Cysteine residues in the transmembrane regions of M13 procoat suggest that oligomeric coat proteins assemble onto phage progeny

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Abstract

The M13 phage assembles in the inner membrane of *Escherichia coli*. During maturation, about 2700 copies of the major coat protein move from the membrane onto a single-stranded phage DNA molecule that extrudes out of the cell. The major coat protein is synthesized as a precursor, termed procoat protein and inserts into the membrane via a Sec-independent pathway. It is processed by leader peptidase from its leader (signal) peptide before it is assembled onto the phage DNA. The transmembrane regions of the procoat protein play an important role in all these processes. With cysteine mutants in the transmembrane regions of the procoat and coat protein we investigated which of the residues are involved in multimer formation, interaction with leader peptidase and formation of M13 progeny particles. We found that most single cysteine residues do not interfere with the membrane insertion, processing and assembly of the phage. Treatment of the cells with copper phenanthroline showed that the cysteines were readily engaged in dimer and multimer formation. This suggests that the coat proteins assemble into multimers before they proceed onto the nascent phage particles.

In addition, we found that when a cysteine is located in the leader peptide at the –6 position, processing of the mutant procoat and of other exported proteins is affected. This inhibition of leader peptidase results in a lethality of the cell and shows that there are distinct amino acid residues in the M13 procoat protein involved at specific steps of the phage assembly process.
Introduction

M13 bacteriophage infects the bacterial cell by binding to the tip of a F-pilus. After binding, the pilus is retracted and disassembled into the inner membrane leaving the phage DNA in the cytoplasm. The single-stranded circular DNA is rapidly converted into a double-strand and replicated for the continuous production of progeny phage. Phage-directed protein synthesis is mainly dedicated to express the five different structural coat proteins, the products of genes III, VI, VII, VIII and IX. All these coat proteins are inserted into the bacterial inner membrane, from where the assembly of progeny particles occurs. The assembly of the particle starts with the formation of the coat cap structure consisting of the gene products VII and IX, and is followed by the assembly of the major coat protein, gp VIII. On the phage particle, units of 5 proteins cover the DNA in a shingle-like arrangement (4, 23). The integrity of the phage particle is based on strong protein-protein interactions and electrostatic protein-DNA interactions.

During the biogenesis of bacteriophage M13, the membrane-inserted major coat protein is processed to the mature form by the leader peptidase before it is assembled onto the extruding single-stranded phage DNA. The precursor protein, termed procoat, spans the membrane twice exposing a negatively charged region in the periplasm. The signal sequence is required for the membrane insertion process, although a coat protein of another filamentous phage exists without a signal sequence (29). The membrane insertion process is independent of SecA and the SecYEG translocase, but requires the membrane insertase YidC and an electrochemical membrane potential (11, 31). The processed and membrane-integrated major coat protein of 50 amino acid residues consists of a negatively charged region of 20 residues in the periplasm, a membrane-spanning region of 19 residues and the 11 residue-long positively charged C-terminal region in the cytoplasm.

The assembly of the progeny phage particle takes place at the inner membrane of E. coli. The products of gene I, III, VI, VII, VIII, IX and XI are integrated into the inner membrane, whereas the gp IV forms a complex of 10-12 subunits in the outer membrane (16) allowing the passage of the nascent phage particle through the outer
membrane. The assembly of the phage is initiated by the proteins VII and IX that bind to the “morphogenic signal” of the newly replicated single-stranded DNA. During the phage assembly process, coat protein (VIII) joins in by binding the positively charged C-terminal region to the ssDNA (5, 7). The hydrophobic region of the coat protein is in an α-helical conformation in the membrane and in the phage particle, whereas the adjacent regions have to undergo a conformational change. Remarkably, the tilt of the helix axis of the coat protein in the membrane remains similar in the phage where it is tilted with respect to the axis of the virion (20, 22).

One open question in the assembly process is how the coat protein binds onto the extruding single-stranded DNA molecule (21). It is possible that coat proteins form multimers in the membrane that bind in a concerted fashion on the DNA, such that lipid molecules are excluded. This implies that the coat proteins already interact in the membrane with each other to form oligomeric complexes that are ready to bind onto the DNA molecule. Previously, Haigh and Webster (6) introduced single cysteines at positions 31, 35, 37 and 39 of the coat protein. The mutant at 31 was able to form dimers when extracted from phage, but was monomeric when extracted from the cytoplasmic membrane. However, the mutant at 35 was found partially dimeric in the membrane, but these proteins were incapable of assembling progeny particles. Since only a single cysteine had been introduced into the coat proteins, higher order oligomers could not be detected. In addition, the membrane environment might be too reductive to observe existing protein-protein interactions.

