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EM structure of a helicase-loader complex depicting a 6:2 binding sub-stoichiometry from *Geobacillus kaustophilus* HTA426

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ABSTRACT

During DNA replication, bacterial helicase is recruited as a complex in association with loader proteins to unwind the parental duplex. Previous structural studies have reported saturated 6:6 helicase-loader complexes with different conformations. However, structural information on the sub-stoichiometric conformations of these previously-documented helicase-loader complexes remains elusive. Here, with the aid of single particle electron-microscopy (EM) image reconstruction, we present the *Geobacillus kaustophilus HTA426* helicase-loader (DnaC-DnaI) complex with a 6:2 binding stoichiometry in the presence of ATPγS. In the 19 Å resolution EM map, the undistorted and unopened helicase ring holds a robust loader density above the C-terminal RecA-like domain. Meanwhile, the path of the central DNA binding channel appears to be obstructed by the reconstructed loader density, implying its potential role as a checkpoint conformation to prevent the loading of immature complex onto DNA. Our data also reveals that the bound nucleotides and the consequently induced conformational changes in the helicase hexamer are essential for active association with loader proteins. These observations provide fundamental insights into the formation of the helicase-loader complex in bacteria that regulates the DNA replication process.

1. Introduction

Accurate and timely DNA replication is crucial for cell proliferation in all organisms, which relies on a number of protein–protein interactions [1,2]. In Gram-negative bacteria, after DnaA mediates replication initiation, DNA helicase hexamers in association with loader proteins are recruited to the origin of replication (OriC) to unwind parental duplexes [3,4]. In Gram-positive species, two accessory proteins (DnaD and DnaB) are also involved in helicase recruitment [5–7]. Subsequently, in both bacterial species, joining of the primase DnaG completes the primosome assembly, by which a short RNA primer is generated on the unwound ssDNA template for strand synthesis by DNA polymerase in the replisome machinery [8]. Interestingly, two mechanisms have been proposed for loading of helicase onto DNA. A ring-forming strategy has been hypothesized for Gram-positive bacteria (e.g. *Bacillus subtilis*), whereby the preformed hexameric helicase-loader complex would first disassemble and then reassemble in the presence of ATP into a functionally-active conformation around the ssDNA [9]. For Gram-negative bacteria (e.g. *Escherichia coli*), a ring-opening strategy has been proposed instead, whereby association of six loader proteins with the helicase hexamer (6:6 stoichiometry) induces a twist that results in an open-spiral conformation, eventually opening the helicase ring and facilitating subsequent loading onto ssDNA [10].

Despite the differences in loading strategies, helicase proteins in both Gram-positive and Gram-negative bacteria share a similar domain organization, including (i) the N-terminal primase binding domain; and (ii) the C-terminal RecA-like domain involved in DNA binding, loader protein interaction and ATPase activity [11,12]. However, although loader proteins share the common C-terminal AAA + ATPase domain, an extra N-terminal domain of ~80 a.a. harboring a unique Zn-binding site has only been characterized in Gram-positive species [13,14]. This Zn-binding site has been shown to be essential for the association of helicase with loader proteins [14], which implies a substantial difference in complex formation between Gram-positive and Gram-negative bacteria.

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Recently, a cross-species crystal structure of a Gram-positive *Bst*DnaG$_{HBD}$-*Bst*DnaB-*Bsu*DnaI (primase$_{(Helicase\ Binding\ Domain)}$-helicase-loader) complex was determined with a 3:6:6 stoichiometry [15]. This nearly undistorted structure describes a state after the recruitment of primase, which might consequently induce a conformational change in the preformed helicase-loader complex. Meanwhile, helicase-loader complexes with sub-stoichiometries (6:1 and 6:2) were also documented in *B. subtilis* species [9,13,16], but their structural detail is lacking. Therefore, the structures of Gram-positive bacterial helicase-loader complexes before loading onto ssDNA remain elusive. Furthermore, in comparison to the open-spiral conformation observed recently in the presence of ADP·BeF_3 [10], earlier EM studies revealed an untwisted architecture of the *Ec*DnaB-*Ec*DnaC complex in the presence of ATP [16,17]. These findings suggest that hydrolyzation of the bound nucleotides in the complex may trigger functionally correlated conformational changes.

Here, we report an EM structure of a helicase-loader complex with a 6:2 binding stoichiometry from the Gram-positive bacteria *Geobacillus kaustophilus* HTA426 (hereafter *Gk*DnaC-*Gk*DnaI complex) in the presence of ATPγS using single particle image reconstruction. The 19 Å resolution EM map shows a distinct hexameric helicase ring in a nearly undistorted conformation. Above the helicase C-terminal domain (*Gk*DnaC$_C$), we reconstructed a density lobe capable of accommodating two *Gk*DnaI loader proteins, revealing the 6:2 binding stoichiometry of the complex. Docking of the homologous models reveals that the helicase hexamer in the complex adopts a near constricted conformation to interact with the loader proteins. Interestingly, the reconstructed *Gk*DnaI density could potentially obstruct the path of the central DNA binding channel in the helicase hexamer. This suggests a possible role of this sub-stoichiometric conformation in acting as a checkpoint to prevent the loading of immature helicase-loader complex onto ssDNA in the early stage of primosome assembly.

