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Effects of amino acid substitution on the thermal stability of MS2 capsids lacking genomic RNA

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The thermal stability of capsids of the bacteriophage MS2, lacking genomic RNA, has been investigated using electron microscopy. Coat protein mutants with amino acid substitutions at residues involved in making contacts at both inter-molecular interfaces and within the coat protein subunit are also capable of forming 'empty' capsids of the same size and symmetry as the wild-type protein. Mutations have been characterised which are neutral, deleterious or advantageous in terms of thermal stability. In some cases, the results can be rationalised by reference to the recently refined X-ray crystal structure of the wild-type particle.

MS2; Capsid assembly; Thermal stability; Electron microscopy

1. INTRODUCTION

Simple viruses have long been used as models for the assembly of macromolecular complexes. In the interests of genetic economy, simple virus capsids are composed of many copies of one or more protein subunits: the resulting structures having largely either helical or icosahedral symmetry. Such structures are often capable of self-assembly, the final capsid representing the lowest free energy state of the component proteins and nucleic acids. Highly symmetrical structures allow the repeated use of the same inter-subunit bonding interactions, the minimum energy state being that which permits the maximum number of the most stable bonds between subunits. In quasi-equivalent spherical virus structures, these requirements are met by relaxing strict icosahedral symmetry, allowing viral shells with a larger number of smaller subunits to be made [1]. Although these ideas about viral architecture are quite old, there have been very few direct experimental tests of the basic postulates. For instance, free energy considerations alone cannot be the sole determinant of inter-subunit bonding in viral capsids since capsids must also allow viral disassembly to occur for biological function.

The simple RNA bacteriophage, MS2, is an excellent model in which to study the physico-chemical basis of capsid stability and architecture. The wild-type phage capsid consists of 180 copies of a single coat protein subunit (M_r = 13.75 kDa) arranged as interdigitated non-covalent dimers in a T = 3 icosahedral surface lattice. The three quasi-equivalent conformers of the protein (A,B, and C) differ primarily in the conformation of the loop connecting the F and G β-strands, the FG loop (Fig. 1). In the A and C subunits, the loops are in similar, extended conformations and interact with each other at the capsid 3-fold axes, but in the B subunit the loop is bent back towards the globular core of the subunit, interacting with other B loops at the particle 5-fold axes [2]. Both in vitro [3,4] and in vivo [5] coat protein molecules are capable of self-assembly into T = 3 shells lacking genomic RNA, so called 'empty' capsids. Aberrant forms, such as T = 1 shells (composed of 60 as opposed to 180 subunits) have also been documented [5].

The refined, high resolution crystal structure of the wild-type phage particle has recently been reported (Golmohammadi, Valegård, Fridborg and Liljas, personal communication). All of the coat protein subunit is visible in the electron density map. It should therefore be possible to use MS2 as a model in which to examine the forces which drive quasi-equivalent shell formation.

Our studies on MS2 have employed mutagenesis of coat protein residues to probe their importance in assembly [4,6]. More recently, this work has been extended to include a study of the product of the assembly process, namely the capsid itself. Here we report the results of experiments aimed at determining the overall thermal stability of the capsid, and the effects of amino acid substitutions at residues involved in intra- and inter-subunit bonding on this stability.

2. EXPERIMENTAL

The MS2 coat protein gene was isolated from a cDNA fragment of the MS2 genome and used to construct the vector, pACP2 [7]. Mutagenesis was carried in two ways, both involving sub-cloning of frag-

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ments of the coat protein gene into the polylinker of M13mp18 or mp19. Random mutagenesis was carried out according to [8], as described [6]. Site-directed mutagenesis was undertaken by a PCR amplification method [9]. DNA sequencing, both in M13 and after subcloning the mutated fragments into the pACP2 expression vector, was carried out using the Pharmacia T7 DNA polymerase kit. This confirmed the presence of only the specific amino acid substitutions expected within the coat protein gene.

In order to obtain quantities of mutant MS2 coat proteins for further studies, the proteins were over-expressed in *Escherichia coli* TG1 (K12 *supE, hsdD5, thi, Δ(lac-pro)* F′ [*traD36 proA⁺B⁺ lacI^q lacZΔM15*] containing the relevant pACP2 expression vector. The mutant proteins were then recovered by sonication of the cells, followed by precipitation with ammonium sulphate to 40% (w/v) saturation and finally purified by centrifugation through sucrose density gradients (15–45% (w/v) linear gradients, with a 60% (w/v) cushion) at 18,500 rpm for 16 h at 4°C in a Beckman SW 28 rotor [3]. Under these conditions, T = 3 empty capsids sedimented to the centre of the gradient and could be detected as an absorption maximum (λ = 280nm). A much smaller peak, corresponding to T = 1 capsids, could also be detected near to the top of the gradient. The presence of capsids of the correct size and symmetry was confirmed by transmission electron microscopy (TEM). Samples for TEM were dialysed at 4°C against 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 100 mM NaCl, 1 mM diaminoethanetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), pH 7.2, to remove sucrose, and subsequently subjected to negative staining [10] using 4% (w/v) uranyl acetate onto formvar/carbon-coated copper grids. These were examined and photographed at 100 kV at a magnification of 50,000×.