We addressed this question by introducing cysteine residues at positions 30, 31, 32, and 33 into the transmembrane region of the coat protein. When only a single cysteine residue was present in the procoat, phage multiplication was normal for most positions. We found that the coat proteins were readily able to form dimers in the membrane when the cells were treated with copper phenanthroline. When the coat protein contained two cysteines, oligomeric forms were found suggesting that the oligomeric forms are accumulating in the membrane ready to assemble onto phage particles. A model is presented how the oligomeric coat proteins are loaded onto the extruding phage particles from the membrane.
Materials and Methods

Plasmid constructions
The plasmid pJF119HE (3) was used to clone gene VIII of M13 phage and used as a template for site specific mutagenesis (10). All mutations were verified by DNA sequencing.

Strains, Phage and Growth Conditions
Escherichia coli K38 (HfrC, T2R, relA1, pit-10, spoT1, tonA22, ompF627, phoA4, λ; 18) was used as a host for M13 phage. To study complementation of plasmid-derived coat expression, phage with an amber mutation at position +2 in gene VIII (M13am8-M2) was used. For some protein expression experiments the strain BL-21 (hsd6, gal(λclts857, ind1, Sam7, nin5, lacUV5-T7gene1; 28) was used. Media preparation and bacterial manipulations were performed according to standard methods (19). Where appropriate, ampicillin (final concentration, 200 µg/ml) was added to the medium.

For the complementation experiments, 10^3 or 10^5 M13am8-M2 phage particles were mixed with E. coli K38 cells and top agar and applied on a plate. Where indicated 1 mM IPTG was added. The plates were incubated at 37°C overnight. The efficiency of plating (E.O.P.) was calculated by comparing the plaque formation of the wild-type phage. Multiple platings resulted in a standard deviation of each value below 20%.

For procoat protein expression, the plasmid-bearing cells were grown overnight at 37°C and backdiluted 1/100. At a density of 2x10^8 cells per ml, 1 mM IPTG was added and the culture was grown further for 4 h (or for the indicated time) at 37°C. To analyze the procoat and coat protein one portion of the culture was treated with 1 mM copper phenanthroline (CuP) for 10 min (or indicated time). The 50 mM CuP stock solution was freshly prepared essentially as described (9) from 225 mM 1,10-phenanthroline monohydrate in ethanol and 150 mM CuSO4 by mixing 2:1 (vol/vol). The reaction was stopped by 10 mM EDTA and 10 mM N-ethylmaleimide (NEM). The samples were precipitated with 10% trichloroacetic acid and subjected to SDS-PAGE.

Membrane isolation
One liter cultures were grown as described above, cells were harvested by centrifugation and the pellets were resuspended in 6 ml 50 mM Na phosphate, 1 mM EDTA. The cells were lysed by adding 0.2 mg/ml lysozyme for 1 h on ice following ultrasonication (3 pulses for 15 sec). After a low speed spin (12 000 x g for 10 min at 4°C) the membranes were pelleted at 350 000 x g for 10 min at 4°C and resuspended in 1 ml 50 mM Na phosphate, 1 mM EDTA. For oxidation, 1 mM CuP was added for the indicated times at 37°C and the reaction was stopped by adding 10 mM EDTA and 10 mM NEM on ice. Rereduction was performed by adding 0.1 M DTT after stopping the reaction. The samples were precipitated with 10% trichloroacetic acid and subjected to SDS-PAGE.

**SDS-gel electrophoresis**

For reductive SDS-PAGE, the samples were incubated in sample buffer containing 0.1 M DTT at 95°C for 3 min, for non-reducing gels no DTT was added to the sample buffer and the samples were incubated at 40°C for 30 min prior to electrophoresis. The 22% SDS polyacrylamide gel was prepared as described (8). For Western blot analysis the proteins were transferred to nitrocellulose (Amersham), incubated for 150 min at 4°C with serum to M13 coat protein (1:5000 in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) followed by peroxidase linked antibody to rabbit (1:5000) for 90 min. The reaction was visualized by enhanced chemoluminescence detection (Amersham).