2. Material and methods

2.1. Cloning, protein expression and purification

Cloning, expression and affinity purification of the *Gk*DnaC-*Gk*DnaI complex was performed as previously described [18]. The Ni-affinity purified fractions were dialyzed against 20 mM Tris–HCl pH 8.5, 200 mM NaCl, 5% glycerol, 5 mM $MgCl_2$ and 1 mM β-mercaptoethanol and then subjected to pre-equilibrated size exclusion chromatography using a Superdex 200-pg column (GE Healthcare). The purity and confirmation of the *Gk*DnaC-*Gk*DnaI complex were assessed by SDS-PAGE and MALDI-MS.

2.2. Specimen preparation and EM data collection

The negatively-stained EM specimens were prepared as described previously [19]. Briefly, the *Gk*DnaC-*Gk*DnaI complex sample was diluted to ~0.02 mg/ml and applied to glow-discharged continuous carbon grids. Uranyl formate (UF) at 0.75% (w/v) was then used for staining. To obtain particle images with several orientations, the carbon grids were pre-treated with 0.1% poly-L-lysine hydrobromide as previously described [10]. All the micrographs were collected on a FEI Tecnai G2 F20 TWIN electron microscope with a Gatan 4k × 4k CCD. Under a working voltage of 200 kV, the images were taken using the low dose mode, with a dosage of 20–25 $e^-/Å^2$. For the random conical tilt (RCT) approach [20], micrographs at $\theta = 0°$ and 50° were imaged pair-wise. Statistical detail for the EM data is tabulated in Table S1.

2.3. Image processing, 3D map reconstruction and homologous model docking

Particle images were selected using EMAN2 [21]. The parameters for the microscope contrast transfer function were estimated by CTFFIND3 [22]. The 2D reference-free alignment was performed using the CL2D algorithm in XMIPP [23]. For the RCT approach, the alignment parameters of the 7607 untilted images were transferred to SPIDER [24] for the reconstruction of the RCT volume from the corresponding tilted images in each 2D class. Due to our observation of more abundant *Gk*DnaI densities, Data 3 (Table S1) was chosen for further EM map reconstruction and refinement. Appropriate isolation of the complex particles from the heterogeneous data was achieved by the 3D classification procedure in RELION [25]. Using a 70 Å low-pass filtered RCT initial model, a total of 19,746 particles were first separated into two sub-classes. In the sub-class comprising 10,214 particles, a distinct *Gk*DnaI density was clearly observed above the helicase hexamer. The remaining 9532 particles were further divided into two sub-classes, both of which only exhibited features of the solitary helicase. Subsequent refinements were also performed in RELION to obtain the final reconstruction map of the captured *Gk*DnaC-*Gk*DnaI helicase-loader complex. The homologous models of *Gk*DnaC and *Gk*DnaI derived from the crystal structures of *Aa*DnaB (PDB: 4NMN) and *Bsu*DnaI (PDB: 4M4W), respectively, were acquired from the web server Phyre2 [26]. The monomeric structures obtained were superimposed onto the corresponding templates to construct the docking models of the *Gk*DnaI dimer, *Gk*DnaC$_N$ dimer and *Gk*DnaC$_C$ hexamer, individually. Model docking and display of the EM maps were performed in Chimera [27].

3. Results and discussion

3.1. Helicase-loader complex of G. kaustophilus is stabilized in the presence of ATPγS

The co-expressed and purified *Gk*DnaC-*Gk*DnaI (helicase-loader) complex in the absence of exogenous nucleotide showed a relatively equal binding ratio at a concentration of ~2.0 mg/ml (Fig. S1). The pre-formed complex was then subjected to EM specimen preparation at a diluted concentration of ~0.02 mg/ml and negatively-stained with 0.75% uranyl formate (UF). For the 17,510 collected particle images, 2D reference-free alignment was applied to extract the representative orientations. Principally, only the top views with a distinct open central channel and the side views depicting features of two stacked layers were observed (Fig. 1A). These traits were also found in the structural projections of the *Gk*DnaC helicase hexamer alone (PDB: 2VYF) [11], where the thick and thin layers in the side views corresponded to *Gk*DnaC$_C$ and *Gk*DnaC$_N$, respectively. Therefore, we surmise that *Gk*DnaI loader proteins may dissociate from the co-purified complex upon dilution, implying a weaker binding affinity toward *Gk*DnaC at lower protein concentrations. This weak interaction between helicase and loader proteins has previously been described in *B. subtilus* species [15,28,29].

It has been previously reported that nucleotides could stabilize helicase-loader complexes in both *E. coli* and *B. subtilis* [9,10,30]. We next inspected whether the affinity between *Gk*DnaC and *Gk*DnaI at the same concentration could be enhanced by the presence of nucleotide. We incubated the same co-purified *Gk*DnaC-*Gk*DnaI sample with 1 mM ATPγS (a non-hydrolyzable ATP mimic) for 30 min before EM specimen preparation. As expected, the 2D reference-free alignment against the corresponding 18,437 collected particles yielded several class averages showing complex-like features, along with some solitary helicase particles (Fig. 1B).