Mutilation of RNA phage Qβ virus-like particles: from icosahedrons to rods

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Abstract Icosahedral virus-like particles (VLPs) of RNA phage Qβ are stabilized by four disulfide bonds of cysteine residues 74 and 80 within the loop between β-strands F and G (FG loop) of the monomeric subunits, which determine the five-fold and quasi-six-fold symmetry contacts of the VLPs. In order to reduce the stability of Qβ VLPs, we mutationally converted the amino acid stretch 76-ANGSCD-81 within the FG loop into the 76-VGGVEL-81 sequence. It led to production in *Escherichia coli* cells of aberrant rod-like Qβ VLPs, along with normal icosahedral capsids. The length of the rod-like particles exceeded 4–30 times the diameter of icosahedral Qβ VLPs.

Key words: RNA phage Qβ; Virus-like particle; Self-assembly; Icosahedron; Rod-like structure

1. Introduction

RNA phages of the Leviviridae family belong to the first classical models of molecular biology, which contributed markedly to the discovery of the genetic code, elucidation of RNA translation and replication mechanisms, virus–host interactions, and self-regulation of biological systems. High-level production in *Escherichia coli* of self-assembled VLPs, morphologically and immunologically indistinguishable from virions, has been achieved after expression of CP genes of RNA phage representatives belonging to four known serological groups: MS2 [1,2] and fr [3] of the group I, and JP34 as an intermediate between groups I and II [4], GA of the group II [5], Qβ of the group III [6], and SP of the group IV [7]. Recombinant capsids of the group I RNA phages MS2 and fr were first proposed as putative VLP carriers of foreign amino acid sequences [2,8–11] and/or as delivery tools of encapsidated nucleic acid [12]. Further, the capsids of the group III RNA phage Qβ were suggested as a more promising VLP carrier, due to their ability to form mosaic particles, therefore accepting longer C-terminal extensions [13–15].

The three-dimensional structures of MS2 [16,17], fr [18], GA [19], and Qβ [20] virions and VLPs have been determined at high resolution by X-ray crystallography and found to be very similar, in spite of marked diversity in primary structure of their monomers. In the virions and VLPs, the 180 CP molecules are arranged in dimers as initial building blocks and form an icosahedral lattice with the triangulation number $T = 3$. The RNA phage CP fold differs from the fold of all other viral coats so far known and consists of a five-stranded β-sheet facing the inside of the particle, and a hairpin and two helices on the outside. The most remarkable structural differences between RNA phage capsids were found in a flexible loop between β-strands F and G, the FG loop, which forms five-fold and quasi-six-fold symmetry contacts [20].

The icosahedrons of Qβ virions and VLPs differ from the other RNA phage structures by the presence of stabilizing disulfide bonds linking the monomeric Qβ subunits together in covalent pentamers and hexamers with a stoichiometric ratio 12:20 [21]. High resolution studies showed that initial Qβ dimers are linked covalently to the adjacent dimers by four disulfide bonds between cysteine residues 74 and 80 [20].

With an aim to reduce the extreme stability of RNA phage Qβ VLPs and facilitate their dissociation/re-association, we mutationally changed the cysteine residue 74 within the FG loop, along with four other neighboring aa residues. Surprisingly, these mutations led to the appearance of rod-like Qβ VLPs in addition to icosahedral particles.

2. Materials and methods

2.1. Construction of plasmids

Plasmids are shown in Fig. 1A. The pQβ10 vector [6], encoding the full-length Qβ CP gene located under the control of *E. coli* tryptophan operon promoter, was used as an initial plasmid for all constructions. The pQβ-TAA plasmid harboring a strong UAA terminator of the Qβ CP, instead of the leaky UGA terminator, was created by site-directed mutagenesis. An upstream primer 5′-TAATACGACTCACTATGGGG-3′ and a downstream primer 5′-TTAAGCTTAATACGCTGGGTTCAGCTG-3′ were used for the synthesis of the mutant (TGA→TAA) PCR fragment, which was reintroduced into the pQβ10 expression vector at the unique restriction sites *Xba*I and *Hin*dIII.

