Structural analysis of an FtsH2/FtsH3 complex isolated from *Synechocystis* sp. PCC 6803

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ABSTRACT

The FtsH2 protease of the cyanobacterium *Synechocystis* PCC 6803 plays an important role in many physiological processes including selective D1 degradation during Photosystem II repair. To clarify the structure of FtsH2, a Glutathione-S-transferase (GST)-tagged derivative was isolated by affinity chromatography from *Synechocystis* 6803. Our results indicate that FtsH2 forms a complex with FtsH3 and that this complex is hexameric.

INTRODUCTION

The cyanobacterium *Synechocystis* sp. PCC 6803 encodes four FtsH homologues, designated FtsH1-4 (Mann et al, 2000), each of which is predicted to contain the AAA^+ (ATP-ase associated with various cellular activities) module and zinc-binding site typical of this class of metalloprotease (Tomoyasu et al, 1993). Of these, FtsH2 (Cyanobase designation slr0228) has been implicated in PSII repair (Silva et al, 2003), the removal of unassembled and misassembled proteins from the thylakoid membrane (Komenda et al, 2006), osmoregulation

(Stirnberg et al, 2007) and the induction of complexes involved in the uptake of inorganic carbon into the cell (Zhang et al, 2007). To address the structural organisation of FtsH2 *in vivo*, we have generated and isolated a C-terminally GST-tagged FtsH2 derivative for analysis by electron microscopy.

MATERIALS AND METHODS

Construction of a GST-Strep II-tagged FtsH2 Synechocystis sp. PCC 6803 strain

Plasmid p0228GSTSTREP containing the modified *ftsH2* gene was used to transform an *ftsH2* deletion mutant (Syn0228GENT) (Barker, 2006). Transformants were selected by restoration of photoautotrophic growth under high-light conditions and their genotype was confirmed by PCR (Barker, 2006).

Isolation of the GST-tagged FtsH2 complex

Cells were grown in BG-11 medium with 5 mM glucose at 70 μmol photons m^{-2} s^{-1} then harvested and exposed to 1,200 μmol photons m^{-2} s^{-1} of white, heat-filtered, light for 6 h to enhance expression of FtsH2 (Barker, 2006). Cells were broken using a French Press and the isolated thylakoids solubilised with 0.5% β-dodecyl maltoside (β-DM), incubated with glutathione agarose beads for 2 h and captured proteins released by glutathione (Barker, 2006). SDS-PAGE and immunoblotting were performed as described by (Silva et al, 2003).

Electron microscopy and single-particle analysis of isolated FtsH2 protein complexes

Samples were applied to glow-discharged copper grids and negatively stained with 1% uranyl acetate. Images were recorded at RT using a Philips CM100 TEM, operating at 80 kV and 50,850x magnification. Micrographs were chosen for minimal astigmatism/drift and scanned using a Nikon LS9000 densitometer. Fourier power spectra for each micrograph displayed first

minima in the range of 19-21 Å. A dataset of ~3,400 particles was compiled using 'boxer' of the EMAN software package (Ludtke et al, 2004). Further processing was performed using Imagic-5 (Image Science GmbH, Berlin) at a sampling frequency of 2.5 Å/pixel on the specimen scale. Reference-free alignment, multivariate statistical analysis and iterative refinement resulted in the class averages presented (Ruprecht and Nield, 2001).

RESULTS

Mutant generation and purification of FtsH2-GST complex

Transformant Syn0228GSTSTREP was constructed to express a tandem GST-Strep II affinity tag attached to the C-terminus of FtsH2 (Barker, 2006). Following a one-step affinity purification procedure, the SDS-PAGE profile of the affinity-purified sample revealed two protein bands (Figure 1). The upper band was the result of the GST-Strep II-tagged FtsH2 protease and a lower band of ~60 kDa was found to be immunoreactive with an *E. coli* FtsH-antiserum but not with an FtsH2-specific antiserum (Figure 1). N-terminal protein sequencing of this band revealed the sequence SKNNKKXXNA which is identical to the N-terminal region of the FtsH3 homologue of *Synechocystis* 6803 (Cyanobase designation slr1604). Control experiments using a non-tagged wild-type strain confirmed that FtsH did not bind non-specifically to the glutathione resin (data not shown).

