulfur bacterium that oxidizes sulfide, elemental sulfur, and thiosulfate for photosynthetic growth. As other anoxygenic green photosynthetic bacteria, *Cba tepidum* synthesizes bacteriochlorophylls for the assembly of a large light-harvesting antenna structure, the chlorosome. Chlorosomes are sac-like structures that are connected to the reaction centers in the cytoplasmic membrane through the BChl *a*-containing Fenna–Matthews–Olson protein. Most components of the photosynthetic machinery are known on a biophysical level, however, the structural integration of light harvesting with charge separation is still not fully understood. Despite over two decades of research, gaps in our understanding of cellular architecture exist. Here we present an in-depth analysis of the cellular architecture of the thermophilic photosynthetic green sulfur bacterium of *Cba tepidum* by cryo-electron tomography. We examined whole hydrated cells grown under different electron donor conditions. Our results reveal the distribution of chlorosomes in 3D in an unperturbed cell, connecting elements between chlorosomes and the cytoplasmic membrane and the distribution of reaction centers in the cytoplasmic membrane.

1. Introduction

Green sulfur bacteria (GSB; order *Chlorobia*) are anerobic, photosynthetic bacteria that utilize reduced sulfur compounds as electron donors to a photosynthetic electron transport chain. The chain provides energy and reduced ferredoxin to drive carbon fixation, biosynthesis, and cell growth [1,2]. *Chlorobia* are widely distributed in aquatic environments where anoxic layers containing reduced sulfur compounds are exposed to light. They predominantly utilize sulfide (S^{2-}), thiosulfate ($S_2O_3^{2-}$), biogenic and abiogenic sulfur (S^0) globules, and hydrogen (H_2) as electron donors to support photoautotrophic growth [3]. Most strains can oxidize S^{2-} and H_2, while oxidation of $S_2O_3^{2-}$ is less commonly encountered in cultivated strains. *Chlorobia* are known to have a high affinity for S^{2-}, and this is usually the preferred substrate. Initially, S^{2-} is generally incompletely oxidized to S^0, which is deposited extracellularly, and oxidized completely to sulfate (SO_4^{2-}) when all of the S^{2-} has been consumed [4]. At present, the mechanisms involved in the formation and consumption of S^0 in *Chlorobia* are largely unknown [5]. *Chlorobaculum* (*Cba*) *tepidum* TLS was isolated from a hot spring in New Zealand and like other *Chlorobia* preferentially oxidizes sulfide to S^0, which is deposited outside of the cell [6]. The ability of *Cba tepidum* to fix atmospheric nitrogen and its dependence on sulfur compounds for the photosynthetic processes, make this species an important model to understand the role microbes play in global nutrient cycles.

Being a Gram negative bacterium, *Cba tepidum* has an outer membrane (OM) and an inner cytoplasmic membrane (CM). The reaction center (RC), which is located in the CM, converts light energy into redox chemical energy, which is further transformed into stable oxidants and reductants [7]. The critical role that the cytoplasmic membrane plays in housing the photosynthetic and respiratory complexes, maintaining a proton gradient for the production of ATP, and maximizing light capture, makes the membrane architecture of *Cba tepidum* of particular interest [8]. Light is primarily captured by chlorosomes, which serve as huge antenna complexes. Chlorosomes are attached to the inner face of the CM and interact with the membrane-embedded RCs (reviewed in [9]) *via* the water-soluble Fenna–Matthews–Olson (FMO) proteins. The latter ~40 kDa protein is present as a homotrimer and associated with bacteriochlorophyll *a* (BChl *a*) [7,10–12]. Chlorosomes are ellipsoid organelles with an interior of self-organized BChl oligomers (BChl *c* and/or *d* or *e*; reviewed in [13]) contained by a

Abbreviations: AFM, atomic force microscopy; 3D, three dimensional; BChl, bacteriochlorophyll; *Cba*, *Chlorobaculum*; CM, cytoplasmic membrane; Cryo-ET, cryo-electron tomography; DLS, dynamic light scattering; EM, electron microscopy; FMO, Fenna–Mathew–Olson; GSB, green sulfur bacteria; OM, outer membrane; PFT-AFM, peak force tapping atomic force microscopy; PG, peptidoglycan; RC, reaction center; TEM, transmission electron microscopy

