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Engineered Mutations Change the Structure and Stability of a Virus-Like Particle

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Abstract

The single coat protein (CP) of bacteriophage Q β self assembles into T=3 icosahedral virus-like particles (VLPs), of interest for a wide range of applications. These VLPs are very stable, but identification of the specific molecular determinants of this stability is To investigate these determinants along with manipulations that confer more capabilities to our VLP material, we manipulated the CP primary structure to test the importance of various stabilizing interactions. Optimization of a procedure to incorporate fused CP subunits allowed good control over the average number of covalent dimers in each VLP. We confirmed that the disulfide linkages are the most important stabilizing elements for the capsid and that acidic conditions significantly enhance the resistance of VLPs to thermal degradation. Inter-dimer interactions were found to be less important for VLP assembly than intra-dimer interactions. Finally, a single point mutation in the CP resulted in a population of smaller VLPs in three structural forms.

Keywords

Virus-like particles; mutation; virus stability; self-assembly

Introduction

Virus-like particles (VLPs) have received increasing attention for their applications in chemistry,^{1–3} materials science,^{4–6} and medicine.^{7–9} They can be highly stable toward extremes of temperature,^{10,11} pH, and variations in solvent composition,¹² and thus are friendly toward chemical modification, while retaining many advantageous characteristics of biological molecules. VLPs have well-controlled composition and structure,¹³ are biocompatible,^{14,15} and can be mutated^{16–18} and/or evolved.^{19,20} Among the most commonly employed VLPs have been the coat proteins (CPs) of MS2, Q β , and PP7, all RNA phages of the *Leviviridae* family. Their three-dimensional structures are very similar in spite of substantial diversity in their protein sequences^{21–23} (see

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Supporting Information. Primer sequences used, and additional discussion of Ellman assay of thiol reactivity. This material is available free of charge via the Internet at <http://pubs.acs.org>.

<http://viperdb.scripps.edu>).²⁴ Expression in *E. coli*^{25,26} or yeast²⁷ results in VLPs assembled from 180 copies of these CPs in high yields. The genetic malleability of *Leviviridae* scaffolds has been demonstrated with exterior display of peptides and proteins,^{28,29} point mutations to add chemical reactivity to specific sites.^{18,30} Many mutations have also been made in MS2 in studies of the mechanisms of natural processes such as RNA binding^{31,32} and assembly.^{33,34}

Here we report a set of rationally designed genetic mutations of the Q β capsid protein that have differential effects on assembly and stability of the resulting VLPs. The X-ray crystal structure of the capsid²¹ is shown in Figure 1; its single small CP (132 amino acids) forms an interdigitated dimer with α -helical domains over adjacent β -sheet regions. In this effort, as in so much else, we build on the studies of Stockley and coworkers, who found modest variations in thermal stabilities for variants of the MS2 VLP.³⁵ The results of the present work identify certain important determinants for assembly and stability which can be exploited to tailor VLPs to specific applications.

Experimental Section

A. Cloning

Point mutations in the coat protein gene were made with overlap extension PCR. For each mutation (X), PCR reactions were performed with primer CP-X-R1 and Primer #1 to amplify the 5' half of the CP gene and a second PCR using primers CP-X-F1 and Primer #2 to amplify the 3' half of the CP gene. (For primer sequences, see Supporting Information, Table S1). PCR products were run on agarose gels to verify size, extracted from the gel and purified. The purified DNA from both reactions were diluted and used as the template for the next PCR reaction with Primers #1 and #2. Exceptions were CP-Y132W-R1 and CP-L128W-R1, which were separately amplified with Primer #1, and CP-L8W-F1, CP-A1S-F1, CP-K2E-F1, and CP-K2Q-F1, which were amplified with Primer #2. The products were purified as above, cut with Nco1/Xho1 restriction endonucleases and ligated onto the similarly cut pCDF or pET28HpR vector. PCR products ligated into pET28HpR were transcribed by T7 RNA polymerase with the RNA sequence coding for the anti-Rev aptamer upstream of the ribosome binding site and start codon and the natural Q β hp downstream of the stop codon.³⁶

The fused dimer was made by silently mutating the endogenous Nde1 (CATAG) cut site in the CP gene sequence using PCR with primers #13 and #14. This mutant CP was then ligated into pCDF as above. Whole-plasmid PCR with primers #16 and #17, followed by blunt end ligation was used to remove the stop codon and insert a Nde1 restriction site at the 3' of the CP gene to make pCDF-CP. Another PCR reaction using WT or any mutant CP gene as the template and primers #15 and #2 was then performed. Purification, digestion with Nde1/Xho1 and ligation into this similarly cut pCDF-CP vector created pCDF-CP₂ or pCDF-CP-(Mutation X). This vector encodes tandem copies of the CP gene or the CP gene and a mutant CP gene with a 5 amino acid spacer (AYGGM).

Ligation products were transformed into DH5 α cells (BioPioneer) and plated on selective media (streptomycin for pCDF and kanamycin for pET28HpR). Colonies were picked and cultured overnight in 3 mL SOB (Amresco) selective media followed by plasmid DNA purification.

