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On the quaternary association of the type III secretion system $HrcQ_B$-C protein: Experimental evidence differentiates among the various oligomerization models

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ABSTRACT

The $HrcQ_B$ protein from the plant pathogen *Pseudomonas syringae* is a core component of the bacterial type III secretion apparatus. The core consists of nine proteins widely conserved among animal and plant pathogens which also share sequence and structural similarities with proteins from the bacterial flagellum. Previous studies of the carboxy-terminal domain of $HrcQ_B$ ($HrcQ_B$-C) and its flagellar homologue, FliN-C, have revealed extensive sequence and structural homologies, similar subcellular localization, and participation in analogous protein–protein interaction networks. It is not clear however whether the similarities between the two proteins extend to the level of quaternary association which is essential for the formation of higher-order structures within the TTSS. Even though the crystal structure of the FliN is a dimer, more detailed studies support a tetrameric donut-like association. However, both models, dimer and donut-like tetramer, are quite different from the crystallographic elongated dimer of dimers of the $HrcQ_B$-C. To resolve this discrepancy we performed a multidisciplinary investigation of the quaternary association of the $HrcQ_B$-C, including mass-spectrometry, electrophoresis in non-reductive conditions, gel filtration, glutaraldehyde cross-linking and small angle X-ray scattering. Our experiments indicate that stable tetramers of elongated shape are assembled in solution, in agreement with the results of crystallographic studies. Circular dichroism data are consistent with a dimer–dimer interface analogous to the one established in the crystal structure. Finally, molecular dynamics simulations reveal the relative orientation of the dimers forming the tetramers and the possible differences from that of the crystal structure.

1. Introduction

Type III secretion system (TTSS)[1] is a protein traffic device used by several plant and animal pathogenic bacteria for injecting virulence factors directly into the eukaryotic cytosol (Hueck, 1998). It is viewed as a 'molecular syringe' composed by an elongated extracellular, needle-like structure and a cylindrical base which is embedded into the two bacterial membranes and ends in a cytoplasmic extension. Low resolution electron microscopy data (He and Jin, 2003; Kubori et al., 1998; Sekiya et al., 2001; Tamano et al., 2000) showed that TTSSs from different pathogens share a common structure and that TTSS and flagellar hook-basal body complex are quite similar. The most prominent architectural similarities are observed between the TTSS cylindrical base and the flagellar basal body. They are both multiprotein complexes assembled by highly symmetrical substructures each of which is constructed by multiple copies of a protein or of a protein complex. In terms of their amino acid sequences, these proteins are broadly conserved among all known TTSSs and the flagellum and they constitute the so called conserved core (Buttner and Bonas,2002, 2003; Rossier et al., 1999; Tampakaki et al., 2004).

$HrcQ_B$ is a conserved protein from the TTSS of the plant pathogen *Pseudomonas syringae* pv. phaseolicola. Genetic and biochemi-

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[1] *Abbreviations:* TTSS, type III secretion system; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; DTT, 1,4-dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; SAXS, small-angle X-ray scattering; CD, circular dichroism; UV, ultraviolet; rmsd, root mean squared deviation; rms, root mean squared.

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cal experiments have shown (Fadouloglou et al., 2004) that the protein is located at the cytoplasmic site of the inner bacterial membrane, interacts with at least one other protein of TTSS (the $HrcQ_A$ protein) and this interaction is mainly established *via* its highly conserved carboxy-terminal domain. The crystal structure of the conserved C-terminal domain, residues 50–128 (hereafter referred to as $HrcQ_B$-C), has been determined to a resolution of 2.3 Å (Fadouloglou et al., 2004). In the crystals, $HrcQ_B$-C forms an elongated, gently curved homo-tetramer. Two monomers fold together in a symmetrical manner to form a compact and highly intertwined dimeric structure (Fig. 1A). Two dimers are packed together to form a dimer of dimers (Fig. 1B). The flagellar homologue of $HrcQ_B$, FliN protein, has been extensively studied and experimental observations which concern its subcellular localization and protein–protein interactions are fully consistent with properties of $HrcQ_B$ (Francis et al., 1994; Mathews et al., 1998; Tang et al., 1995; Zhao et al., 1996). Thus, both proteins are mainly located in the cytoplasm and they adopt a similar pattern of protein–protein interactions. For example, the $HrcQ_B$ interacts with the $HrcQ_A$ protein and the analogous interaction has also been reported between the flagellar FliN and the, $HrcQ_A$-analogue, FliM protein. Moreover, it has been shown (Zhao et al., 1996) that FliN is a major component of the cytoplasmic extension of the basal body, called the C-ring. In addition, Spa33, the $HrcQ_B$ homologue from *Shigella*, has also shown to be an essential C-ring (Morita-Ishihara et al., 2006) component. Even though there is no direct experimental evidence for the existence of the C-ring in plant pathogens, the extensive similarities between $HrcQ_B$, FliN and Spa33 do suggest that the TTSS of plant pathogens may include a cytoplasmic structure analogous to flagellar C-ring. In that case, it would be difficult to predict the extent of the structural correspondence. However, it seems rational to presume that the $HrcQ_B$ would participate in this ring as a major building block. The hypothesis of functional and structural analogies between $HrcQ_B$ and FliN was strengthened by the crystal structure determination (Brown et al., 2005) of the conserved C-terminal domain of the FliN (hereafter referred to as FliN-C), residues 68–154 (1yab.pdb) or residues 59–154 (1o6a.pdb) which revealed similar tertiary structure with $HrcQ_B$-C (the C_α-rmsd for 138 residues is 2 Å, and 1.1 Å if surface loops are excluded).