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lipid envelope. They have a specific region termed the baseplate comprised of primarily CsmA-BChl *a* protein–pigment complexes organized in a two dimensional paracrystalline array [14]. Baseplates are exclusively located on the chlorosome surface facing the CM [15], into which the C-terminus of CsmA is thought to insert [16]. The N-terminus of the CsmA proteins is inserted in the lipid monolayer of the chlorosome envelope [16].

Various attempts have been made to visualize structural details of the light harvesting and energy transfer-mediating systems of *Chlorobia*. The 2.2 Å crystal structure of the FMO protein has trimeric crystallographic symmetry [10]. Single particle electron microscopy (EM) has given structural information for *Cba tepidum* RC core complexes, RCs associated with FMO protein and FMO protein complexes [11,12,17]. Chlorosomes isolated from *Cba tepidum* have been investigated using EM, light scattering and proteomics tools [13,18] and the structure of the FMO protein is available at 1.9 Å resolution [19]. Cryo-EM and X-ray scattering indicate a lamella organization of pigments within chlorosomes [20–22]. Isolated chlorosomes from three different phyla, *Chloroflexus aurantiacus*, *Cba tepidum* and the newly discovered *Candidatus Chloracidobacterium thermophilum* have also been analyzed under close to native conditions using peak force tapping atomic force microscopy (PFT-AFM) [23].

To date, descriptions of the localization of chlorosomes relative to the CM and the organization of the OM and CM of the whole bacterium, are based on thin-section electron micrographs, freeze–fracture studies [8,24] and mass spectroscopy-based methods [25]. The first studies employed the freeze–fracture technique to investigate the photosynthetic machinery of *Chlorobium limicola* [26]. Some replicas revealed 10 nm-wide rod-like elements arranged in hexagonal arrays. Others showed regularly spaced planar arrays that were associated with the CM and often with the rod-like structures. Later work employing a different sample fixation protocol, reported that the interface between chlorosomes and the CM is occupied by repetitive structural elements [24]. These structures were shown to connect the chlorosomes to the CM, creating a space between them. Further, elements connecting the CM and the OM in the periplasmic space under the chlorosomes were also observed [24]. However, such studies have led to conflicting interpretations because the data were obtained by a variety of fixation techniques and protocols.

3D electron tomographic (ET) imaging of non-fixed, non-stained, hydrated biological material has allowed insights into the *in situ* organization of cells at macromolecular resolution [27,28]. Examination of chlorosomes isolated from *C. aurantiacus* (green nonsulfur bacteria) by cryo-ET confirmed the overall dimensions estimated by cryo-EM, but did not reveal internal structural details [21].

In the present paper, we use cryo-ET to define the three dimensional (3D) organization of the green sulfur bacterium *Cba tepidum* grown in the presence of sulfide (S^{2-}) and thiosulfate ($S_2O_3^{2-}$). Further, we employ cryo-ET to systematically analyze cellular structures of bacteria grown in the presence of S^{2-} alone.

2. Materials and methods

2.1. Culture media and growth

Cba tepidum was cultured as described previously [6]. The medium contained 7.7 mM Na_2S (S^{2-}) and 4 mM $Na_2S_2O_3 \cdot 5H_2O$ ($S_2O_3^{2-}$) as electron donors. Cultures (1 L or 500 mL) were incubated at 47 °C under continuous illumination from 2 W illuminating tubes at 20 μE m^{-2} s^{-1}. Cells were harvested 1–2 days after inoculation with 20 mL of old cell culture.

2.2. Sulfide transition

Cultures were inoculated into 500 mL of medium as described previously [6] except that $S_2O_3^{2-}$ was omitted. The concentration of S^{2-} was 9.5 mM. Cultures were incubated at 47 °C under continuous illumination from 20-W illuminating tubes at 20 μE m^{-2} s^{-1}. Cells were harvested 1–2 days after inoculation with 20 mL of cells cultured in the presence of 7.7 mM S^{2-} and 4 mM $S_2O_3^{2-}$.