B. Expression

DNA sequence-verified plasmids were used for transformation into BL21(DE3) (Novagen) or T7 Express (NEB) cells for expression. For co-expressions, compatible plasmids (pET28 and pCDF, Novagen) were maintained under double selection. VLPs were expressed and

purified as previously described.³⁶ All VLPs were assayed for purity and uniformity by size-exclusion chromatography (SEC), dynamic light scattering (DLS), and transmission electron microscopy (TEM). The protein content of each sample was analyzed with a Bioanalyzer 2100 Protein 80 microfluidics chip (Agilent). The average number of CP and CP₂ gene products per particle was determined from the ratio of the integration of the Bioanalyzer electrophoretic peaks corresponding to the proteins, correcting for their relative masses and multiplying by 180. Total protein concentration was measured using the Coomassie Plus Protein Reagent (Pierce) with bovine serum albumin as standard.

C. Cysteine reactions

WT, C74S and C74S/C80S mutated particles (70 μ M in total protein) were reduced with DTT (3 mM) or tris(2-carboxyethyl)phosphine (TCEP, 10 mM) for 1 hour in deoxygenated 0.1 M phosphate buffer (pH 7.0). The reducing agent was removed by exchanging with buffer using 100,000 MWCO centrifugal filters (Millipore). The concentration of accessible protein thiol groups was assayed by Ellman's reagent against an *N*-acetylcysteine standard curve.³⁷ *N*-ethylmaleimide (10x excess) was incubated with pre-reduced VLPs overnight at room temperature. Attachment was verified with MALDI mass spectrometry.

D. Labeling with OND electrophile

Various Q β mutants were reduced and purified as described above. A solution of Q β (approx. 1 mg/mL = 70 μ M in protein; 100 μ L) in deoxygenated 0.1 M pH 7.0 phosphate buffer was treated with dihomopropargyl 1-dansylamino-methyl-7-oxanorbornadiene-2,3-dicarboxylate,³⁸ **1** (3 mM, 3 μ L), and the fluorescence increase was monitored on Varioskan Flash plate reader (λ_{ex} 332 nm, λ_{em} 550 nm). Fluorescence emission intensities were corrected for differences in particle concentration and normalized to 100% for the most extensive reaction (with rC74S) before plotting in Figure 6.

E. UV-vis measurements of aggregation

VLPs (0.1 mg/mL = 7 μ M protein = 0.4 μ M particle) in PBS (pH 7.1) or MES (pH 5.5 and 6.5) buffers were interrogated with a Varian Cary 1 Bio UV-visible spectrometer in 1 cm quartz cuvettes. Oil was layered on top of the solution to minimize evaporation and the samples were heated from 25°C to 90°C at a rate of 1°C/min. The solution absorbance at 310 nm and 350 nm was measured every 0.5°C. These wavelengths were chosen because the non-heated samples were transparent at these values, meaning that absorption caused by aggregation upon heating could be readily detected. Cloudy precipitates were clearly visible by eye at the end of each experiment. Apparent T_m values were calculated by plotting the optical density (OD) against temperature (T) and fitting the curve to equation (1) where A_1 is the initial OD, A_2 is the final OD, and dx is the time constant. Triplicate runs were independently analyzed and the reported data are the average values $\pm$ standard deviations.

$$OD = A_2 + \frac{(A_1 - A_2)}{1 + \exp((T - T_m)/dx)} \quad (1)$$

F. Other spectroscopy

Mutant VLPs were assayed at roughly equivalent concentrations with a Dynapro dynamic light scattering plate reader (Wyatt Technologies, Santa Barbara, CA) in a 384 well plate with 10% laser power and 30% attenuation with light mineral oil layered on top of the solution to minimize evaporation. The instrument was set to the indicated temperature and the radius was recorded at regular intervals up to 80 minutes. The radius data was plotted vs

time Q β VLPs have a radius of 14.5 nm). Circular dichroism was performed as described in 39.

G. Electron microscopy

Samples were prepared for CryoEM through preservation in vitreous ice via rapid-freeze plunging (FEI Vitrobot) onto plasma cleaned C-flat grids. Electron micrographs were acquired using a Tecnai F20 Twin transmission electron microscope operating at 120 kV using a dose of $\sim 12 \text{ e}^-/\text{\AA}^2$ and a nominal underfocus ranging from 1.5 to 3 μm . For wild type Q β , 195 images were automatically collected at a nominal magnification of 80,000X, corresponding to a pixel size of 0.0975 nm at the specimen level. For mini-Q β (L35W), 1,177 images were at 50,000X, representing a pixel size of 0.164 nm. All images were recorded with a Tietz F415 4k $\times$ 4k pixel CCD camera (15 μm pixel) utilizing the Leginon data collection software.⁴⁰ Experimental data were processed by the Appion software package,⁴¹ which interfaces with the Leginon database infrastructure. The contrast transfer function (CTF) for each micrograph was estimated using the ACE package.⁴² For Q β , two templates created from 500 manually selected and centered particles were used to automatically pick 13,408 particles from the micrographs using a template-based particle picker.⁴³ The particles were extracted at a box size of 400 $\times$ 400 pixels. For mini-Q β , eight templates, created using images of the sample preserved in negative stain (data not shown), were used pick 29,892 particles. The particles were extracted at a box size of 256 $\times$ 256 pixels. CTF correction was achieved by phase correction of individual particles. After pruning the stack to eliminate obvious junk and contamination, the final Q β stack contained 11,671 particles, which were then aligned and separated into 32 classes using Xmipp clustering 2D alignment.⁴⁴ The final mini-Q β stack contained 29,445 particles, which were aligned and separated into 64, 32, 16, and 8 classes.

