doi:10.1016/S0022-2836(03)00950-1 J. Mol. Biol. (2003) 332, 777–782

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Comprehensive Mutagenesis of the C-terminal Domain of the M13 Gene-3 Minor Coat Protein: The Requirements for Assembly into the Bacteriophage Particle

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Filamentous bacteriophage assemble at the host membrane in a non-lytic process; the gene-3 minor coat protein (P3) is required for release from the membrane and subsequently, for recognition and infection of a new host. P3 contains at least three distinct domains: two N-terminal domains that mediate host recognition and infection, and a C-terminal domain (P3-C) that is required for release from the host cell following phage assembly and contributes to the structural stability of the phage particle. A comprehensive mutational analysis of the 150 residue P3-C revealed that only 24 side-chains, located within the last 70 residues of sequence, were necessary for efficient incorporation into a wild-type coat. The results reveal that the requirements for the assembly of P3 into the phage particle are quite lax and involve only a few key side-chains. These findings shed light on the functional and structural requirements for filamentous phage assembly, and they may provide guidelines for the engineering of improved coat proteins as scaffolds for phage display technology.

Keywords: phage display; protein engineering; combinatorial mutagenesis; M13 bacteriophage

M13 bacteriophage is a member of the Inoviridae family of viruses, which encapsulate their single-stranded DNA (ssDNA) genome within a cylindrical, proteinaceous coat.[1] Virion length depends on the size of the packaged genome, but typically ranges from 700 nm to 2000 nm; in contrast, the virion has a diameter of only 6–7 nm, hence, the designation as filamentous bacteriophage.[2–4] The filament length is covered by several thousands copies of the gene-8 major coat protein (P8), while the ends are capped by approximately five copies each of four minor coat proteins (two different proteins at either end).

Filamentous bacteriophage are non-lytic and assemble by a membrane-associated mechanism.[5–7] Coat proteins initially reside as integral inner membrane proteins with a single transmembrane domain, and assembly occurs at sites formed by multimers of additional phage-encoded proteins; protein-4 (P4) in the outer membrane interacts

Supplementary data associated with this article can be found at doi: 10.1016/S0022-2836(03)00950-1

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Abbreviations used: ELISA, enzyme-linked immunosorbant assay; hGH, human growth hormone; hGHbp, hGH-binding protein; P3, M13 gene-3 minor coat protein; P3-C, C-terminal domain of P3; P3-C1, C-terminal domain 1 of P3; P3-C2, C-terminal domain 2 of P3; P8, M13 gene-8 major coat protein; Pn, (where $n = 1$, 4, or 5), M13 gene-n protein; Pn, (where $n = 6$, 7, or 9), M13 gene-n minor coat protein; ssDNA, single-stranded DNA; wt, wild-type.

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with protein-1 and protein-11 (P1 and P11) in the inner membrane to form an assembly site through which phage are extruded. While in the cytoplasm, the ssDNA is sequestered with another phage-encoded protein (protein-5, P5) which is stripped off during assembly. It is believed that assembly is initiated when a signature sequence within the ssDNA, the packaging signal, interacts with two different minor coat proteins (P7 and P9) and with P1 at the assembly site. The association of the ssDNA with P7 and P9 forms the leading end of the phage, which is elongated by extrusion from the host and concomitant incorporation of P8. When the end of the ssDNA is reached, assembly is completed by the addition of minor coat proteins P6 and P3, and the particle is released from the host cell membrane.

Studies of filamentous phage structure and biology have shown that both the genome and coat are quite malleable, and these findings have paid major dividends in the development of molecular biology methods. Very large inserts can be made in the ssDNA genome, as they are accommodated readily by a corresponding increase in the particle length, and this property has led to the development of versatile cloning vectors.[8] The coat proteins can tolerate insertions of foreign polypeptides, allowing for the development of phage display vectors that permit the display of polypeptides on the surfaces of phage particles that also contain the encoding DNA.[9–11] More recently, phage display methods have been used to study the phage itself, in order to better understand phage biology and structure, and to further improve phage display technology.[12–14]

Unlike the other phage coat proteins, which are small and hydrophobic, mature P3 contains 406 residues and consists of several distinct domains that together mediate both entry into and release from the host. Initially, P3 was divided into three domains separated by glycine-rich linkers: two N-terminal domains (P3-N1 and P3-N2) required for host cell infection, and a C-terminal domain (P3-C) required for the release of newly assembled virions from the membrane and for structural stability of the phage particle.[15–17] However, detailed analysis of deletion mutants revealed that P3-C contains two functionally distinct subdomains: C-terminal domain 1 (P3-C1, residues 257–313), which is involved in capping and stabilizing the assembled phage particle, and C-terminal domain 2 (P3-C2, residues 314–406), which is required for incorporation into the phage coat and subsequent release of the assembled virion from the host membrane.[18] While the three-dimensional structures of P3-N1 and P3-N2 have been determined,[19–22] the structure of P3-C has not.

