

## Spontaneous insertion of gene 9 minor coat protein of bacteriophage M13 in model membranes

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Received 23 October 2000; received in revised form 17 January 2001; accepted 30 January 2001

### Abstract

Gene 9 minor coat protein from bacteriophage M13 is known to be located in the inner membrane after phage infection of *Escherichia coli*. The way of insertion of this small protein (32 amino acids) into membranes is still unknown. Here we show that the protein is able to insert in monolayers. The limiting surface pressure of 35 mN/m for 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol lipid systems indicates that this spontaneous insertion can also occur in vivo. By carboxyfluorescein leakage experiments of vesicles it is demonstrated that protein monomers, or at least small aggregates, are more effective in releasing carboxyfluorescein than highly aggregated protein. The final orientation of the protein in the bilayer after insertion was addressed by proteinase K digestion, thereby making use of the unique C-terminal location of the antigenic binding site. After insertion the C-terminus is still available for the enzymatic digestion, while the N-terminus is not. This leads to the overall conclusion that the protein is able to insert spontaneously into membranes without the need of any machinery or transmembrane gradient, with the positively charged C-terminus remaining on the outside. The orientation after insertion of gene 9 protein is in agreement with the 'positive inside rule'. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Spontaneous insertion; Bacteriophage M13; Model membranes

### 1. Introduction

Cleavable leader peptides or uncleaved signals can function to initiate translocation of proteins across membranes [1]. However, translocation of short domains with no or a small number of charged residues often does not require such signals. Examples of short proteins that translocate in this way include the Pf3 and M13 major coat proteins [2]. Whether gene 9 minor coat protein (g9p) of bacteriophage M13 is also able to translocate across membranes will be addressed in this paper.

Abbreviations: g7p, g8p, g9p, respectively gene 7, gene 8 and gene 9 protein; C16S, g9p with mutation Cys<sub>16</sub> to Ser; C16S/S32C, g9p with mutations Cys<sub>16</sub> to Ser and Ser<sub>32</sub> to Cys; CF, 5,6-carboxyfluorescein; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; L/P, lipid to protein molar ratio; LUV, large unilamellar vesicle; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; TFA, trifluoroacetic acid; TFE, trifluoroethanol

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g9p is a minor coat protein of bacteriophage M13. The amino acid sequence of this 32-residue protein (molecular mass 3681 Da) is the following [3]: HCO-Met-Ser-Val-Leu-Val-Tyr-Ser-Phe-Ala-Ser-Phe-Val-Leu-Gly-Trp-Cys-Leu-Arg<sup>+</sup>-Ser-Gly-Ile-Thr-Tyr-Phe-Thr-Arg<sup>+</sup>-Leu-Met-Glu<sup>-</sup>-Thr-Ser-Ser-COOH.

A few copies of the g9p are located together with g7ps at one end of this 880 nm long cylindrically shaped filamentous phage [4]. The main constituents of the bacteriophage are the single stranded DNA, surrounded all along the cylinder with approx. 2700 copies of g8ps, also known as major coat proteins. Five copies each of the minor coat proteins, which are the products of gene 3 and gene 6, cover the other end.

Bacteriophage M13 uses *Escherichia coli* cells as a host, entering by the F-pilus. Coat proteins, as well as non-structural viral proteins, such as gene 1, gene 4, gene 11 proteins, and host proteins (thioredoxin) play a role in the assembly process of newly formed bacteriophages (for reviews see [5–9]). The gene 9 and gene 7 minor coat proteins were shown to interact with a hairpin loop in the viral DNA [10], thereby playing an important role in initiating the assembly process. Because of this interaction with the packaging signal the phage end containing g9ps and g7ps is the first to emerge from the cell [11]. Prior to incorporation in the bacteriophage g9p is known to be located in the *E. coli* inner membrane [12], probably mainly  $\alpha$ -helical, as has been demonstrated in model membranes [13].

The protein is synthesised and inserted into the inner membrane [12] without any signal sequence. The finding that the N-terminus of the g9p contains a formyl group, and therefore is not processed [14], gives rise to the idea that the protein must insert rapidly into the membrane, before proteolytic processing can occur. g9p does not contain a cleavable signal peptide. However, when looking at the sequence there are similarities with the general features of signal peptides, although the sequence is in a reversed order (positive charges are located C-terminally from the hydrophobic region). Similar to signal peptides [15,16], g9p contains a hydrophobic stretch followed by a hydrophilic basic region with two arginine residues. The structure of signal peptides seems to be designed for interaction with lipids, as

demonstrated from studies of the interaction with and translocation into phospholipid vesicles [17–19]. The N-terminal side of g9p is the most likely side to insert, since the N-terminus remains formylated after membrane insertion and is still formylated after assembly in the viral particle [14].

In the present paper we contribute to the model presented above showing the penetrative strength of g9p by monolayer and vesicle leakage experiments. The orientation of g9p, after reconstitution and insertion, is addressed by proteinase K digestion, thereby making use of the C-terminal location of the antigenic site.