Results

The short form of the Q β coat protein (amino acids 1–132), lacking the natural A1 extension, was used to make standard VLPs designated here as wild type (WT). Monomers of all the *Leviviridae* RNA phages form tightly-associated noncovalent dimers.^{34,45} Genetically fusing the two monomers together has been done previously with PP7⁴⁶ and MS2⁴⁷ to facilitate incorporation of larger domains into a loop region or the terminus.^{34,48} We fused two Q β CP monomers with a 5 amino acid spacer (AYGGM) to form a construct designated CP₂. *E. coli* expression of CP₂ gave VLPs that were structurally indistinguishable from wild-type particles by SEC, DLS, and TEM (data not shown).

A novel capsid was created by co-expression of the CP₂ gene with the wild-type CP gene on separate plasmids. The two resulting proteins self-assembled to form “hybrid” particles both forms of the CP₂₉ in ratios matching the relative expression levels in the *E. coli* cell. This is consistent with the expectation that the noncovalent and fused dimers behave similarly in the capsid assembly process. The ratio of CP to CP₂ was varied by changing the expression of the gene sequence of one of these proteins. This was done by appending the sequence coding the Q β RNA packaging hairpin (hp, known as TR for MS2) to the 3' end and an aptamer (coding for Rev-binding element, designated “R”) to the 5' end of the transcribed mRNA, as was previously performed to package proteins.^{36,49} When the CP gene sequence was flanked by these functional RNA motifs (CPHpR), its protein product was expressed less abundantly than the coexpressed CP₂ protein product, and this difference was reflected in the composition of the resulting assembled particles. The reverse construction, involving coexpression of CP and CP₂HpR genes, had the expected effect, diminishing the amount of CP₂ expressed and thereby incorporated relative to CP (Figure 2).

The stability of many of the capsids against thermal decomposition was assessed by monitoring the optical density of the solution by UV absorption spectroscopy as a function of temperature. Disruption of the capsid structure is accompanied by aggregation and precipitation the coat protein. As shown in Figure 3, an apparent melting temperature (T_m) was defined as the temperature at which 50% of the aggregation-related absorbance (310 nm) was reached. The correlation of the aggregation phenomenon with capsid protein unfolding was established by circular dichroism (CD) spectroscopy³⁹ of WT, CP₂ and hybrid particles, showing loss of secondary structure at transition temperatures very similar to those found by UV absorption. Disassembly and aggregation have also been shown by electrophoretic methods to be concurrent processes for VLPs derived from MS2 coat protein.¹¹ Aggregation, precipitation and loss of secondary structure was irreversible under these conditions: cooling did not allow for refolding the protein and re-establishment of the capsid structure.

Aggregation of the VLPs could also be monitored by DLS. In this assay, the temperature was held constant and the radius of the species in solution was monitored over time (Figure 4). The rapid increase in size is assumed to indicate the initiation of aggregation, which ultimately leads to protein precipitation. Although the DLS and UV-absorption aggregation methods correlated well, the latter was more convenient and robust and so was used for subsequent experiments.

A variety of point mutants were made to test the importance of interactions identified in examination of the X-ray crystal structure of Q β .²¹ In part, these studies were intended to give us a fuller picture of the relationship between the static image derived from crystallography and the behavior of the VLP as a dynamic entity in solution. Our guiding hypothesis is that an important mechanism of VLP decomposition is the exposure of hydrophobic patches, usually buried in interfaces between subunits, which both destabilizes the capsid and allows subunits to aggregate in an irreversible manner.³⁴ Thus, anything that assists in the exposure of buried hydrophobic residues, such as disruption of favorable inter-subunit interactions (disulfide linkages, salt bridges, hydrogen bonds, *etc.*), can contribute to this process by making it easier to separate or unfold subunits.⁵⁰

Mutants containing single amino acid substitutions at ten different positions (L8, L19, L35, C80, R86, F94, F96, E104, E111, and L128) did not provide intact VLPs in spite of robust expression in *E. coli*. (Table 1). Several of these mutants were also tested in a less stringent by making the mutation in only one of the two subunits in the CP₂ gene, but these structures also failed to provide any purified VLPs. The designation of “assembly-defective” denotes only that robust expression of each modified coat protein was observed (by electrophoretic analysis of cell lysates) without isolation of any particles. It is possible that these systems were defective not in particle assembly (quaternary structure), but rather in the achievement of the proper secondary or tertiary structure of capsid subunits, but we regard this as less likely for single-point mutations. experiments were performed to test these competing hypotheses.