For thermal stability studies, 1 ml samples of T = 3 assembled coat protein, at approximately 1 mg/ml, were freshly dialysed as above. 100 μl aliquots, in Eppendorf vials, were placed into a heating block at specific temperatures for 10 min periods prior to immediate processing for TEM as described above.

Wild-type MS2 phage was a gift from H.R. Hill, University of Leeds.

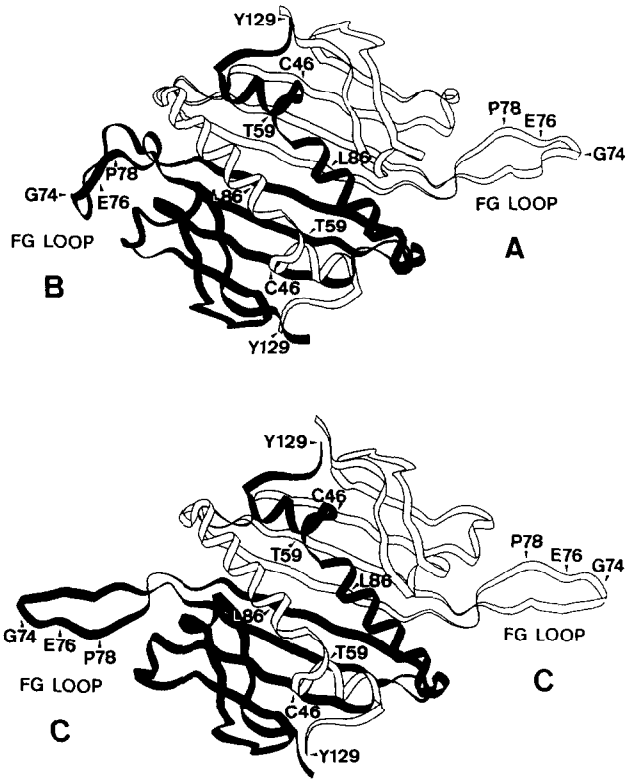

Fig. 1. Ribbon representations of the two types of coat protein dimer found in the MS2 phage capsid (top, an A/B dimer; bottom, a C/C dimer). The FG loop (connecting the F and G β-strands) exists in an extended conformation in the A and C subunits, but is bent back towards the body of the protein in B subunits. The positions of the amino acid substitutions described in the text are indicated by the arrowheads.

3. RESULTS

Studies initially concentrated on the stability of wild-type T = 3 empty capsids. These could be clearly visualised by TEM at temperatures up to 68°C, as illustrated in Fig. 2. No capsids were seen, however, if the temperature was increased by just 1°C, the sample incubated at 69°C appearing as amorphous aggregates. Subsequent lowering of the temperature of samples incubated at 69°C yielded a small number of re-assembled capsids, although the majority of the material remained as aggregates. Once heated to 70.5°C, however, even this limited reassembly was no longer possible.

In order to investigate the role of MS2 RNA on capsid stability, the experiment was repeated with wild-type phage particles, prepared for electron microscopy in the same way as the empty capsids. A similar result was obtained for the denaturation temperature (data not shown).

To examine the interactions involved in the maintenance of stable capsids, the investigation was extended to include coat protein mutants. Mutants with single amino acid substitutions at either inter-molecular interfaces or within coat protein monomers, and which still formed capsids of the correct size and symmetry, were selected for thermal stability studies (Fig. 1). Wild-type empty capsids were included as controls. Three of the mutants, L86S, G74A and C46R, were found to exhibit identical thermal stabilities to wild-type. Another group of mutants showed a small but significant 2–4°C increase in denaturation temperature (T59S, Y129C and C46W), whereas a third group (P78N and E76D) showed a 2°C decrease. These results are illustrated by the electron micrographs shown in Fig. 2 and are listed in Table I.