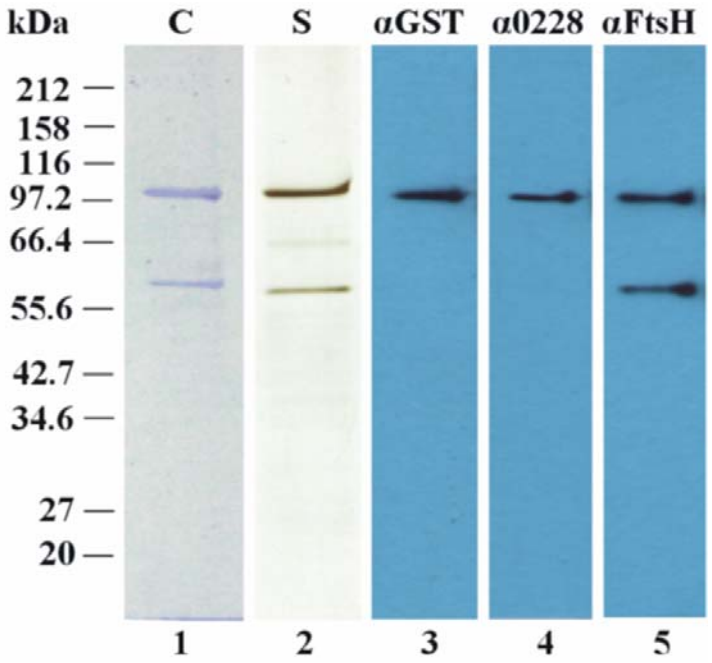

Figure 1. One-step purification of GST-tagged FtsH2. Sample was analysed by SDS-PAGE followed by Coomassie-blue staining (lane 1) or silver staining (lane 2) and by immunoblotting using antibodies specific for GST (lane 3), FtsH2 (lane 4) and *E. coli* FtsH (lane 5).

Electron microscopy and single particle analysis

The oligomeric structure of the FtsH2-GST-Strep II complex was probed by negatively staining samples and imaging by TEM at 50,850x (Figure 2A). A dataset of ~3,400 single particles was built. Reference-free alignment and classification resolved these particles into distinct populations of a uniform median size and treated *de novo*. Iterative multi-reference alignments improved their signal-to-noise ratios and removed poorly correlating particles. No symmetry operators were applied. A sub-population of 2,964 particles contained structures with ~ diameter of 12 nm (Figure 2 b,c). Some of these structures exhibited hexameric-type detail. An overlay of the NSF-D2 AAA domain crystal structure (PDB: 1D2N, (Lenzen et al, 1998)) illustrates that the scale is consistent with the FtsH2-GST-Strep II complexes existing in a hexameric form.