Bacteriophages in the *Leviviridae* family package their genomic RNA by virtue of a strong interaction of the capsid protein with the cognate packaging hairpin (hp) stem-loop sequence.⁵¹ In our hands VLPs also encapsidate other cellular RNA in both the absence and presence of this packaging sequence.⁵² In order to probe the role of cognate and non-cognate RNA in assembly and stability, we expressed some of the mutants in the presence and absence of the natural Q β hp (Tables 1 and 3). It was anticipated that hp could add stabilizing interactions to overcome the destabilizing effect of the mutant, but none of the assembly-defective mutations yielded VLPs even in the presence of co-transcribed hp RNA (Table 1).

Mutations at nine positions (A1, P23, Q65, Y62 D81, D91, Y99, V108 and Y132) did form VLPs identical in size and shape to wild-type particles. Some positions (2, 16/18, 35, 62 and 74) gave varied results depending on the substitution: K2Q, K13Q/K16Q, L35F, L35W, Y62F, Y62R, Y62W, and C74S all yielded intact particles, whereas K2E, K13E/K16E, L35G, L35H, L35A, L35V, Y62E and C74H did not. Figure 5 shows the placement of these positions on the structure, and Table 2 lists the yields of the mutant VLPs, which ranged from poor to excellent. These results are discussed below in terms of the putative stabilizing interaction targeted by the mutation.

Disulfide bonds

An obvious difference between Q β and other bacteriophages of the *Leviviridae* family are the presence of inter-subunit disulfide linkages in the FG loop at the five-fold and quasi-six-fold symmetry axes (Figure 5b). To test the importance of the FG loop disulfide bond, we removed it with a C74S single mutation and a C74S/C80S double mutation. The single C74S mutation contained 180 free thiols from the position 80 cysteine and required storage in the presence of reducing agent (1 mM dithiothreitol (DTT)) to prevent aggregation and precipitation. Both particles were obtained in good yields and displayed T_m values approximately 20°C lower than the WT particle (Table 2, entries 2, 3, 8–13), confirming the substantial impact these disulfide bonds have on capsid stability. This same level of destabilization was observed when the disulfide bonds were disrupted with DTT, which had no further effect on mutant VLPs already missing the C74–C80 disulfide linkage (see Table 4 and associated discussion).

The Cys80 free thiol of the C74S mutant was unreactive with maleimides or disulfides (Ellman's reagent) until it was pre-reduced by treatment with DTT or TCEP. After reduction, at least 90% of the thiol sites (162 per particle) were chemically accessible. The disulfide bonds of WT VLPs could also be reduced to the free thiol and reacted with maleimides and Ellman's reagent. The number of accessible thiols was found to be approximately 280 per particle, representing 78% of the total. Before reduction, the wild-type VLP was also unreactive with *N*-ethylmaleimide or Ellman's reagent on the same timescale.

Additional insights into cysteine reactivity were provided by the fluorogenic oxanorbornadiene (OND) reagent **1**^{38,53} (Figure 6). In this case, the reduced C74S particle proved to be significantly more reactive than reduced WT (rWT). This may be due to two factors: the sterically demanding character of the OND electrophile, which would make it difficult for the reagent to access all of the closely-spaced thiols of rWT, and the potential for adjacent rWT thiols to re-form disulfide bonds in competition with OND trapping (Supporting Information). The double cysteine knockout mutant C74S/C80S showed no reaction with **1** even after reductive treatment, consistent with the much greater reactivity of **1** with thiols vs. amines under these conditions.

Inter-dimer interactions

The hydroxyl of Tyr62 in the F-strand forms an inter-dimer hydrogen bond with the guanidine group of Arg47 on the E strand of an adjacent subunit (Figure 5b, 7a). This H-bond was shown to be relatively unimportant to particle stability, since its removal (Y62F) had no effect on the T_m of the VLP (Table 2, entry 4). Two other mutations at position 62 (Y62R, Y62W) did form particles, but the T_m values were significantly reduced compared to WT (entries 14, 15). Surprisingly, the Y62E mutant was found to be assembly defective, suggesting that the 62 position will not accept an alternative H-bond partner or salt bridge, or that protein folding and/or particle assembly was somehow compromised by the installation of a negatively charged side chain at this position.

The structure of Q β also shows another inter-dimer interaction between Arg86 in the G strand and Asp81 on the FG loop (Figure 5b,7b). This salt bridge is also apparently not necessary for particle stability, as the D81N mutant forms VLPs in normal yields with only a slight decrease in T_m value (Table 2, entry 5).