**Pulse-label experiments and protein mapping**

_E. coli_ K38 cells were grown overnight in minimal medium lacking methionine and diluted 1/100 into fresh minimal medium. After 2 h growth the cells were induced with 1 mM IPTG and pulse-labeled with 35S-methionine (10 µCi/ml) for the indicated times. To generate spheroplasts, the pulse-labeled cells were collected by centrifugation at 4°C and resuspended in 40% sucrose 33 mM Tris-HCl, pH 8.0. Lysozyme (5 µg/ml) and 1 mM EDTA, pH 8.0 were added and kept on ice for 20 min. Where indicated proteinase K was added (0.5 mg/ml) and incubated on ice for 1 h. Immunoprecipitation was performed as previously described (10). The samples were acid-precipitated and analyzed by SDS-PAGE.
Results

Cysteine mutants in the M13 procoat protein allow normal phage multiplication

To study whether an intramolecular disulfide bond between the leader and the mature region of the M13 procoat protein affects membrane insertion, processing by leader peptidase or phage assembly, single TGC or TGT codons were introduced encoding cysteine residues at positions -6 to -12 in the leader sequence and at positions +30 to +33 in the mature sequence using site-directed mutagenesis (Fig. 1A). They were introduced into an IPTG-inducible plasmid into the cloned gene VIII encoding the procoat protein. E. coli K38 cells bearing the respective plasmids were then infected with M13 phage harbouring an amber mutation in the gene VIII (M13am8-M2). The phage only multiplied, forming plaques on plates when a functional plasmid-derived coat protein was expressed (Table 1). In the absence of induction or in the absence of the gene, less than $10^{-2}$ plaques were formed per infecting particle as a result of revertants of the amber mutation or low level protein expression. When the expression of the plasmid-encoded wild-type protein was induced, phage progeny were assembled and an efficiency of plating (E.O.P.) of about 1 was measured. A similar efficiency was reached (E.O.P. of 0.6 to 0.8) for the mutants coding for a procoat protein with a single cysteine residue at positions -7, -8, -9, -11, +30, +31, +32, +33. A slightly reduced E.O.P. was observed for the mutants with a cysteine at -10 and -12. Except for the mutant with a cysteine at -6, all single cysteine mutants allowed productive phage multiplication.

Bacterial growth inhibition after induction of cysteine mutants at -6, -10 and -12.

To investigate why the single cysteine mutants at -6, -10 and -12 show a low complementation efficiency for the amber VIII phage, we followed the bacterial growth of the induced and non-induced cultures in the absence of phage (Fig. 1C). The wild type and all the other cysteine mutants were not affected in their growth when the coat protein was induced. However, when the expression of the cysteine mutants at -6, -10 and -12 was induced, cell growth was clearly affected. Two hours after induction the mutants at -10 and -12 showed a bacteriostatic reaction, whereas for the cysteine mutant at -6 lysis was observed. This explains why in these cases we observed a lower phage production (Table 1).
Cysteine-containing procoat mutant proteins are inserted into the membrane

To investigate whether the single cysteine mutants were affected for membrane insertion or processing by leader peptidase, the expression of the mutant coat proteins was induced for 2 h and analysed by a Western blot (Fig. 2). All the single cysteine mutant proteins were well expressed and efficiently processed with the exception of the -6C mutant. It is well known that the proline at residue -6 in the wild-type is important for the recognition by leader peptidase (15). We conclude that the single cysteine residues at all the other positions have no inhibitory effect on the membrane insertion and cleavage of the M13 coat protein.

The cysteine mutants were found as monomers on a non-reducing gel (Fig. 2A). A treatment of the cells with copper phenanthroline changed the membrane to an oxidative milieu. This allowed efficient disulfide bond formation of two closely positioned cysteines in the mature regions which led to dimeric structures (Fig. 2B). The efficiency to form disulfides was highest for the cysteines at the positions +30 and +31, suggesting that these residues are closely exposed and interact with each other. Cysteines at +32 and +33 showed dimers with an efficiency of only 64% and 18%, respectively. As expected, only the mature coat proteins with cysteines might oligomerize, whereas the proteins that have a cysteine in the signal sequence and no cysteine in the mature region (lanes 2 to 7) remain as a monomer. The -6C mutant, however, that was only partially cleaved showed dimers with the precursor form (lane 1). We suspect the membrane-inserted procoat proteins might oligomerize when their cleavage is inhibited. However, when the procoat protein is normally cleaved by leader peptidase, the formation of cysteine dimers is prevented by their rapid processing to coat.