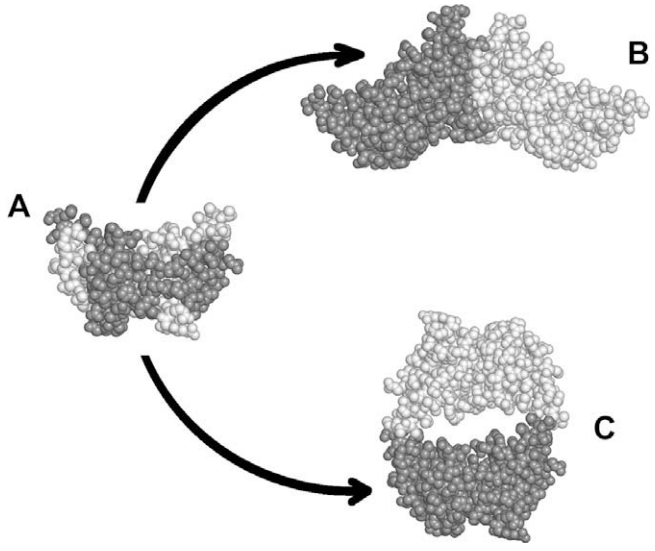

Fig. 1. Possible oligomerization states of the $HrcQ_B$-C protein. (A) The dimer. (B) The elongated tetramer (dimer of dimers) found in the crystals of the protein. (C) A donut-like tetramer formed accordingly to the model proposed for the FliN-C.

Except from the similarities mentioned above the two proteins might have also significant functional differences. Thus, for example, it has been shown that FliN is related with the flagellar motility and that it is closely involved in the process of the direction switching, functions which could not have correspondence to the injectisomes. At the present, it remains unclear whether $HrcQ_B$ and FliN are organized into similar quaternary associations, which is a critical property for the formation of higher-order structures. Given the functional differences, the possibility of important structural differences at the level of quaternary association is particularly valid. The crystal structure of $HrcQ_B$-C revealed a homo-tetrameric dimer of dimers (Fig. 1B) while FliN-C from *Thermotoga maritima* is a dimer, roughly equivalent to a $HrcQ_B$-C dimer (Fig. 1A), both in crystals and in solution (Brown et al., 2005). On the other hand, in analytical ultracentrifugation experiments the FliN protein from *Escherichia coli* behaved as a tetramer with a shape factor indicating an elongated shape (Brown et al., 2005) in agreement with the $HrcQ_B$-C model. However, more recent biochemical/mutagenesis data for the *E. coli* FliN have been interpreted as being consistent with a donut-like homo-tetrameric association (Paul and Blair, 2006). A hypothetical donut-like association for the $HrcQ_B$-C is shown in Fig. 1C.

In the present study, we investigate the solution quaternary association of the $HrcQ_B$-C protein from *P. syringae* pv. phaseolicola and compare our results with existing models for the FliN proteins. For this purpose we used a variety of biochemical, biophysical and computational techniques i.e. SDS–polyacrylamide gel electrophoresis (SDS–PAGE), cross-linking by glutaraldehyde, size-exclusion chromatography, mass spectroscopy, mutagenesis, circular dichroism, small angle X-ray scattering and molecular dynamics simulations. Our results suggest (i) a tetrameric association with the overall dimensions and the elongated shape of the crystallographic $HrcQ_B$-C tetramer and (ii) a probable rearrangement of the dimers with consequences on the interface area.

2. Materials and methods

2.1. Overexpression and purification

$HrcQ_B$-C was overexpressed and purified as described earlier (Fadouloglou et al., 2001). The I41W-G74W mutant of $HrcQ_B$-C was produced by PCR reactions using the appropriate sets of primers according to the method described by Fisher and Pei (1997). The gene of the full length $HrcQ_B$ was initially mutated and the isoleucine residue corresponding to Ile 41 of the $HrcQ_B$-C was changed to Trp in the first PCR reaction. The template in this PCR was the *hrcQ$_B$* gene in the vector pT7-7 and the primers which were used were: upper (mutagenesis primer) 5′-tggCTTGAAGTCACCGG CATTTCGC-3′, lower 5′-AGTCCCGGCATCTAGACGGCGCAGTTCGG-3′. The mutation site is indicated by the lowercase letters. The lower primer contains a XbaI restriction site, which is underlined. The final product from the first PCR was used as the template of a second PCR for the production of the I41W mutant of *hrcQ$_B$*-C. The primers used were: upper 5′-CAGCGCAGGATCCACAGGACG AGCCC-3′, lower 5′-CAAGAAACAGCGCCAGGATCCTCGG-3′. The BamHI fragment (BamHI restriction sites is underlined) was cloned into the vector pPROEX-HTa. For the final construct a third PCR followed. The I41W mutant of *hrcQ$_B$-C* in the pPROEX-HTa vector was used as template. The primers were: upper 5′-CAGATTACC CGCCTGGTGACCCGA-3′, lower 5′-CAGccaCAGGCGACCCTCGACATC CACCAG-3′. The mutation site is indicated by the lowercase letters. The PstI fragment (PstI restriction sites is underlined) was cloned into the vector pPROEX-HTa and then the pPROEX-HTa/I41W-G74W-*hrcQ$_B$-C* plasmid was used to transform DH5 *E. coli* cells. Mutation was confirmed by restriction digestion and sequencing.