Intra-dimer interactions

Prominent sidechain-mediated intra-dimer contacts in Q β include a salt bridge at E104-R86 and a charge-dipole interaction between E111 and N70 (Figure 5a,7c). Mutation of E104 and E111 separately to isosteric glutamine removed the anionic charge, but potentially allowed H-bonding interactions to remain. Both mutant CPs, expressed at normal levels but did not assemble into VLPs. This is in agreement with other studies on related phages,^{16,20,54} but is in contrast with one report.³⁵

Hydrophobic interactions

Two α -helices lay on top of the FG β -sheet strands of the continuous β -sheet structure of the *Leviviridae* capsid dimers. The interior area of this tertiary structure is quite hydrophobic, with F94 and F96 on the G-strand providing prominent side (Figure 5a, 7d). These residues proved to be important in particle assembly and stability, as F94H, F94S, F94W, F96H, and F96W mutants were all assembly defective. Pairing the F94H change with V108I to increase the bulk of a contacting sidechain at position 108 did not rescue assembly, although V108I alone formed WT-like VLPs.

Since Q β has no tryptophans in its primary sequence, we wished to insert Trp residues in order to provide for more convenient characterization of the resulting particle by increasing absorbance at 280 nm and building in a structure-dependent optical signal. As seen in Table 1, no VLPs were recovered when Trp was substituted for residue L8, L19, or L128, perhaps unsurprising given the large chemical and steric differences between Leu and Trp and the positioning of L8 and L19 into a hydrophobic pocket. The MS2 structure contains a Trp residue in the position corresponding to residue Arg86 of Q β . However, we also found the R86W to be assembly defective, further highlighting the sensitivity of capsid structural programming to the introduction or removal of hydrophobic residues.

The more structurally conservative replacement of tyrosine by tryptophan was tolerated at three positions (Y62, Y99, Y132); all three particles exhibited yields comparable to WT VLPs, but Y62W and Y99W were slightly less stable than WT (Table 2, entries 15,16). The last residue of the short coat protein (Y132), found on the external surface of the capsid, tolerated the Trp replacement, and displayed no difference in stability compared to WT. This was somewhat unexpected, since the C-terminal tyrosine residue is conserved among the *Leviviridae* and is implicated in VLP assembly for related phages.⁵⁴

Mini-Q β

The MS2 bacteriophage also has a tryptophan in the position structurally equivalent to Leu 35 of Q β . Replacement of this residue with Trp gave VLPs in high yield that were significantly smaller than the WT particles, designated “mini-Q β ”. The difference in size was verified by significant differences in ultracentrifugation sedimentation through sucrose gradients and elution volume on size-exclusion chromatography (Figure 8a), indicative of lower density smaller diameter particles, respectively. Dynamic light scattering showed L35W VLPs to have average diameter of approximately 21 nm compared to 28 nm for WT (Figure 8b), which was confirmed by transmission electron microscopy (Figure 8c). The L35F mutation also displayed mini-Q β phenotype, but mutation of this residue to amino acids bearing smaller aliphatic sidechains (G,A,V) did not yield any intact particles.

A close examination of the TEM data revealed significant heterogeneity in the sizes and of particles in both the L35W and L35F samples. L35W particles formed large crystals under typical crystallization conditions, but these did not diffract X-rays to an appreciable degree, presumably because of this heterogeneity. Cryo-electron microscopy proved to be the most informative structural technique. Images of approximately 30,000 individual L35W particles segregated by size and shape using Leginon software,⁴⁰ revealing three structural classes. Half of the VLPs form the smallest class of particles having a hexagonal two-dimensional projection an average radius of 16.5 nm (Figure 9a). The second class of particles, representing about 40% the population, had a similar shape but a larger size, with an average radius of 20 nm (Figure 9b). The third class of particles were elongated (23.5 × 20 nm), comprising approximately 10% of the total population (Figure 9c).

RNA interacting residues

The residues Q65 and D91 have been reported to be important for directing specific binding of the Q β CP to the genomic packaging hairpin (hp); mutations Q65H and D91N increase the affinity of the CP-hp interaction.³¹ Q β VLPs comprised of these mutant CPs were expressed and isolated in good yields. The two single-point mutants and the double mutant each exhibited thermal stabilities slightly lower than WT VLPs (Table 2, entries 6–8). Little change was observed when these mutants were co-expressed with RNA containing the hp sequence: the D91N mutant showed a slight increase in stability ($T_m = 83.6^\circ\text{C}$ vs. 79.4°C), the Q65H/D91N double mutant a slight decrease ($T_m = 79.5^\circ\text{C}$ vs. 81.6°C), and Q65H did not appreciably change (79.5°C vs. 78.6°C). Several other mutants discussed here were also expressed with the cognate packaging hp, but no differences were observed in the stabilities of these particles relative to their hp-free analogues (Table 3).

Chemical determinants of stability

To assay the tolerance of VLPs toward other conditions relevant to their manipulation and potential use, we measured the thermal stability of WT and C74S particles in the presence of increasing concentrations of dimethyl sulfoxide (DMSO) (Table 4). Concentrations of DMSO greater than 10% had a significant deleterious effect on thermal stability. At 40% or more DMSO, the simple UV-absorbance assay could not be used, as the samples did not exhibit an increase in 310 nm absorbance with heating, suggesting that the unfolded protein did not aggregate under these conditions.