Procoat -6C inhibits leader peptidase

Since the growth of cells expressing the cysteine mutant at -6, -10 and -12, respectively, was inhibited, we analyzed at which stage of the pathway the procoat protein was blocked. *E. coli* K38 bearing the respective plasmid was grown to exponential phase and the mutant protein was induced with 1 mM IPTG. The cells grew normally without a plasmid or with a plasmid without induction. In comparison cells expressing procoat -6C stopped growing 2 h after induction and started to lyse 30 min later (Fig. 1C). Similarly, the cysteine mutants at -10C and -12C, respectively,
showed an arrest of cell growth 2 h after induction, however no lysis was observed. At this time, cells expressing the mutant proteins were then tested for procoat processing by leader peptidase and translocation across the membrane. The processing of proOmpA was taken as an indicator of leader peptidase function. The cells were labeled with $^{35}$S-methionine for 1 min and analyzed for their processing to OmpA. Whereas cells expressing the wild-type (not shown) and the –7C mutant, respectively, showed normal processing of proOmpA and procoat (Fig. 3A, lane 2), the cells expressing the mutants at -6C and -12C were affected in their processing of proOmpA (lanes 1 and 3). Also, the processing of the -12C procoat protein (lane 3) was inhibited. Likewise, the -6C procoat mutant was completely blocked (lane 1). The observed defect of processing might be caused by an enzymic inhibition of the leader peptidase or by an inhibition of membrane translocation. To test this, we analyzed the membrane translocation of the –6C coat protein with protease mapping (Fig. 3B, left panels). Proteinase K added to the periplasmic side of the inner membrane showed that most of the accumulated procoat protein was accessible and was therefore translocated (Fig. 3B, lane 2). This was also observed for most of the proOmpA protein (upper panel, lane 2) that had accumulated. In the proteolysis of the –6C procoat, a 3 kDa fragment (arrow pfg) was immunoprecipitated suggesting that the uncleaved procoat protein was partially protected to the outside added protease. This fragment was not observed in cells expressing the -7C procoat mutant that is normally processed (lanes 4 to 6). Estimating from the molecular weight and the antigenic region of coat (residues +2 to +8) the fragment most likely contains the region between –23 to +10. This suggests that the mutant protein remains bound to the leader peptidase and is protected for the region of –23 to +10 that includes the antigenic region. This indicates that the activity of the leader peptidase is affected in these cells. Intriguingly, a double mutant combining the –6C with the non-cleavable H5 mutation at –3, which inhibits the binding to the leader peptidase had no lytic effect on the bacterial growth (data not shown).

**Oligomer formation of coat protein with two cysteines**

When two cysteines were placed into the membrane-spanning region of the procoat protein, oligomeric forms were observed (Fig. 4). The cysteines at +30 and +31 were combined in a double mutant and the protein was analyzed on a non-reducing gel system. The coat proteins with the two cysteines were found mainly in their
monomeric form (lane 6). When the cells were treated with copper phenanthroline (lane 7-9) oligomeric forms were readily observed, whereas for the single cysteine mutant +30C, only dimers appeared (lane 2-4). We conclude from this result that the coat proteins accumulate in the membrane in oligomeric clusters and that the residues, +30 and +31, are able to stabilize intermolecular contacts.

Cysteine procoat mutants can form dimers in the membrane

To see whether the precursor form, the procoat protein, can also form dimers, each cysteine mutant was combined with the H5 mutation that prevents the processing of the protein to its mature form. The H5 mutation substitutes the serine at -3 with a phenylalanine that interferes with the leader peptidase recognition (13). Each of the mutant plasmids was transformed into *E. coli BL-21*, the cells were grown at 37°C to exponential phase and the coat protein expression was induced for 4 h. The cells were acid-precipitated and analyzed by a Western blot (Fig. 5A). To exactly localize the position of the dimeric form, a mutant procoat was genetically constructed that encodes a twin-fused procoat protein of 146 amino acid residues (not shown). A minor amount of the H5 procoat proteins that had a cysteine at the positions -7, -8, -10 and -12, respectively, was found at the dimeric position (lanes 3, 4, 6, 8). Intriguingly, the same mutants showed some processing to the coat protein (see coat arrow). The procoat proteins with a cysteine in the mature region did not spontaneously form dimers (lanes 9 – 12).

When the samples were analyzed under oxidizing conditions, most of the protein was shifted to the dimeric position (Fig. 5B). Whereas the control, the H5 procoat protein without a cysteine residue did not shift (lane 1), all the cysteine mutants, including the -6C mutant were now found as dimers. Intriguingly, the mutants -9C, -10C, -12C and +31C showed in addition to the dimer band higher molecular weight bands that most likely represent contacts to other proteins or procoat multimers. Since a single cysteine in a protein can participate in only one disulfide bond and thereby give rise to only one dimer (between two procoat proteins), the additional bands must derive from other interactions that might be stabilized by disulfide bonds.