Here, we map in fine detail the side-chains required for the assembly of P3 into the phage coat, independent of the other functions of P3. To facilitate this goal, we used a phagemid-based phage display system analogous to a system used to study the assembly requirements of P8.[23] The phagemid vector was designed to direct the secretion of a fusion protein consisting of human growth hormone (hGH) fused to the N terminus of P3-C. Co-infection of *Escherichia coli* with the phagemid and a helper phage resulted in monovalent phage display of hGH; that is, the production of phage particles containing predominantly wild-type P3 from the helper phage and no more than one copy of the hGH-P3-C fusion. Thus, the level of hGH display served as an indicator of the incorporation efficiency, while the excess wt P3 from helper phage supplied the other functions of P3 (i.e. release and stabilization of the assembled virion, and host cell recognition and infection).

Side-chain contributions to P3-C incorporation were determined by measuring the effects of truncating mutations. Ala mutations were used because this substitution removes side-chain atoms past the β-carbon atom, yet it is unlikely to introduce flexibility into the protein backbone.[24] At positions where Ala was the wild-type (wt) residue, Gly substitutions were used to remove even the side-chain β-carbon atom. The entire P3-C sequence was scanned using an extremely rapid "shotgun scanning" combinatorial mutagenesis strategy applied previously to the study of P8[23] and several other proteins.[25–28] Our results offer insights into the relationships between viral structure and function, and they may provide useful guidelines for the design of P3-C variants as improved scaffolds for phage display technology.

Shotgun scan of P3-C

A comprehensive mutational analysis of P3-C was conducted using ten shotgun scanning libraries within a phagemid vector designed to display hGH fused to the N terminus of P3-C.[14] Each library covered nine to 21 contiguous residues and there was no overlap between the different libraries. Together, the libraries mutated the entire sequence of P3-C, with the exception of two cysteine residues that likely form a disulfide bond critical for proper folding. Shotgun scanning codons were used to mutate residues to either the wt or Ala in an equimolar ratio (Ala or Gly in cases where the wt was Ala), although the redundancy of the genetic code necessitated two other substitutions at some positions.[26] The diversity of each library was at least tenfold greater than the number of possible amino acid combinations, thus ensuring complete representation of the theoretical diversity.

Phage from each library were cycled through rounds of binding selection with immobilized growth hormone-binding protein (hGHbp) as the capture target ($K_d = 1.6$ nM for binding to hGH),[29] as described for a similar analysis of P8.[23] Approximately 100 selected clones were sequenced for each library, the sequences were aligned, the occurrence of wt or each designed mutation was tabulated at each position, and the wt/mutant ratio was

calculated for each mutation at each position. The magnitude of the wt/mutant ratio correlates with the effect of each mutation on hGH display, which in turn is dependent upon the efficiency of P3-C incorporation into the bacteriophage particle. Thus, ratios greater than or less than 1 indicate mutations that decrease or increase incorporation efficiency, respectively.

Only 23 Ala mutations and one Gly mutation (A361G) affected P3-C incorporation significantly (wt/m1 > 4 in Table 1), and amongst these, only eight Ala mutations had large effects (wt/m1 > 16). These 24 residues consist of 16 hydrophobic residues, three small residues (Ala361, Gly362 and Gly379), one negatively charged residue (Glu353), and four positively charged residues (Lys373, Arg378, Arg402 and Lys404). Notably, all of the residues that affect incorporation are located amongst the last 70 residues of P3-C. The results are in agreement with those reported by Rakonjac *et al.*, who found that, while the entire 93 residue P3-C2 was required for release of the assembled phage from the bacterial membrane, the last 83 residues were sufficient for efficient incorporation into the phage particle.[18] Since the 83 residue fragment was the smallest construct tested by Rakonjac *et al.*, our results suggest that an even smaller 70 residue C-terminal fragment is the minimum element required for the assembly of P3 into the bacteriophage particle (Figure 1).