The pQβ-FG plasmid was designed by two-step PCR, in order to convert the aa stretch 76-ANGSCD-81 into 76-VGGVEL-81. The 5′-end upstream and 3′-end downstream primers were the same as for the construction of the pQβ-TAA plasmid. The mutations were created by inverse PCR. The inverse primers were designed in inverted tail-to-tail directions: 5′-TCGGTGGTGTCGAGCTCCCATCCGTTACTCGCC-3′ and 5′-AGCTCGACACCACCGACAGTGCATGCGGTCGGG-3′. The products of the first PCR were used as templates for the second PCR reaction, in which the upstream and downstream primers were the same. The product of the second PCR was digested with *Xba*I and *Hin*dIII and cloned into the pQβ10 expression vector, which was cleaved by the same restriction enzymes. All PCR reactions were performed with PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

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Abbreviations: aa, amino acid; CP, coat protein; PAGE, polyacrylamide gel electrophoresis; VLP, virus-like particle; wt, wild-type

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2.2. Expression and purification of Qβ CP

E. coli JM109 cells harboring the appropriate plasmids were grown overnight in M9 medium supplemented with 10 g/l Casamino acids (Difco). *E. coli* lysates were prepared by grinding with alluminium oxide (Alcoa A-305, Serva) in a lysis buffer containing 10 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 2 mg/ml lysozyme. After centrifugation, proteins were precipitated from the supernatant with ammonium sulfate at 50% saturation for 1 h at 4°C. The precipitates were resuspended in a minimal volume of lysis buffer without lysozyme and loaded onto a Sepharose CL-4B (Pharmacia Biotech) column. Fractions containing the appropriate VLPs were combined, concentrated by ammonium sulfate precipitation and stored at −20°C.

2.3. SDS–PAGE and Western blotting

Laemmli's SDS–PAGE, silver staining, and Western blotting were performed according to standard protocols. The SDS–PAGE was run in a slab gel (150×150×0.75 mm) apparatus with a gradient 12–18% running gel and a 4% stacking gel. For Western blotting, the nitrocellulose sheets (0.2 μm, Millipore, Bedford, USA) were incubated with polyclonal anti-Qβ antibodies, which were generated by immunization of rabbits with purified wt Qβ VLPs, in dilution of 1:1000 overnight, followed by incubation with protein A-peroxidase conjugate (1:1000, Sigma) for 1–2 h at room temperature. The reaction was developed with 3,3′-diaminobenzidine.

2.4. Electron microscopy

E. coli cell slices and purified Qβ VLPs were stained in 2% aqueous phosphotungstic acid, or in 2% aqueous uranyl acetate. The grids were examined with a JEM 100C electron microscope operated at 80 kV.

3. Results

3.1. Expression vectors for wt and mutated Qβ VLPs

Starting pQβ10 plasmid ensures high-level synthesis of 133 aa long Qβ CP and 329 aa long A1 protein, which is generated by addition of a 195 aa C-terminal extension in consequence of translational read-through of a leaky UGA stop codon of the Qβ CP (Fig. 1A). The Qβ CP and A1 proteins give rise to mosaic Qβ10 VLPs containing 1–2% of the A1 protein [6]. In order to obtain 'pure' wt Qβ VLPs, named here Qβ-TAA, which do not contain the A1 protein, the leaky UGA stop codon of the Qβ CP was converted into the strong UAA stop codon by site-directed mutagenesis. Construction of the Qβ-FG gene with mutated FG loop pursued an idea to eliminate cysteine residue 80, which is responsible for the inter-dimeric Qβ disulfide bonds. Moreover, we tried to structurally adjust the FG loop of the Qβ CP to corresponding regions of CPs of RNA phages from the serological groups I and II (Fig. 1B). For this reason, the following aa exchanges were accomplished: Ala-76→Val-76, Asn-77→Gly-77, Ser-79→Val-79, Cys-80→Glu-80, Asp-81→Leu-81. It allowed to restore the FG loop structure 76-VGGVELP-82, which is typical for the FG loop of the MS2 CP and homologous to the corresponding regions of the fr and GA CPs (Fig. 1B).