For some of the single cysteine mutants in the mature region a portion shifted to a slightly lower position than the dimer (lanes 9 to 12). These protein bands might
represent disulfides between a procoat and a coat molecule (PC-C) which is possible if the cysteine residue is in the mature region. In these cases, the H5 mutation had not fully blocked the processing to coat protein.

We then analyzed whether the dimers and multimers can also be found in isolated membranes. To do this, the exponential growing cells were induced for 4 h. The cells were harvested and treated with lysozyme and by sonication. The membranes were isolated by differential centrifugation. When the membranes with the procoat mutant -6C were treated with 1 mM copper phenanthroline, dimers readily appeared in a time-dependent manner (Fig. 6A). Similar, the -7C mutant showed efficient dimer formation of the procoat form (Fig. 6B). In this case, a portion of the protein was cleaved to coat protein, possibly due to the long expression time (4 h) and the membrane isolation procedure. The resulting coat protein was not converted to a dimer and remained as a monomer. The mutants -8C, -9C (not shown) and -10C (Fig. 6C) had already some dimers in the untreated samples. This is similar to what we had observed in the results shown in Fig. 5A. The treatment of these mutants with copper phenanthroline showed the appearance of higher molecular weight bands with a simultaneous disappearance of the monomeric and dimeric procoat band. When the treated membranes were rereduced the higher molecular weight bands disappeared and the monomeric procoat band increased in intensity (Fig. 6C, lane 9). In all cases, the portion of the mature coat protein remained at the monomer position since all these mutants have the cysteine in the leader sequence.

Procoat proteins with two cysteine residues affect phage multiplication

The single cysteine mutants that supported phage assembly were combined with a second cysteine mutation (Table 2). To see whether a possible intramolecular disulfide between the leader peptide and the mature hydrophobic region could interfere with phage multiplication, the mutants were analyzed for their capability to support plaque formation of a M13am8-M2 infection. As shown in Table 2, most of the double cysteine mutants allowed normal phage growth. However, the mutants with cysteines at +32 and a second cysteine at -7, -9 or -11 and mutants with a cysteine at +33 and -9 or -11 were strongly inhibited for phage growth and did not allow plaque formation. The double cysteine mutants were tested for their expression and processing on SDS-polyacrylamide gels (Fig. 7). Most of the mutants migrated
at the position of the coat protein showing that the cleavage by leader peptidase was not generally inhibited by two cysteine residues. We found that the amount of coat protein corresponded to the efficiency of phage propagation (Table 2). For the mutants with one cysteine at +32 or +33 and the second cysteine at -9 or -11 only a weak coat protein band was visible suggesting that these proteins were unstable. We verified in a pulse chase experiment that these mutant proteins were processed to a mature form, but were degraded (data not shown). Since the single cysteine mutants were stable, the conformation of the double mutants might be different and defective for phage assembly. We conclude that the instability of the protein mutants is related to the observed inhibition of phage assembly.

Procoat proteins with two cysteine residues form multimers

The double cysteine mutants were combined with the H5 mutation to see whether the precursor proteins can form intramolecular disulfides and multimeric forms in the membrane as was observed for the coat protein (Fig. 4). It is expected that intramolecular disulfides are migrating faster than the monomer (35). When analyzing the procoat protein on a Western blot, most of the mutant procoat proteins had identical mobility (Fig. 8A). However, the mutants H5/32C-8C, H5/32C-9C, H5/32C-10C, H5/33C-8C and H5/33C-10C showed some of the protein with an increased mobility, possibly representing some cleaved coat protein. The relative amount, however, was less than 10% of the procoat protein and most of the protein was at the normal position. These protein mutants were also partially present in dimeric forms. We conclude that these procoat protein mutants are engaged in intermolecular and not in intramolecular contacts under the normal growth conditions.