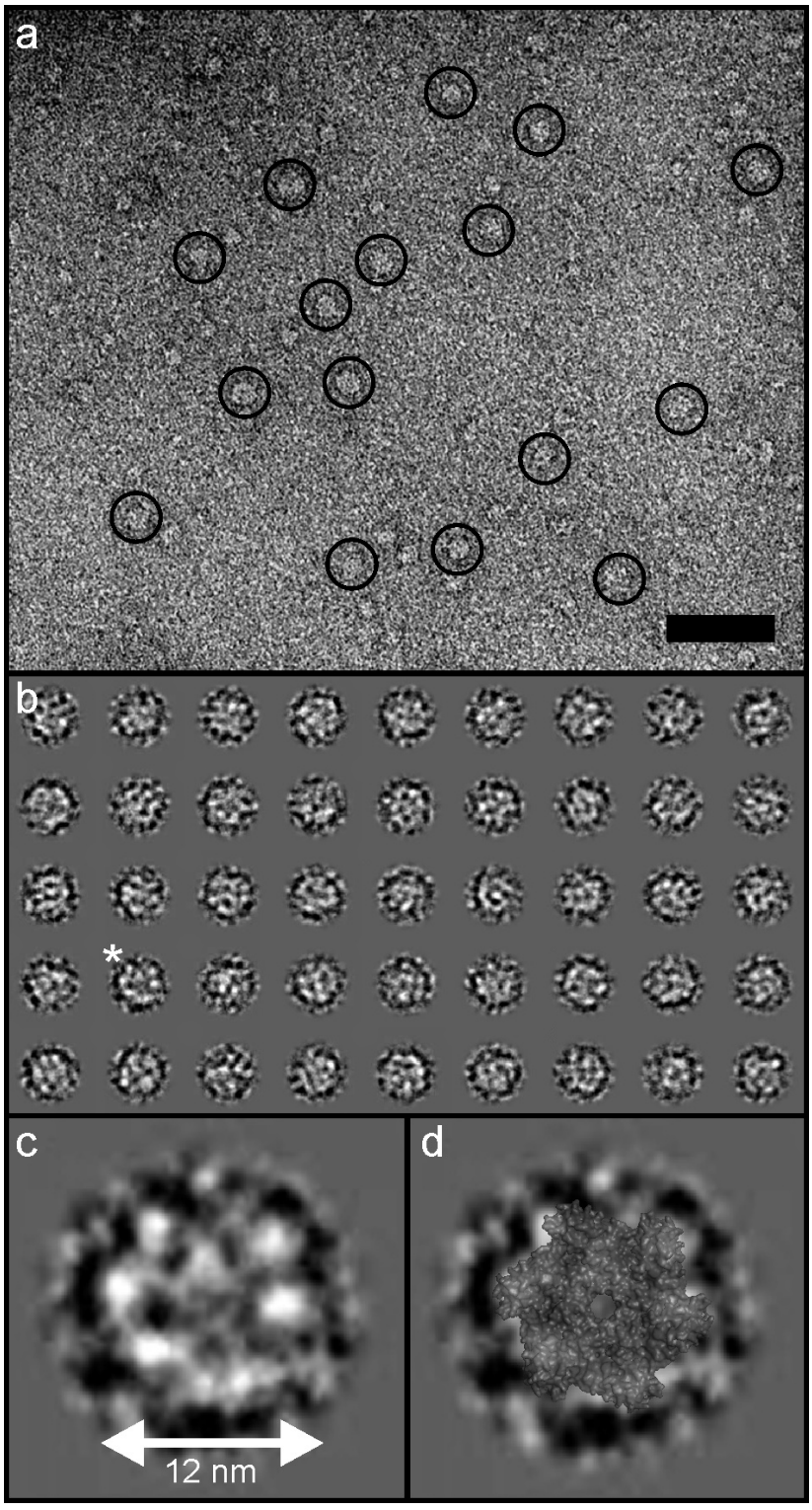

Figure 2. Electron microscopy and single-particle analysis of negatively stained FtsH2-GST-STREP complexes. (a) Typical micrograph region, FtsH particles circled. Black bar represents 50 nm. (b) Single particle 2D projection averages of the FtsH complex. (c) Magnified average, see asterisk in (b). (d) The average shown in (c) plus overlay of the NSF-D2 AAA domain (PDB: 1D2N, (Lenzen et al, 1998)).

DISCUSSION

We describe for the first time the isolation and structural characterisation of a FtsH complex from the thylakoid membrane. Our approach involved the tagging of the FtsH2 protein with a GST-Strep II tag and affinity purification using a glutathione column. Although the monomeric affinity tag is large (~26 kDa) the strain expressing the FtsH2-GST fusion protein restored photoautotrophic growth to the *ftsH2* null mutant at high-light intensities suggesting retention of some if not all activity (data not shown).

FtsH3 (Slr1604) co-purifies with FtsH2-GST-Strep II

We have found that FtsH2-GST-Strep II co-purifies with another member of the FtsH family of proteases, namely FtsH3, probably in the form of a hetero-oligomeric complex. In the case of chloroplasts, genetic and immunoprecipitation experiments have suggested that FtsH forms hetero-oligomeric complexes consisting of two classes of FtsH subunit: an A-type (FtsH1 and FtsH5) and a B-type (FtsH2 and FtsH8) (reviewed by (Adam et al, 2006)). Significantly, sequence comparisons indicate that *Synechocystis* FtsH2 is a B-type subunit whereas FtsH3 is an A-type (Sakamoto, 2003). Interestingly, the FtsH3 subunit, unlike the FtsH2 subunit, appears to be absolutely required for *Synechocystis* 6803 viability (Mann et al, 2000), which would suggest that FtsH3 is not restricted to forming complexes with FtsH2 but might form homocomplexes or heterocomplexes with other members of the FtsH protease family.

Purified FtsH2-GST-Strep/FtsH3 complexes form ring structures

Recent crystal structures of *E. coli*-expressed soluble portions of FtsH have indicated that FtsH forms a hexameric structure of ~12 nm in diameter (Suno et al, 2006). However a high-resolution structure of the intact FtsH complex (including the two transmembrane helices and interconnecting loop) has not been determined, so it remains unproven that FtsH is hexameric

in vivo. Indeed an early analysis of *E. coli* FtsH by electron microscopy indicated a size of only 6-7 nm, much smaller than that for a hexamer (Shotland et al, 1997). In contrast the FtsH complexes isolated here are ~12 nm in diameter and of the appropriate size to accommodate a hexamer (Figure 2). Our results therefore provide important experimental evidence to support the assumption that intact FtsH is indeed hexameric.

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