3.2. Formation of wt and mutated Qβ VLPs in vivo

The difference in the intracellular self-assembly of Qβ VLPs encoded by the wt and mutated CP genes was initially revealed by cross-sections of VLP-producing *E. coli* cells (Fig. 2). If the Qβ10 product is seen in such sections as paracrystalline areas of icosahedric 20–25-nm particles (Fig. 2A), the Qβ-FG VLPs are represented by mixture of icosahedral and tubular particles of the same diameter, but different, up to 600 nm, in length (Fig. 2B).

3.3. Structural and immunological properties of wt and mutated Qβ VLPs

The wt Qβ10, and Qβ-TAA VLPs, as well as mutated Qβ-FG VLPs were purified on Sepharose CL-4B columns and examined by SDS–PAGE, Western blot with polyclonal anti-Qβ antibodies (not shown), and electron microscopy (Fig. 2C–F).

The SDS–PAGE and Western blots proved highly efficient

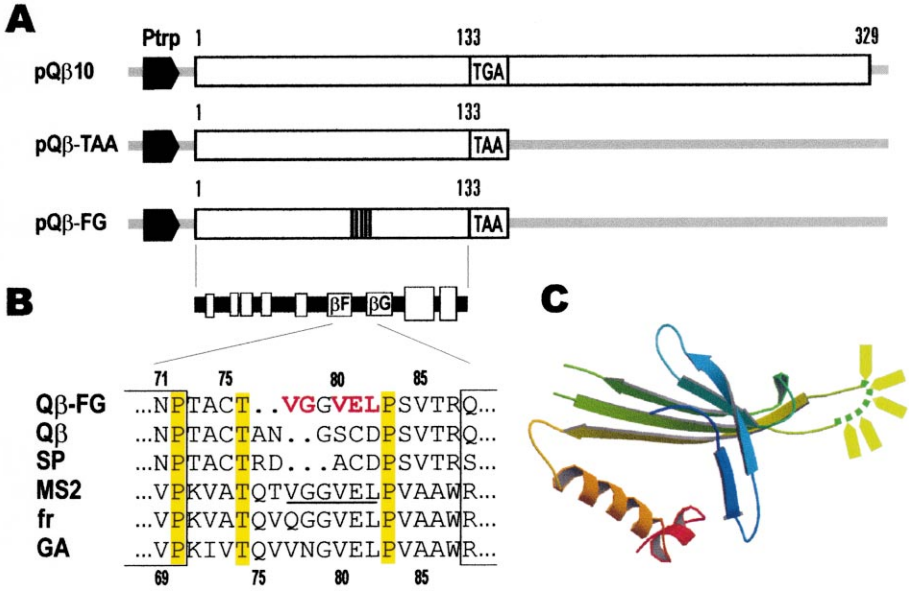

Fig. 1. Construction of Qβ CP genes encoding synthesis of wt and mutated Qβ VLPs. A: Schematic illustration of constructed plasmids. The vertical strips within the Qβ-FG CP gene indicate location of mutated aa residues. B: Location of β-sheets (small boxes) and α-helices (large boxes) on the RNA phage CP and aa alignment of the FG loop of the CPs of the RNA phages belonging to four serological groups with the corresponding region of the mutated Qβ-FG CP. Qβ belongs to group III, SP to group IV, GA to group II, and MS2 and fr to group I. The mutated Qβ CP aa are shown bold red. C: Three-dimensional map of the monomeric form of the Qβ CP [20]. The arrows locate the aa changes within the FG loop, which is shown dashed. The map is a generous gift of Vijay Reddi.