The minimal requirements for P3-C incorporation

While the absence of a three-dimensional structure precludes any definitive statements regarding the functional roles of the 24 residues required for efficient P3-C incorporation, our results should prove useful in guiding further structural and functional analyses of M13 bacteriophage assembly. In particular, it is possible that smaller fragments of P3-C designed on the basis of our studies and the results reported by Rakonjac *et al.*[18] may be more amenable to structural analysis than the entire P3-C, especially if the 70 C-terminal residues can be subdivided into two structural regions consisting of a fairly hydrophilic domain followed by a hydrophobic domain that includes the transmembrane anchor (Figure 1).[18] Computer-modeling methods may prove useful in generating and testing structural and functional hypotheses, and we have used machine learning algorithms to derive a preliminary model of P3-C, which can be viewed on the internet†.

Implications for natural and artificial viral evolution

Here, we have used shotgun scanning mutagenesis to identify the side-chains required for P3

† http://www.ics.uci/edu ~ pfbaldi/P3

Table 1. P3-C shotgun scan

	wt/mutant Ratios[a]		
Residue[b]	wt/m1	wt/m2	wt/m3
D257	1.1		
F258	1.9	2.2(S)	1.2(V)
D259	1.3		
Y260	1.1	1.1(D)	2.7(S)
E261	1.4		
K262	0.88	3.1(E)	1.2(T)
M263	1.0	1.5(T)	0.77(V)
A264	0.79		
N265	0.81	0.77(D)	1.1(T)
A266	0.74		
N267	1.2	0.81(D)	1.9(T)
K268	0.67	1.8(E)	1.3(T)
G269	1.8		
A270	0.71		
M271	0.63	0.81(T)	0.63(V)
T272	0.61		
E273	0.83		
N274	1.2	1.4(D)	1.1(T)
A275	0.87		
D276	0.89		
E277	0.81		
N278	0.81	0.59(D)	0.94(T)
A279	1.4		
L280	1.4	1.2(P)	1.3(V)
Q281	0.55	0.89((E)	0.73(P)
S282	1.1		
D283	0.93		
A284	1.6		
K285	0.88	1.2(E)	1.2(T)
G286	1.2		
K287	0.56	2.4(E)	0.86(T)
L288	2.3	1.6(P)	0.96(V)
D289	1.4		
S290	0.81		
V291	1.3		
A292	2.2		
T293	0.83		
D294	1.2		
Y295	0.78	1.0(D)	0.62(S)
G296	1.1		
A297	0.76		
A298	0.63		
I299	0.82	1.6(T)	1.0(V)
D300	0.76		
G301	2.5		
F302	1.7	2.2(S)	0.93(V)
I303	1.1	2.3(T)	0.83(V)
G304	1.5		
D305	0.93		
V306	2.0		
S307	0.98		
G308	1.8		
L309	1.0	1.5(P)	1.0(V)
A310	0.98		
N311	0.62	1.2(D)	1.3(T)
G312	1.3		
N313	0.92	1.0(D)	1.3(T)
G314	1.1		
A315	0.66		
T316	0.62		
G317	2.5		
D318	1.4		
F319	1.3	2.2(S)	1.1(V)
A320	0.67		
G321	1.8		
S322	0.69		
N323	1.4	0.85(D)	1.1(T)
S324	0.56		
Q325	0.92	1.2(E)	1.4(P)
M326	1.3	2.5(T)	1.5(V)

(*continued*)