## 2. Materials and methods

### 2.1. Materials

Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany), and trifluoroethanol (TFE) was obtained from Acros (Pittsburgh, PA, USA). 1,2-Dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Proteinase K was from Boehringer (Mannheim, Germany). Alkaline phosphatase was from Boehringer. Polycarbonate filters (200 nm) were from Avestin (Ottawa, Canada). The mini-extruder device was from Milsch Equipment (Laudenbach, Germany). 5,6-Carboxyfluorescein (CF) was obtained from Kodak, and used after purification. The M13mp18rf template was from Pharmacia (Uppsala, Sweden). The Quikchange protocol was from Stratagene (La Jolla, CA, USA). The g9p was prepared by solid phase synthetic techniques and characterised as described before [13]. The N-terminus was synthesised with a formyl group.

### 2.2. Solubilisation of G9p in TFE

The protein was first dissolved in a small volume of TFA (10–20  $\mu$ l/mg of protein) and dried under a stream of nitrogen. Next, the protein was washed two times with TFE, followed by evaporation of the TFE under a stream of nitrogen, to remove residual TFA. Finally the protein was dissolved in a

known volume of TFE. The protein concentration was determined by the procedure of Peterson [20] with bovine serum albumin as a standard.

### 2.3. Monolayer experiments

For the monolayer experiments, the pressure was measured by the Wilhelmy balance method using filter paper in a thermostatically controlled box, using a Cahn D202 microbalance [21]. This method is based on the determination of the weight of the filter paper, which is partially submersed in the aqueous subphase. Differences in plate weight are directly related to surface pressure. The measurements were performed as follows. The lipid monolayers were spread from a chloroform solution to give an initial surface pressure between 15 and 40 mN/m on a subphase solution of 10 mM Tris-HCl, 0.2 mM EDTA, pH 8.0 (TE8). During the measurements the temperature was kept at  $22.5 \pm 1^\circ\text{C}$ , and the subphase was continuously stirred with a magnetic bar. Instead of a teflon dish a glass dish with a teflon coated rim was used, which allows more rigorous cleaning of the dish between the separate experiments. The glass dish had a volume of 4 ml and a surface of 4.41 cm<sup>2</sup>. Protein, dissolved in 30  $\mu\text{l}$  of TFE/TE8 (1:1) was added through a hole on one side of the dish. Unless stated otherwise, a saturating amount of protein was injected into the subphase. The pressure changes were followed until the surface pressure increase had reached a maximal value, usually within 30 min.

### 2.4. Reconstitution of *g9p* into lipid vesicles

To reconstitute the protein into lipid bilayers, desired amounts of lipids (in chloroform) and protein (in TFE) were mixed, dried under a stream of nitrogen and lyophilised overnight to remove residual solvent. The sample was rehydrated in the desired volume of buffer and freeze-thawed several times. To prepare large unilamellar vesicles (LUVs) the sample was extruded 31 times through a polycarbonate filter with 200 nm pores. The filters were mounted in a mini-extruder device as described in the literature [22]. Vesicles without protein were prepared in the same way.

### 2.5. Sucrose gradient centrifugation

Samples were brought on top of a linear 5–40% (w/w) sucrose gradient in 10 mM Tris, 0.2 mM EDTA, 150 mM NaCl, pH 8.0, and centrifuged for 18 h at  $110\,000 \times g$  at  $4^\circ\text{C}$  in a Beckman SW41 rotor. To enable visualisation of the lipid-protein complexes, the gradient was fractionated and analysed by gel electrophoresis and Western blotting as described below.

### 2.6. Preparation of carboxyfluorescein loaded vesicles and determination of release of carboxyfluorescein

An amount of 12.5  $\mu\text{mol}$  of lipid (DOPC or DOPG), dissolved in chloroform, was dried under a stream of nitrogen followed by high vacuum for several hours. CF (121 mM) in 2 ml of a solution of 10 mM Tris-HCl, 0.2 mM EDTA, pH 8.0 was used to rehydrate the lipids. The solution was vortexed and subjected to several cycles of freezing in liquid nitrogen and thawing in hot water to form multilamellar liposomes. Subsequently, the vesicles were converted into LUVs by extrusion as described above. External CF was removed by passing the vesicles over a P10 column ( $1.5 \times 20$  cm), pre-equilibrated with 10 mM Tris-HCl, 0.2 mM EDTA, 50 mM NaCl, pH 8.0. After collection of the CF-loaded vesicles, they were diluted with the same buffer to give a maximal optical density at 490 nm between 0.09 and 0.1 in a 1 cm cuvette. Protein was added in a 1:1 mixture of TFE:buffer to 1 ml of the vesicle solution and stirred. The release of CF was followed in time on a Perkin-Elmer LS-5 fluorescence spectrometer (excitation slit 5 nm, emission slit 5 nm) at an excitation wavelength of 490 nm, and an emission wavelength of 520 nm. The measurements were performed at room temperature. Maximal (100%) fluorescence was obtained by addition of 20  $\mu\text{l}$  of 10% (v/v) of Triton X-100. To check for background leakage, an equal volume of solvent without protein was added. The lipid to protein molar ratios (L/P) of the samples were calculated from the known amount of protein added, and the phospholipid concentration determined by total fluorescence intensity of CF after addition of cholate (final concentration 50

mM) to release all CF from the vesicles. An average diameter of the vesicles of 200 nm is assumed.