We also assayed the stability of WT and some mutants in a reducing environment and at different pH values (Table 5). In the presence of reducing agent, WT particles lost resistance to heating to the same degree as the C74S or C74S/C80S particles in neutral buffer. Surprisingly, the stability of all of the particles tested increased as pH was lowered, even by a modest amount to 6.5.

Discussion

The tolerance of the Q β capsid protein to changes in sequence is an important parameter to understand for its adaptation to different environments and target functions. We describe above the results of a series of experiments designed to probe interactions thought to contribute to the extraordinarily stability of this non-thermophilic capsid.

While many reports exist that describe random or rational mutation in the coat protein of related coliphages,^{10,16,20,31,32,34,35,54–58} most focus on the ability of the mutant CP to dimerize and bind RNA;^{31,32,55–58} quaternary assembly is usually considered separately or not at all. Here we examine the outcome from a product-oriented point of view, measuring the stabilities of VLPs assembled from mutant Q β CPs without determining if assembly-

defective mutants undergo CP dimerization. We find significantly larger changes in thermal stability for Q β mutants than were observed for MS2 VLPs,³⁵ but we start with a particle stabilized by disulfide crosslinks that account for most of that extra stability.

The fusion of capsid proteins into covalent dimers allowed for mutational changes to be propagated over half the number of sites (90 dimers per particle) as the WT particle (180 monomers per particle).⁴⁷ We expect that this will be useful when investigators wish to attach functional molecules to the VLP with greater spacing. We controlled the relative amounts of covalent dimer (CP₂) and monomer (CP) subunits within each VLP by appending the packaging hp to the transcribed mRNA coding for either of these proteins. The mRNA bearing the hp produced less protein, perhaps due to the ability of the hp to direct the mRNA into the assembling VLP, removing it from the pool of translatable RNA during expression. The ratio of CP to CP₂ incorporated in the VLPs was found to correspond directly to their cellular concentrations, consistent with the idea that noncovalent dimers form very early in the assembly process as the true capsid building blocks,¹⁰ and that the covalent dimer has the same structure and self-assembling properties as its natural noncovalent counterpart.

However, the inclusion of CP₂ components was found to weaken the Q β VLP in direct proportion to their number, with T_m decreasing from 85°C for the wild-type particle to 74°C for particle composed of 90 CP₂ subunits (Figure 2). A similar genetic fusion was previously described with the related bacteriophage MS2 with a two-residue linker (AM) between the CPs.³² This construct folded correctly and bound to its RNA genome packaging signal, but was for VLP assembly. The authors suggested that the additional two amino acids may have with inter-dimer contacts at the quasi-3-fold axis. A mutant designed to fix this problem the first two residues of the second CP gene) allowed quaternary assembly of the covalent dimer, with the resulting VLP being more stable to urea-induced unfolding than the wild-type form,³² slightly less stable to heating.¹¹ A similar effect was also reported for capsids derived from the coat protein and its fused dimer.⁴⁶ Although the Q β VLP assembly mechanism is able to tolerate our five-residue linker, the extra bulk may impose some cost on inter-dimer interactions.

The results reported here from point mutations in the CP sequence suggest that the particle assembly process is more sensitive to changes in intra-dimer interactions (that is, to the structure and conformation of the key dimeric building block) than it is to inter-dimer interactions (contacts between dimeric building blocks). For example, most of the mutations made to the central hydrophobic core of the dimer, designed to disrupt intra-dimer interactions among these interior residues, gave rise to protein that did not form VLPs. We believe that achievement of the conserved folded dimer structure is compromised in these cases, as has been reported for other phages.^{16,20}

The removal of inter-dimer contacts was better tolerated. Many mutations described in the literature that disrupt inter-dimer interactions are unable to form VLPs,^{20,50} but most changes made at Y62 and D81 (to disrupt inter-dimer H-bonding or salt bridge interactions) were able to assemble correctly. Note, however, that the relatively low stability of the Y62R mutant particle (loss of approximately 15°C in thermal tolerance; Table 2, entry 14) and the failure of the Y62E mutant CP to form VLPs suggests that charges in this region are not well tolerated.

The greatest change in VLP stability (T_m diminished by 20°C) was induced by elimination of inter-dimer disulfide bonds by mutation or reduction. A similar observation was made for the related PP7 VLP upon reduction of its crosslinking disulfide bonds,⁴⁶ and MS2, which lacks such disulfide crosslinks, decomposes at a temperature roughly 15 degrees lower than

Q β .¹⁰ However, the C74S Q β VLP still represents a robust structure, which is addressable by thiol-reactive electrophiles only after pre-reduction. This suggests that the reactive C80 sidechain is isolated as mixed disulfide with cellular thiols or that it is oxidized to the sulfenic acid.⁵⁹

Some evidence exists that capsid protein association with packaged RNA confers additional stability to the particle against urea-mediated denaturation.¹⁶ Accordingly, we hypothesized that strengthening the Q β CP-RNA interaction further might stabilize the VLP to thermal denaturation, but this was not observed: the D91N and Q65H particles, containing mutations known to increase the affinity of CP for internally packaged RNA, exhibited no enhancement against denaturation and aggregation upon heating (Table 4).