Table 1 Continued

Residue[b]	wt/mutant Ratios[a] wt/m1	wt/m2	wt/m3
A327	0.89		
Q328	1.3	1.3(E)	1.8(P)
V329	1.6		
G330	2.4		
D331	1.7		
G332	2.6		
D333	1.8		
N334	1.1	0.92(D)	1.4(T)
S335	0.76		
P336	0.56		
L337	**4.3**	**8.6(P)**	2.4(V)
M338	2.3	2.9(T)	1.8(V)
N339	0.80	3.0(D)	1.3(T)
N340	0.85	3.2(D)	**4.1(T)**
F341	**8.8**	**53(S)**	2.7(V)
R342	3.6	2.4(G)	**7.2(P)**
Q343	1.0	1.7(E)	1.6(P)
Y344	2.4	**11(D)**	3.6(S)
L345	3.9	**18(P)**	1.1(V)
P346	3.3		
S347	1.3		
L348	**21**	**62(P)**	**5.6(V)**
P349	2.9		
Q350	0.83	**6.3(E)**	1.2(P)
S351	1.0		
V352	**4.3**		
E353	**5.7**		
C354	ND		
R355	1.4	**6.0(G)**	**4.8(P)**
P356	1.5		
F357	**8.3**	**25(S)**	1.3(V)
V358	**24**		
F359	**19**	**37(S)**	3.7(V)
G360	1.0		
A361	**12**		
G362	**50**		
K363	0.28	0.63(E)	0.56(T)
P364	1.3		
Y365	0.86	2.7(D)	1.7(S)
E366	0.86		
F367	**11**	3.1(S)	1.8(V)
S368	1.7		
I369	1.7	2.8(T)	1.7(V)
D370	1.6		
C371	ND		
D372	2.3		
K373	**4.0**	2.7(E)	**4.4(T)**
I374	**41**	**5.1(T)**	1.6(V)
N375	2.6	2.2(D)	12(T)
L376	3.3	**4.9(P)**	2.6(V)
F377	2.8	**5.7(S)**	1.6(V)
R378	**10**	2.8(G)	**8.3(P)**
G379	**17**		
V380	**4.4**		
F381	**4.0**	**4.5(S)**	1.8(V)
A382	0.85		
F383	**4.9**	**8.2(S)**	1.8(V)
L384	2.3	**4.4(P)**	2.3(V)
L385	**4.1**	**5.9(P)**	2.9(V)
Y386	3.9	3.3(D)	**7.2(S)**
V387	3.2		
A388	0.79		
T389	1.6		
F390	3.3	**17(S)**	2.0(V)
M391	3.6	3.9(T)	1.1(V)
Y392	**25**	**76(D)**	5.1(S)
V393	**23**		
F394	3.4	**44(S)**	3.4(V)
S395	1.9		
T396	0.31		
F397	**13**	**76(S)**	**6.3(V)**

(*continued*)

Table 1 Continued

Residue[b]	wt/mutant Ratios[a] wt/m1	wt/m2	wt/m3
A398	0.94		
N399	1.9	1.6(D)	6.8(T)
I400	1.6	**15(T)**	0.65(V)
L401	1.0	**8.0(P)**	0.29(V)
R402	**4.4**	0.91(G)	40(P)
N403	2.9	2.1(D)	3.4(T)
K404	**12**	**6.4(E)**	**10(T)**
E405	2.3		
S406	1.5		

The shotgun scan was conducted as described for an analogous scan of P8,[23] using a previously described phagemid (pS1602) designed for the display of hGH fused to the N terminus of P3-C.[14] Ten non-overlapping libraries were constructed and named 1 to 10, starting from the N terminus. The libraries were cycled separately through rounds of binding selections with immobilized hGHbp to capture hGH-displaying phage. After one (libraries 1 to 4) or three (libraries 5 to 10) rounds of selection, greater than 50% of the clones exhibited positive phage ELISA signals at least twofold greater than signals on control plates coated with bovine serum albumin. These positive clones were subjected to DNA sequence analysis (one 96-well plate for each library). The sequences were analyzed with the program SGCOUNT as described,[23,26] and the tabulated occurrences of each natural amino acid at each position were used to calculate the wt/mutant ratios.

[a] The wt/mutant ratio provides an estimate of the effect of each mutation on the incorporation of P3-C into the phage coat. Deleterious mutations are indicated by wt/mutant ratios greater than 1, and mutations that have large deleterious effects (wt/mutant > 4) are shown in boldface. The mutation m1 was an Ala substitution in all cases, except in the case of wt Ala, where a Gly substitution was used. The identities of the mutations m2 and m3 depend on the particular shotgun codon used,[26] and are shown in parentheses to the right of each wt/mutant ratio. ND indicates that these values were not determined, because Cys residues were not mutated.

[b] Each wt residue is represented by the single-letter amino acid code followed by the position in the mature P3 sequence.

assembly, and previously we used a similar approach to investigate the assembly of P8.[23] P8 is a 50 residue α-helix, and it was found that only 13 side-chains (including those of four alanine residues) were required for P8 assembly. While P3 is much larger and structurally more complex than P8, it is remarkable that its assembly requires only a small number of side-chains. Furthermore, these P3 side-chains are located within the last 70 residues of the P3 sequence and, thus, the region of P3 that mediates incorporation into the phage coat is similar to that of P8 in terms of size and complexity. However, the assembly requirements of P3 differ from those of P8, in that, while the incorporation of P8 was essentially independent of side-chains within the transmembrane domain, the incorporation of P3 requires eight transmembrane side-chains (Figure 1).