4. DISCUSSION

Wild-type MS2 T = 3 empty capsids are clearly very stable thermally, denaturing completely, however, if the temperature is raised from 68°C by just one degree. Presumably this sharp denaturation temperature means that the conformation of the disassembled subunits is less stable than the capsid itself. The denaturation temperature is clearly not related to the physiological process of disassembly, since MS2 RNA uncoating in vivo is mediated by the maturation A protein, which interacts both with the RNA and with the pili of target cells [11,12]. Previous studies on the MS2-related phage, f2

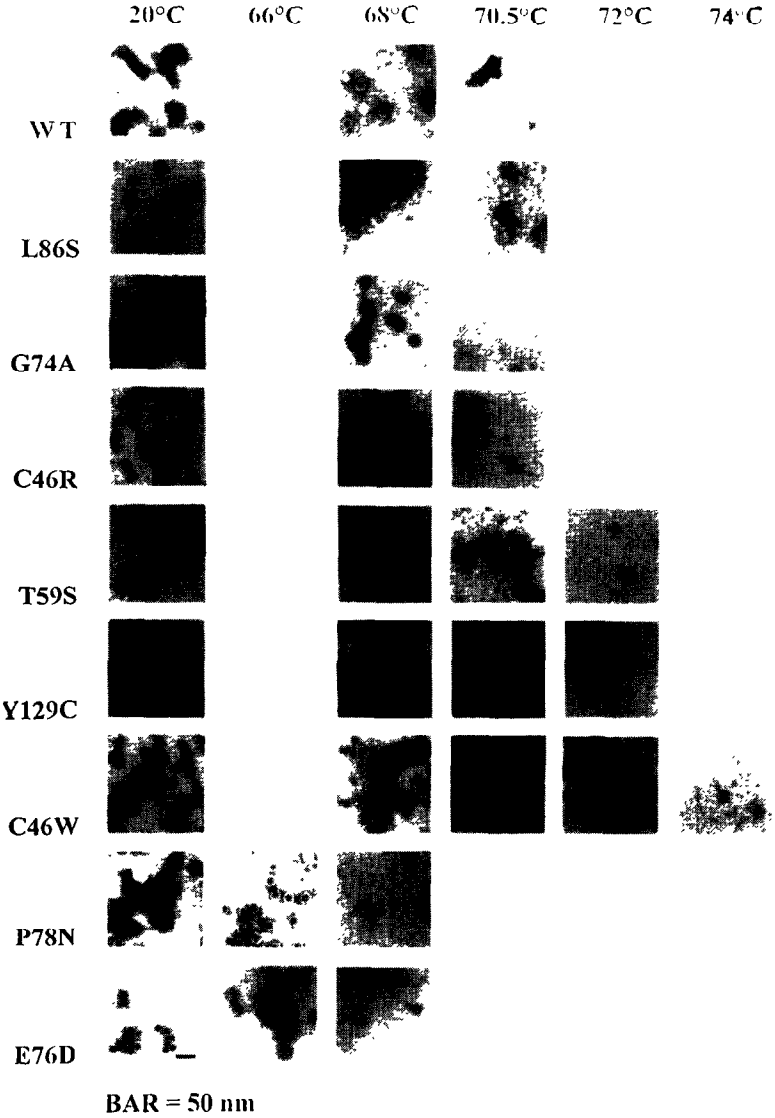

Fig. 2. Thermal stability of 'empty' MS2 capsids. Samples of assembled MS2 coat protein were heated to the temperatures shown for 10 min periods, prior to immediate processing for electron microscopy, as described in the text. Bar = 50 nm.

[13], are consistent with our result. These authors obtained denaturation temperatures, based on UV spectra, of between 55–70°C for capsids, assembled with or without RNA and 60–65°C for phage. Our more precise value of 69°C maybe a consequence of working with purified T = 3 shells. In our hands, phage and recombinant capsids denatured at identical temperatures, implying that RNA–protein interactions do not significantly stabilise the viral capsid. This is consistent with the fact that nucleic acid charge neutralisation in RNA phages is accomplished largely by interaction with polyamines [14] and the three-dimensional structure of the phage particle, which does not show any evidence for the presence of RNA at protein–protein interfaces [2].

The empty MS2 shells would therefore appear to be an ideal model in which to study the effects of amino acid substitutions on overall capsid stability and architecture. In principle, altering residues involved in inter-subunit contacts could shift the equilibrium of capsid shell formation in favour of the T = 1 structure. The mutants studied here all behaved exactly like wild-type in terms of assembly and their distribution between T = 1 and T = 3 shells. Kinetic factors influencing the production of T = 3 shells must therefore be similar in each case. The mutants can be classified into different groups depending on the types of interaction made (Table I). Effects on stability result from changes in contacts made within a coat protein monomer (intra-subunit), between monomers in a dimer (intra-dimer) or between dimers (inter-dimer).