Structural plasticity in VLPs derived from *Leviviridae* coat proteins is well known, including the formation of rod-shaped particles from a Q β loop mutant,⁶⁰ rods from mixtures of coat from bacteriophages *φ*r and GA,⁶¹ small spherical particles in low yield from a *φ*r point mutant (I104T),⁵⁴ and small amounts of T=1 particles observed for mutants of the MS2 coat protein.^{35,62} These structures all bear mutations in the extended FG loop that clusters at the five-fold and quasi-six-fold axes.⁶³ However, residue 35, the position that keys the formation of a discrete set of smaller (mini-Q β) particles in high yield, is not near the FG loop. A closer analogy might be to smaller variants of the cowpea chlorotic mottle virus (CCMV) capsid, formed upon N-terminal peptide truncation.^{64,65} Detailed structural analysis showed that truncation changed interdimer subunit angle, leading to aberrant particle assembly. Residue 35 of Q β is on the D-strand at the end of the 5-strand β -sheet that faces the interior of the VLP (Figure 5a). The natural Leu at this position occupies a hydrophobic pocket defined by the A–B strands and the α -helix of the intra-dimer CP. A tryptophan at this position may be too large for this pocket, forcing the D-strand “down” relative to the rest of the monomer and changing the way that associate with one another. Proline 23, located on the α -helix directly above residue 35, is one of the few residues conserved among most of the leviviruses (excluding PP7 and AP205).⁶⁶ Replacing this residue with alanine (P23A) gave a VLP of standard size, but in substantially lower yields.

Of the three classes of particles formed in the mini-Q β preparation, the smallest may represent a T=1 form containing 60 subunits, given its size and regular structure. However, we have been unable to model such a particle using only modest changes to the natural (T=3 particle) subunit X-ray crystal structure. From a practical point of view, the availability of mini-Q β particles in large amounts will allow us to explore how size can affect the nanoparticle’s interactions with eukaryotic cells and mammalian immunological and clearance mechanisms.

Finally, another unexpected result was provided by the assessment of particle stability as a function of pH. A modest decrease in pH from 7.1 to 6.5, far from the isoelectric point of the Q β capsid (5.3),⁶⁷ resulted in a significant increase in resistance to heating for both the untreated particle and variants in which the C74–C80 disulfide had been reduced or removed (Table 5). Relatively little further change was observed at pH 5.5. Sensitivity of protein structure to pH changes in this range often derives from protonation of key histidine residues,⁶⁸ but the Q β sequence does not contain this amino acid. The origin of this effect will therefore require further study to identify. Less surprising was the finding that increasing amounts of DMSO decreased stability in a concentration-dependent fashion.^{12,69}

In combination, the above results allow us to control capsid stability over quite a large range, with T_m values varying by almost 40°C at the two extremes. This represents a key

parameter in the development of targeted vehicles that deliver the contents of its interior to a desired destination or in response to environmental triggers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The Q β capsid protein used in this study (PDB 1qbe),²¹ in ribbon diagram representation showing quasi-equivalent subunits in different colors with the C74–C80 disulfides in yellow.

Figure 2.

Construction and composition of hybrid VLPs containing the fused dimeric coat protein. “% CP₂ expressed” is the amount of the CP₂ protein relative to the CP protein present during expression as determined by electrophoretic analysis of cell culture 4 hours after induction. T_m is the decomposition temperature (°C) as defined in the text, determined by circular dichroism. All values are averages from independently expressed triplicates; error values are standard deviations of mean. Note that these errors report on the reproducibility of *average* values of CP₂ subunits in the capsids, but do not reflect the width of the distribution of these values. In other words, samples having a value of 50 ± 1 CP₂ units per capsid (56 ± 1%) could include a narrow or a broad distribution about that average value when comparing different particles in the same sample. T_m value error was 0.3°C.

Figure 3. Representative analyses of thermal stability of Q β VLPs. (a) UV-Vis absorption of WT VLPs before and after heating-induced aggregation. (b) Change in optical density at 310 nm vs. temperature.

Figure 4. Aggregation of mutant VLPs assayed by dynamic light scattering at a constant temperature of 60°C.

Figure 5.

Positions of the residues mutated in this study shown in (a) the Qβ dimer (top and side view) and (b) the surface of the VLP. Mutated positions are displayed as space-filling spheres or colored sticks: red = positions at which mutations abrogated assembly; orange = positions that allowed VLP formation with some but not all amino acid substitutions; yellow = positions for which VLPs were formed with all substitutions tested. The N- and C-termini are highlighted in green. One loop of the dimer (between C74 and C80, around the 3-fold symmetry axis) is not resolved in the published structure, due presumably to dynamic flexibility, and is not modeled here.

Figure 6. Labeling of Q β variants with fluorogenic OND reagent **1**. “SS” denotes the C74S/C80S double mutant; “r” before each designation indicates samples freshly reduced prior to reaction with **1**.