In conclusion, our studies with P3 and P8 have demonstrated that the requirements for protein incorporation into the filamentous phage coat are quite lax. For both proteins, efficient incorporation requires only the recognition of a few key side-chains located within a small stretch of primary

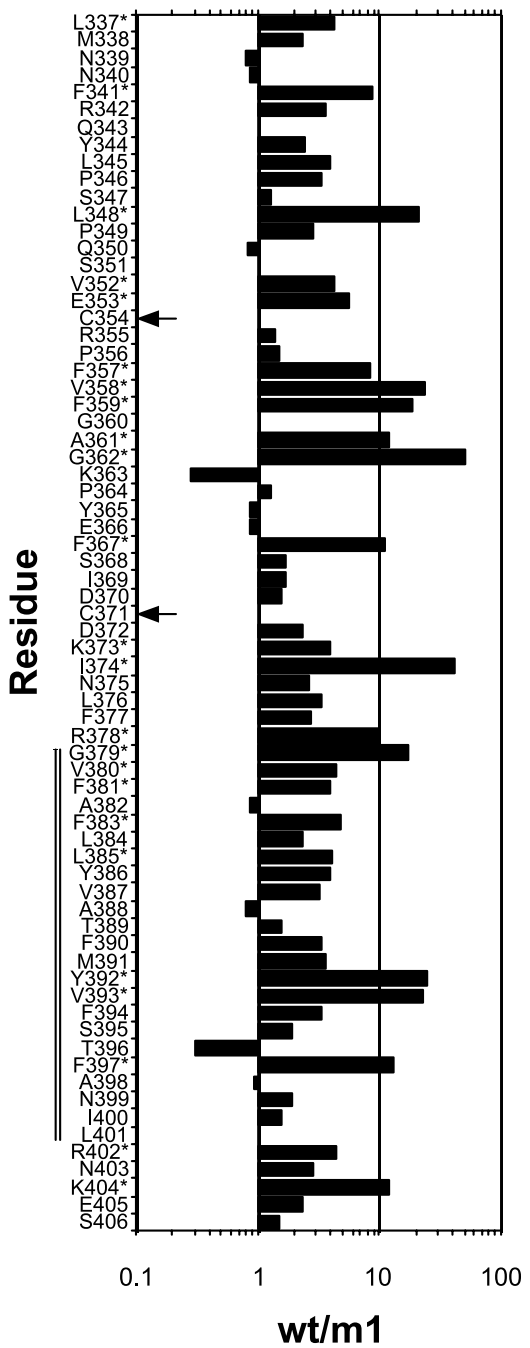

Figure 1. The minimal region required for P3-C incorporation. The plot shows wt/mutant ratios measuring the effects of P3-C mutations on the efficiency of incorporation into the wt phage coat. For each P3-C residue (vertical axis), the wt/m1 ratio from Table 1 is plotted (horizontal axis). The m1 substitution was Ala, except when the wt residue itself was Ala, in which case a Gly substitution was used. Asterisks ($*$) indicate mutations that have large deleterious effects on P3-C incorporation ($wt/m1 > 4$). Black arrows indicate Cys residues that were not mutated. The vertical double line indicates the transmembrane region.[18]

sequence. Thus, in the context of a fully functional viral genome that encodes all of the proteins necessary for viral assembly and structural stability, it seems likely that the recruitment of new coat proteins may be a facile process. Heterologous proteins that present epitopes similar to those of P3 or P8 could be incorporated as additional components of the phage coat, and this could facilitate the evolution of new viral functions. Previously, we used the promiscuous nature of the phage assembly process to engineer P8 variants as improved scaffolds for phage display,[12–14] and we hope that the present results will prove useful for the development of improved phage display scaffolds based on P3.

Acknowledgements

We thank Alan Zhong for DNA sequencing, and the Genentech DNA synthesis group for oligonucleotides.

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Edited by W. Baumeister

(Received 15 April 2003; accepted 24 June 2003)

Supplementary Material comprising four views of a P3-C computational model is available on Science Direct