Residues which make only intra-subunit main chain contacts were initially intended as controls. These were C46, T59 and P78. Both C46 and T59, however, are located close to the intra-dimer interface and may therefore have indirect effects on this interaction. Although C46R behaved like wild-type, both C46W and T59S

mutants were 2–4°C more stable. The side chain of C46 is located in a hydrophobic region of the main β-sheet of the subunit. It is possible that substitution of cysteine with the more hydrophobic tryptophan could increase the hydrophobic interactions in this area, thus increasing stability. Despite its location in the β-sheet at the intra-dimer interface, the effects of substitution of T59 with serine would be expected to be minimal. The increased denaturation temperature of this mutant is therefore somewhat perplexing. P78 exists in two isomeric forms in the phage capsid; as a *trans* isomer in A and C subunits and as a *cis* isomer in B subunits. This proline residue makes contacts with the side chain of S39 in A and C subunits only, and may therefore be involved in ensuring the correct, extended, orientation of the loop at the particle 3-fold axes. The cyclic nature of proline confers distinct constraints on the conformation of a polypeptide chain and it is unlikely that another residue could make equivalent contacts. This may explain the 2°C reduction in the denaturation temperature of P78N mutant shells.

Residue E76 is also involved in intra-subunit contacts, the side chain hydrogen bonding with the side chain of Q40 and the main chain of T71 in the B subunits only. Together with inter-dimer solvent-mediated contacts, these interactions may be responsible for the pinning back of the FG loop in B subunits. The E76D mutation resulted in a 2°C reduction in shell stability. Glutamate-to-aspartate is not a dramatic substitution, but the shorter side chain of aspartate is probably incapable of forming the contacts to Q40 and T71.

Two of the substituted residues are involved in intra-dimer interactions, L86 and Y129. L86 makes hydrophobic contacts to L90, the loss of which by substitution with serine is probably not significant, accounting for the wild-type stability of L86S. Y129 is involved in many different contacts with residues at the N-terminus of its dimer partner. It was therefore surprising to find that substitution of Y129 by the smaller cysteine resulted in an increased denaturation temperature. The formation of novel disulphide bonds, unlikely owing to the presence of DTT, was discounted by reference to the crystal structure.

Residue G74 is involved in main chain inter-dimer contacts with T71. The wild-type behaviour of G74A suggested that substitution with alanine creates little disturbance of the polypeptide chain at this position.

The data reported for the stability of wild-type empty MS2 capsids are in agreement with differential scanning calorimetry (DSC) studies, which gave a thermal denaturation temperature of 68.8°C (Cooper and Robertson, personal communication). DSC has also been employed to study the stability of the heads of the DNA phages T4 [15,16] and P22 [17]. These structures have denaturation temperatures in excess of 80°C, proheads being significantly less stable than mature particles. The lack of three-dimensional models of these systems together with their structural complexity, however, makes more detailed interpretation in these viruses difficult.

Monomeric proteins, such as T4 lysozyme, appear to permit a wide range of structural re-adjustments, allowing the protein to be very tolerant of amino acid substitutions [18]. Perhaps surprisingly, our data show that even in complex self-assembling structures such as the viral capsid, a wide range of amino acid substitutions can be tolerated, even at inter-molecular interfaces, with only modest effects on overall stability. Clearly the MS2 capsid has not been selected as the lowest free energy form of the assembled coat proteins, suggesting that other factors were important in the evolution of this structure. The factors which control the choice of the symmetry of the final assembly product, i.e. a T = 3 shell instead of a T = 1, are most interesting from an evolutionary standpoint, and the MS2 system appears an ideal model in which to study the molecular basis of this choice.

Table I

A summary of the thermal stability of specific coat protein mutants relative to wild-type, together with the molecular contacts made by these residues in the capsid, as determined by X-ray crystallography[a]

Residue	Contacts	Mutant	Stability
C46	Intra-subunit H-bonding (main chain)	C46R	WT
		C46W	+4°C
T59	Intra-subunit H-bonding (main chain)	T59S	+2°C
G74[b]	Inter-dimer H-bonding (main chain to T71 main chain) in A/B dimers only	G74A	WT
E76[b]	Intra-subunit H-bonding (side chain with Q40 side chain and with T71 main chain) in B subunits only Inter-dimer solvent mediated H-bonding (main chain) to T69 (main chain) near 5-fold and 3-fold axes	E76D	−2°C
P78[b]	Intra subunit H-bonding (main chain) Intra-subunit H-bonding (main chain with S39 side chain) in A and C subunits only	P78N	−2°C
L86	Intra subunit H-bonding (main chain) Intra-dimer hydrophobic interactions with L90	L86S	WT
Y129	Intra-dimer H-bonding (main chain with N3 side chain. Side chain with S2 side chain, and with N3 and F4 main chains) Intra-dimer solvent mediated H-bonding (main chain) to A1 (main chain) in C/C dimers only	Y129C	+2°C

[a] Golmohammadi, Valegård, Fridborg and Liljas, personal communication.

[b] Denotes an amino acid residue located within the FG loop.

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