The same cells were treated with copper phenanthroline to investigate whether all the procoats can efficiently interact with each other under oxidative conditions. All double cysteine mutants showed multimeric forms (Fig. 8B). This suggests that already the procoat proteins are in close contact with each other which allows multiple disulfide bonding within the membrane under oxidizing conditions.
Discussion

The assembly of the filamentous phage at the cytoplasmic membrane is a fascinating process that is still not well understood. Transmembrane coat proteins are loaded most likely as oligomers onto the single-stranded DNA and seal the virion into a tightly folded structure. To address the question if oligomers of the coat protein are already formed in the membrane of *E. coli* prior to the virus assembly, we generated a collection of mutant coat proteins that contain one or two cysteine residues within their hydrophobic domains. We anticipated that a cysteine residue in the leader sequence would form procoat dimers and could block the processing step. Likewise, a cysteine in the mature region would form coat dimers that could be blocked in the assembly of the phage (6). Surprisingly, the cysteines did not generally inhibit the multiplication of the phage (Table 1). The analysis of the procoat and coat proteins localized in the cytoplasmic membrane by SDS-PAGE revealed that only few procoat protein mutants were found as dimers (Fig. 5A). This suggests that the membrane environment is rather reductive and will not allow disulfide bridges to form. When we treated the cells with copper phenanthroline, a hydrophobic oxidizing reagent, procoat dimers (Fig. 5B) and coat dimers were readily formed (Fig. 2B). Since this reaction occurred instantly (Fig. 6), we conclude that both the membrane-inserted procoat and coat proteins are organized in clusters of multimers. Indeed, multimers of procoat were found with the mutants containing two cysteines (Fig. 8B) and multimers of coat were found when two cysteines were present in the mature region (Fig. 4).

Copper phenanthroline has been previously used to demonstrate a close contact between two transmembrane helices. The Tar chemoreceptor of *E. coli* was shown to dimerize by its TM1 region, but not by TM2 (17). For the Trg chemoreceptor intramolecular disulfides were found and the interacting faces of TM1 and TM2 matched on a helical wheel (14). The maximum distance between two helical axes is 7-12 Å to allow the formation of a disulfide bond (1). When the disulfides are formed instantly, the two partner helices must be in close contact. When the formation of disulfides takes a longer time, the molecules possibly require movement within the membrane or the two cysteine residues are shielded to react with copper phenanthroline.
Previously, it has been shown that a cysteine mutant at position +35 of the coat protein forms a disulfide when it is integrated in the membrane (6). We now find that residues at the positions +30 and +31 efficiently interact (Fig. 2B), whereas the residues at +32 and +33 are less reactive. This is consistent with the protein-interactive region that was proposed by Deber et al. (2). Such an interactive region suggests that the coat proteins in the membrane are organized in an ordered fashion and are not freely mobile. An intermolecular interaction of the hydrophobic regions is also implicated from the presence of a GxxxG motif that is found at +34 to +38 within the transmembrane region of coat. The GxxxG motif has been found in glycophorin A to promote helix-helix interactions (24, 30). Taken together, the proposed contacts between two coat proteins can be summarized in a model, where the two proteins are tilted. There are indications for such a tilted conformation of coat protein in a lipid bilayer and it has been shown that the protein is tilted with about 20° to the membrane normal (16). When two interacting coat proteins are tilted against each other by 40°, residues +23, +27, +31 and +35 are in close contact (Fig. 9A). The tilted helices could then interact with two additional neighbouring helices resulting in additional close contacts (Fig. 9B). As a consequence, a multimeric protein sheet structure is formed involving more than two proteins.

Remarkably, in the phage particle, the coat proteins are also arranged in a tilted fashion of about 20° compared to the phage axis (22, 26). However, in the phage the coat proteins form an interdigitated helical array, such that one protein comes into contact with 10 neighbouring subunits. All the coat proteins are tilted at the same angle. As a result, many residues (among them the residues +30, +31 and +35) are not exposed to the outside of the phage (22). Given that the tilted coat helix is found in the membrane-anchored protein as well as in the phage particle, we conclude that little structural rearrangement is necessary during the phage assembly process. Thereby, the protein can keep its basic structural arrangement from the host membrane to the free phage particle such that the assembly process possibly occurs from binding multimeric sheets of coat proteins out of the membrane (Fig. 9C). This assembly model contrasts a previously proposed mechanism proposing a major topological change from the membrane to the phage (21).
Whereas the formation of multimers of coat proteins makes sense regarding the phage assembly process, we were surprised to see that also the procoat protein was found in oligomeric forms (Fig. 8). Since all procoat mutants were capable to initiate the formation of multimers, we conclude that the proteins can rotate in the membrane. However, we found that at positions -7, -8, -10 and –12 spontaneous dimers were formed (Fig. 5A) suggesting that there might be a preferred region of interaction between two procoat proteins.