S^0 and BChl *c* were determined spectrophotometrically. The cell pellet was extracted with methanol, and the concentrations in the supernatant were determined at 265 nm and 669 nm, respectively, using absorption coefficients of 23.9 L g^{-1} cm^{-1} [29] and 86 L g^{-1} cm^{-1} [30]. S^{2-} was measured using the colorimetric methylene blue method [31]. SO_4^{2-} was measured by ion chromatography using a Dionex AG4A-SC 4 mm pre-column and a Dionex AS4A-SC 4 mm column attached to a Dionex GP50 gradient pump. The column was equilibrated by a buffer containing 3.4 mM $NaHCO_3$ and 3.6 mM Na_2CO_3, at 1.5 mL/min. SO_4^{2-} was detected by a Dionex CD20 conductivity detector.

2.3. Cryo-ET

Cryo-electron tomography (cryo-ET) was performed essentially as described before [32]. Bacteria in their respective growth medium were anerobically centrifuged for 3 min at 300 *g*. The pellet containing ~20 μL of media was mixed with 1 μL of 10 nm colloidal gold particles and rapidly transferred onto glow-discharged holey carbon Quantifoil EM grids. Grids were rapidly plunge frozen in liquid ethane cooled to liquid nitrogen temperature, using a Vitrobot (FEI Corp, Hillsboro, USA). The grids were observed in a Titan Krios (FEI) operating at 300 kV and equipped with a Gatan post column energy filter and a 2 k Ultrascan 1000 CCD camera (Gatan, Pleasanton, USA). Single axis tilt series were acquired with an increment of 2° covering −60° to +60°. The cumulative dose was under 10,000 electrons/nm^2 and the defocus was −5 to −10 μm.

A total of 19 tomographic tilt series were recorded for *Cba tepidum* grown in the presence of S^{2-} and $S_2O_3^{2-}$ and 6 tomographic tilt series for *Cba tepidum* grown in the presence of just S^{2-}. The tomograms were reconstructed by weighted back-projection using the *eTomo* (Boulder Laboratory for 3D Electron Microscopy) [33]. Volume-rendered segmentation were performed manually using the Amira package (FEI Corp., Hillsboro, USA).

2.4. Connections between the IM and chlorosomes

198 sub-volumes from 2 × 2 × 2 voxel binned volumes (resulting pixel size = 1.08 nm) were extracted from 8 tomograms of bacteria grown in the presence of S^{2-} and $S_2O_3^{2-}$ and recorded at a defocus of −5 μm. The particles were manually translationally and rotationally aligned to a common center and orientation using the dynamo_gallery tool of Dynamo (www.dynamo-em.org) [34]. The particles then were separated to the even and odd subpopulations and processed independently: the particles were aligned to a template that was generated summing up the manually aligned particles. The average was iteratively refined applying high rotational symmetry; the final resolution was obtained by measuring Fourier ring correlation between the final averages from the odd and even subpopulations, using the 0.143 threshold criterion. No correction for the contrast transfer function was performed, as it was not limiting for the resolution [35].

3. Results

In *Cba tepidum* cultures that contain both S^{2-} and $S_2O_3^{2-}$, S^{2-} is oxidized preferentially and S^0 globules are formed [4]. Following S^{2-} depletion, $S_2O_3^{2-}$ and S^0 globules are oxidized to SO_4^{2-}. In order to study whether the presence of $S_2O_3^{2-}$ affects the formation of S^0 globules, we grew the bacteria in the presence of both S^{2-} and $S_2O_3^{2-}$ and in the presence of just S^{2-}. The amount of S^{2-} ions initially present was the same in both cases. Fig. 1A shows the growth rate of the bacterial cells indicated by the BChl *c* concentration. At the same time, we measured the transformation of the sulfur compounds to SO_4^{2-}, which is