Figure 7. Location of selected interactions in the Q β capsid targeted by point mutations. (a) inter-dimer: Y62 and R47. (b) inter-dimer: D81 and R86. (c) intra-dimer: E104 and R86; E111 and N70. (d) hydrophobic core: F94, F96 and V108.

Figure 8. Initial characterization of L35W mutant VLPs. (a) Size-exclusion chromatography (Superose-6). (b) Dynamic light scattering of L35W particles. (c) TEM of L35W particles.

Figure 9. Representative two-dimensional images and class average representations (far left) of the three populations resulting from the L35W mutation, compared to WT VLPs. (a) small particles (16.5 nm diameter), 49% of the population; (b) medium particles (20 nm diameter), 39%; (c) prolate particles (23.5×20 nm), 12%; (d) WT (28 nm diameter). Scale bar = 20 nm.

Table 1

Assembly-defective mutants of the Q β VLP.

In standard particle			
L8W	L19W	L35G	L35H
L35A	L35V	Y62E	C74H
C80H	R86W	F94H	F94L
F94W	F96H	F96W	E104Q
E111Q	L128W	C74S/V108I	K2E
K13E/K16E			
In CP2 particle			
C74H	F94H	F96H	E111Q
With hairpin RNA			
C74H-hp	E111Q-hp	F96H-hp	F94H-hp
F94L-hp	C74S/V108I-hp		F96H/V108I-hp

Table 2

Assembly-competent mutants of the Q β VLP, and measurements of their expression yield and thermal stability as described in the text.

#	Mutation(s)	Category ^a	Yield (mg/L) ^b	T _m (°C) ^c
1	WT		++	83.3
2	C74S	Remove disulfide	+	61.7
3	C74S C80S	Remove disulfide	++	61.0
4	Y62F	inter-dimer H-bond	+	82.8
5	D81N	Inter-dimer salt bridge	++	78.7
6	Q65H	RNA binding	++	78.6
7	D91N	RNA binding	+	79.4
8	Q65H D91N	RNA binding	+	81.6
9	Y62F C74S C80S	combination	++	60.4
10	D81N C74S C80S	combination	++	62
11	Q65H C74S	combination	+	60.7
12	D91N C74S	combination	+	62.1
13	Q65H D91N C74S	combination	++	61.4
14	Y62R	inter-dimer H-bond	+	67.3
15	Y62W	Trp replace	+	77.0
16	Y99W	Trp replace	+	74.8
17	Y132W	Trp replace	+	80.9
18	L35W	Trp replace	++	72.2
19	P23A	structure	–	73.8
20	K2Q	exterior charge	++	74.5
21	K13Q K16Q	exterior charge	++	75.6
22	A1S	conjugation handle	+	73.9

^aType of capsid interaction or feature targeted by the mutation, or reason for its inclusion (see text).

^bmg of purified VLP per liter of standard *E. coli* culture. ++: >80 mg/L, +: 20–80 mg/L, –: <20 mg/L.

^cDetermined by monitoring aggregation with UV-vis spectrophotometry, in PBS buffer at pH 7.1. Values are the average of at least three independent runs; standard deviations of the mean were less than 0.6°C for all experiments.

Table 3

Assembly-competent mutants of the Q β VLP expressed with the hp, and measurements of their expression yield and thermal stability.

entry	mutation(s)	yield (mg/L) ^a	T _m (°C) ^b
1	C74S-hp	20–80	61.2
2	C74S C80S-hp	>80	61.0
3	Q65H-hp	>80	79.5
4	D91N-hp	<20	83.6
5	Q65H D91N-hp	20–80	79.5
6	Q65H C74S-hp	20–80	61.3
7	D91N C74S-hp	>80	63.7
8	Q65H D91N C74S-hp	<20	63.0

^a mg of purified VLP per liter of standard *E. coli* culture.

^b Determined by monitoring aggregation with UV-vis spectrophotometry, in PBS buffer at pH 7.1. Values are the average of at least three independent runs; standard deviation of the mean was <0.8 °C.

Table 4

Thermal stabilities (T_m , °C) of WT and C74S VLPs in the presence of increasing amounts of DMSO.

% DMSO	T_m (°C)	
	WT	C74S
0	83.3 ± 0.3	61.7 ± 0.2
10	82.3 ± 0.2	57.5 ± 1.1
20	73.9 ± 0.1	53.9 ± 0.2
30	76.7 ± 0.2	46.3 ± 0.1
40	n.d.	n.d.

Values are the average of at least three independent runs ± the standard deviation of the mean. n.d. = not determined

Table 5

Thermal stabilities (T_m , °C) of WT and mutant VLPs in the presence of reducing agent (DTT) and at acidic pH values.

sample	pH 7.1	+ DTT	pH 6.5	pH 5.5
WT	83.3	59.2	88.5	85.2
C74S	61.7	58.8	69.5	67.1
Q65H C74S	60.7	59.6	71.0	71.2
D91N C74S	62.1	63.3	70.7	70.5

Values are the average of at least three independent runs; standard deviations were <0.8 °C.