We did not find a major fraction of intramolecular contacts in the procoat protein mutants. In the normal pathway, cleavage by leader peptidase occurs rapidly, and only cysteines in the mature region were able to form disulfides (Fig. 2). These dimers were mainly found when we treated the cells with the hydrophobic oxidant copper phenanthroline (Fig. 2B). In untreated cells, the intramembrane milieu is reductive and only minor amounts of dimers were observed (Fig. 5A, 8A). This also explains why the cysteine mutants had generally no effect on the multiplication of the phage (Tables 1 and 2). The instances where the phage production was reduced had reasons other than the interference by disulfide formation. For the single cysteine mutants, the expression of –6C and –12C had a lethal or static effect on the bacterial growth, respectively. Interestingly, when the same mutations were combined with H5, a mutation that prevents cleavage (-3F), no growth defect was observed (data not shown). Topological analysis with proteinase K showed that the Sec-independent procoat proteins and also the Sec-dependent proOmpA accumulated in a translocated form (Fig. 3B) suggesting that the processing of the precursor proteins by leader peptidase, but not the translocation across the membrane was inhibited. We suspect that the cysteine mutants remain bound to leader peptidase and block the enzyme. Binding to leader peptidase is prevented with the H5 mutation which explains why the expression of the H5 cysteine mutants had no lethal effect.

In contrast, we did not find intermolecular disulfides of the –6C procoat protein with leader peptidase. This is plausible, since leader peptidase has no cysteine residues in the center of its two transmembrane segments (27). The formation of protein disulfides in the membrane has also been used to analyse the interactions between two different proteins. The interaction between SecE and SecY was studied by cysteine-scanning (33) and also between SecG and SecY (21). Intramolecular
contacts between two transmembrane regions were found within the Tar receptor protein (28), the lactose permease (34) and leader peptidase (35).

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Table 1
Single cysteine mutants affecting the efficiency of plating of M13am8-M2 on *E. coli* K38

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$^1$ Efficiency of plating
Table 2
Double cysteine mutants affecting the efficiency of plating of M13am8-M2 on *E. coli* K38

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Figure legends

Figure 1. Cysteine mutants in M13 procoat protein

(A) Amino acid sequence of the M13 procoat protein. The bold letters depict the positions which were mutated to single cysteine residues. The leader sequence is numbered with –23 to –1, the mature region from +1 to +50. The boxes highlight the membrane-spanning regions. (B) Membrane insertion and assembly pathway of M13 procoat. (C) Expression of the cloned procoat protein from plasmids bearing single cysteine mutants affects the growth of *E. coli* K38. Exponentially growing cultures were induced (open symbols) at the 2 h timepoint (arrow) and the optical density was monitored at 600 nm. As controls the uninduced cultures (full symbols) were monitored. Wild type procoat (●, ○), mutant -6C (●, ○), mutant -7C (▲, △), mutant -10C (■, □), and mutant -12C (●, ○) are shown. The culture without a plasmid is shown as a dotted line.

Figure 2. Processing and dimerization of M13 single cysteine procoat mutants

*E. coli* BL-21 cells bearing a plasmid to express a single cysteine procoat mutant was (A) induced for 4 h, analyzed on a non-reducing SDS-gel and visualized by a Western blot. (B) After 4 h induction, the samples were oxidized with 1 mM copper phenanthroline and analyzed as in (A). The cysteine mutants at –6 (lanes 1), -7 (lanes 2), -8 (lanes 3), -9 (lanes 4), -10 (lanes 5), -11 (lanes 6), -12 (lanes 7), +30 (lanes 8), +31 (lanes 9), +32 (lanes 10) and +33 (lanes 11) were analyzed.

Figure 3. Expression of procoat -6C inhibits leader peptidase

*E. coli* K38 cells bearing a plasmid to express procoat -6C, -7C or -12C, respectively, were grown in minimal M9 medium and induced for 2 h with 1 mM IPTG. 35S-methionine was added for 1 min and the sample was immediately chilled by mixing with ice cold medium. (A) The cells were acid precipitated and analysed for their processing of proOmpA (upper panel) and procoat (lower panel) by immunoprecipitation, SDS-PAGE and phosphorimaging. Cells expressing procoat –6C (lane1), procoat –7C (lane 2) and procoat –12C (lane 3) are shown. The position of proOmpA, OmpA, procoat and coat protein are indicated by arrows. (B) To analyze the membrane translocation of the proteins the cells were converted to spheroplasts and 1 mg/ml proteinase K was added to the outside of the cells for 1 h on ice (lanes 2, 5). Cells expressing procoat –6C (lanes 1-3) and procoat –7C (lanes
4-6) are shown. As a control, the cells without proteinase K (lanes 1, 4) and cells treated with TritonX-100 and proteinase K (lanes 3, 6) are shown. The cytoplasmic GroE protein was analyzed to indicate that the spheroplasts remained intact during proteolysis.