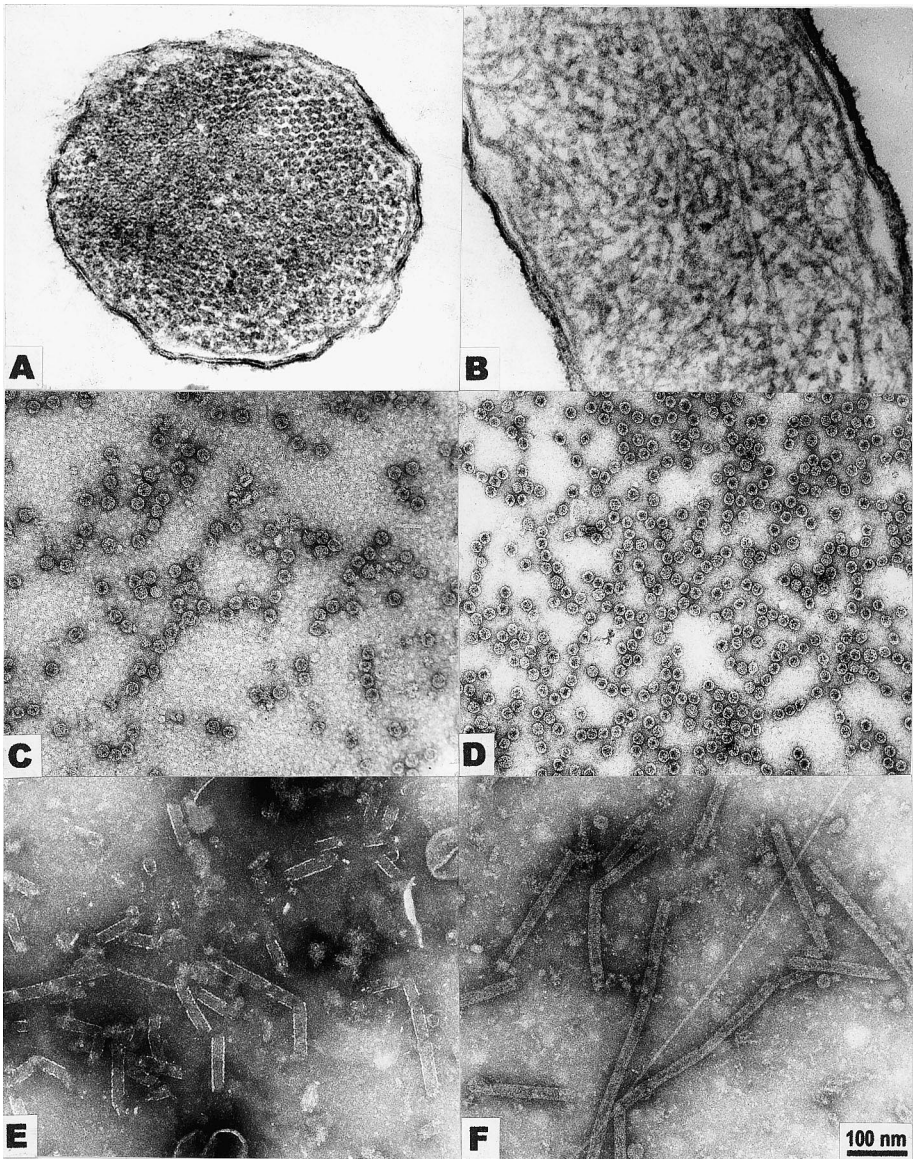

Fig. 2. Electron micrographs of recombinant wt and mutated Qβ VLPs. A, B: Cross-sections of *E. coli* JM109 cells producing Qβ-TAA (A), and Qβ-FG (B) VLPs. C–E: Purified VLPs of Qβ10 (C), Qβ-TAA (D), and Qβ-FG (E). F: Crude preparation of Qβ-FG VLPs in the lysozyme lysates of the bacterial cells.

synthesis of the 14-kDa product in the case of Qβ-TAA and Qβ-FG, which comigrated with the Qβ10 CP as a control. Both Qβ-TAA and Qβ-FG were recognized by polyclonal anti-Qβ antibodies with the same efficiency as the Qβ10 protein (not shown).

As revealed by electron microscopy, the Qβ-TAA CP is able to self-assemble into the VLPs, which are indistinguishable in shape and immunological properties from the Qβ10 VLPs produced by the native full-length Qβ CP gene (Fig. 2C and D), or from the infectious Qβ virions [6].