Figure 4. Oligomerization of the M13 coat protein in the membrane

E. coli BL-21 cells bearing a plasmid to express procoat +30C (lanes 1-5) or +30C/31C (lanes 6-11) were induced for 3 h (lanes 1 and 6) or induced for 3 h and treated with 1 mM copper phenanthroline for 1 min (lanes 2 and 7), 10 min (lanes 3 and 8) or 30 min (lanes 4 and 9). For a control, the copper phenanthroline-treated sample was rereduced by adding 100 mM DTT (lanes 5 and 10) or pretreated with 10mM NEM and 10 mM EDTA before the treatment with copper phenanthroline for 10 min (lane 11). The samples were acid-precipitated and analyzed by non-reducing SDS-PAGE and Western blotting.

Figure 5. Dimerization of H5 procoat mutants

E. coli BL-21 cells bearing a plasmid to express a single cysteine procoat H5 mutant protein was (A) induced for 4 h (A) or induced for 4 h and treated with 1 mM copper phenanthroline for 10 min (B). The cells were acid-precipitated and analyzed by non-reducing SDS-PAGE and Western blotting. PC-C depicts the dimer of a coat and procoat protein.

Figure 6. Oxidation kinetics with copper phenanthroline

The formation of procoat dimers was studied depending on the time of phenanthroline exposure. E. coli BL-21 cells bearing plasmids coding for procoat H5/-6C (A), H5/-7C (B) and H5/-10C (C), respectively, were induced for 4 h. The cell membrane fraction was collected and exposed to 1 mM copper phenanthroline for the indicated times. The reaction was stopped by addition of 10 mM EDTA and 10 mM NEM. The samples were acid-precipitated, analyzed by non-reducing SDS-PAGE and Western blotting. Lanes 1 show the samples pretreated with 100 mM DTT, lanes 2 the untreated samples (-CuP), lanes 4 to 8 the copper phenanthroline-treated samples and lanes 9 correspond to lane 8 but were rereduced with 100 mM DTT. The arrows indicate the position of the procoat dimer.
Figure 7. Expression and processing of procoat double cysteine mutants

_E. coli_ K38 cells bearing a plasmid to express the double mutants with a cysteine at position +31 (A), +32 (B) and +33 (C), respectively, and a second cysteine at the position indicated in the abscissa were induced for 3 h, acid-precipitated and analyzed by SDS-PAGE and Western blotting.

Figure 8. Oligomerization of H5 procoat double cysteine mutants

_E. coli_ BL-21 cells bearing a plasmid to express procoat H5 with two additional cysteine mutations at the indicated positions were induced for 4 h (A) or induced for 4 h and incubated with 1 mM copper phenanthroline for 10 min (B). The samples were acid-precipitated and analyzed by non-reducing SDS-PAGE and Western blotting with anti coat. One cysteine residue was at +30 (lanes 1 to 6), at +31 (lanes 7 to 12), at +32 (lanes 13 to 18) and at position +33 (lanes 19 to 23). The second cysteine was at –6 (lanes 1), -7 (lanes 2,7,13,19), -8 (lanes 3, 8, 14, 20), -9 (lanes 4, 9, 15, 21), -10 (lanes 5, 10, 16, 22), -11 (lanes 6, 11, 17, 23) and –12 (lanes 12, 18).

Figure 9. Model for the assembly of coat oligomers onto the nascent phage particle

(A) Two coat proteins interact joining the residues +27, +30, +31 and +35. (B) Multimeric sheets are formed. (C) The coat proteins retain their tilt and their interprotein contacts from the membrane to the viral particle.
A

-23
MKKSLVLK  ASVAVATLVP  MLSFA  AEGDDPAKAAYNLSLQASATE  YIGYAWAMVVIVGATIGI  KLFKKFTSKAS

B

Insertion  Processing  Oligomerization  Assembly into phage

C

Bacterial Growth (A6000)

0  0.2  0.4  0.6  0.8  1  1.2

Time (h)

0  1  2  3  4  5  6  7

K38  wt ind.  wt  wt 6C ind.  wt 6C  wt 7C ind.  wt 7C  wt 10C ind.  wt 10C  wt 12C ind.  wt 12C