The purified Qβ-FG VLPs appeared as a mixture of normal icosahedral VLPs, 25 nm in diameter, and tubules of the same diameter, but of different lengths (Fig. 2E and F). It is evident that the longest rod-like VLPs are seen in the cross-sections of freshly grown *E. coli* cells (Fig. 2B) and in the fresh bacterial lysates (Fig. 2F). In the course of purification, the rod-like Qβ-FG VLPs become slightly shorter, but their proportion to the icosahedral VLPs was retained.

4. Discussion

Assembly of viral capsid and coat proteins is a highly regulated process that rarely leads to formation of aberrant particles in vivo. Occasional presence of rod-like particles within icosahedral viral preparations has been described previously for the representatives of the Papovaviridae family, papilloma- and polyomaviruses [22]. Appearance of tubular structures of either 25–30 nm or 50–60 nm in diameter with variable length, in addition to predominant icosahedral VLPs 50–60 nm in diameter, was described also in high-yield baculovirus-driven expression of human papillomavirus 33 capsid proteins L1 and L2 [23]. Interestingly, the L1 molecules are also cross-linked by intermolecular disulfide bonds within the HPV-33 capsid.

Both infectious virions and non-infectious VLPs of Leviviridae RNA phages were characterized as yet exceptional icosahedrons. Infectious virions of RNA phages of serological

groups I and II (*Levivirus* genus) are composed of 180 copies of CP and one copy of A, or maturation, protein, whereas the infectious particles of RNA phages of serological groups III and IV (*Allolevivirus* genus) contain some copies of an additional read-through protein A1. The present investigation clearly shows that not only RNA phage virions, but also homogeneous non-infectious VLPs deprived of any component other than the CP molecules, exist in the form of icosahedral shells.

RNA phage virions and VLPs are formed by 90 very similar CP dimers. The only conformational difference between the dimeric CP subunits is found in a long FG loop responsible for the five-fold and quasi-six-fold contacts of the CP dimers. The interactions around the five-fold and six-fold axes differ markedly for the representatives of different RNA phage serological groups [19,20]. The most evident differences in the FG loop region were found in the Qβ VLPs, where cysteine 74 of each FG loop interacts with cysteine 80 of the adjacent FG loop, forming covalent hubs around the five-fold and quasi-six-fold symmetry axes [20]. In this way, each Qβ CP dimer is linked to the neighboring dimers by four disulfide bonds, making the Qβ capsid extremely stable. The two characteristic cysteine residues involved in disulfide bond formation are present in the CP sequences of all known representatives of the RNA phage serological groups III and IV. Therefore, it seems to be an inherent prerequisite of their correct self-assembly.

A number of MS2 and fr CP mutants with different kinds of insertions, deletions, and substitutions within the FG loop was constructed, many of which failed to self-assemble into VLPs (for review see [24]). The fr CP mutant with deletion of 70-QVQG-73 within the FG loop retained its ability to form VLPs of normal shape and size, but such VLPs had a significantly lower thermal stability than the wt VLPs [24]. The conserved proline 78 residue (corresponding to the proline 82 residue in the Qβ CP) was found to be responsible for the isomerization of the FG loop around the five-fold symmetry axis, thus creating a channel [17,25].

In the mutated Qβ-FG structure, we replaced the intrinsic FG loop region 76-ANGSCD-81 with the 72-VGGVEL-77 motif from the MS2 FG loop, ignoring the obstacle that the MS2 FG loop is two aa residues longer than the Qβ-FG loop (Fig. 1B). The main idea of this approach was to reduce the stability of the Qβ VLPs. Surprisingly, the resulting Qβ-FG VLPs gained the ability to appear in the alternate icosahedral and rod-like forms. Although purification led to remarkable shortening of the rod-like Qβ VLPs, their proportion to the icosahedral VLPs remained stable. Therefore, the FG loop was experimentally approved as being of major importance for the direction of VLP self-assembly. Detailed structural investigations are necessary for tackling the question, which Qβ self-assembly intermediates, pentamers or hexamers, or both, are affected by the FG loop mutation.

Since RNA phage Qβ VLPs attracted remarkable attention as a possible target for introduction of foreign epitopes and packaging of nucleic acids, appearance of their alternate rod-like VLP forms may play an important role for further development of this class of VLP carriers.

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