### 2.7. Overexpression and mutagenesis of g9p

Overexpression of g9p was achieved by introducing M13 gene 9 into the pT7-7 vector [23]. By PCR, using M13mp18 RF as a template, *Nde*I and *Bam*HI sites were introduced respectively preceding and following the gene 9 protein-coding gene. After insertion in the respective sites of pT7-7, the plasmid was introduced in *E. coli* BL21(DE3) strain and expression of g9p was obtained in LB medium after induction by IPTG to a final concentration of 0.25 mM. Cells were harvested 1 h after induction. The presence of g9p before and after induction was analysed as described below. Mutant g9ps were produced using the Quikchange protocol. By sequencing the plasmid DNA the presence of the mutation was checked.

### 2.8. Protein analysis by sodium dodecyl sulphate (SDS) gel electrophoresis and Western blotting

To verify the presence of the g9p after overexpression, gel electrophoresis on a tricine SDS polyacrylamide gel was performed [24]. Western blotting [25] and immunodetection were used to verify the reaction with antibody, using goat-anti-rabbit IgG conjugated with alkaline phosphatase as the secondary antibody. Primary antibodies were raised against synthetic protein in female New Zealand white rabbits. Spot intensities were determined by image analysis after white light scanning of the blot, thereby using a calibration curve from a series created by blotting of known relative amounts of protein.

### 2.9. Cleavage of g9p by proteinase K

Samples of reconstituted g9p in 200 nm lipid vesicles, as well as the 200 nm lipid vesicles used in the insertion experiments, were produced as described above. For insertion, g9p, dissolved in a small volume of TFE, was added to the lipid vesicles and left at room temperature for 1 h. 1 ml of the mixtures contained 1.6  $\mu$ mol lipid and 3.2 nmol g9p, resulting in an L/P ratio of 500. Vesicles with protein attached were purified from non-interacting protein by sucrose gradient. The clear visible band was iso-

lated and dialysed for 16 h against buffer. After dialysis proteinase K was added, at a concentration of 50  $\mu$ g/ml, and the samples were incubated at room temperature during 1 h. The reaction was stopped by adding phenylmethylsulphonyl fluoride (PMSF; final concentration 5 mM), an equal volume of gel electrophoresis sample buffer (double concentration) and immediately putting in a boiling water bath for 3 min. The samples were then further analysed by gel electrophoresis and Western blotting as described above.

## 3. Results

### 3.1. Monolayer experiments

The interaction of g9p with lipid monolayers was monitored by measurement of the surface pressure increase in a constant area setup. Since extrinsic proteins and other non-penetrative agents do not induce an increase of the monolayer surface pressure [26], this effect can be related to the degree and nature of penetration. The highest initial pressure allowing penetration, the limiting surface pressure, is often used as a measure of the penetrative force. The g9p is surface active itself, spontaneously forming a protein monolayer to a surface pressure of 23 mN/m after introduction into the subphase. The injection

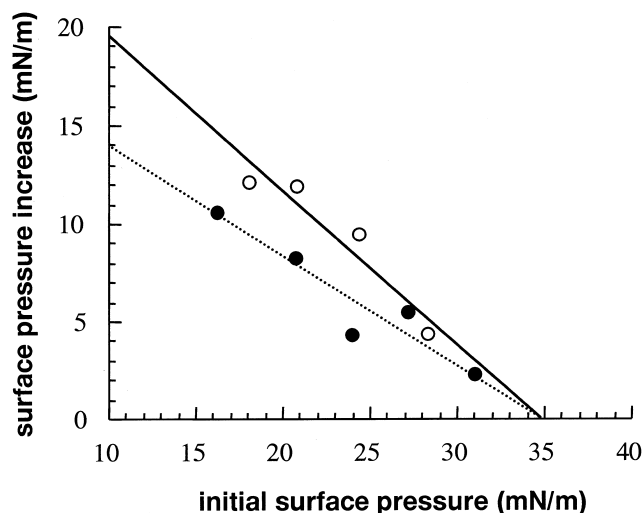


Fig. 1. Surface pressure increase after injection of 11.6 nmol (resulting in a 2.9  $\mu$ M concentration in the subphase) g9p in the subphase of monolayers of DOPC (●, dotted line) and DOPG (○, solid line) as a function of the initial pressure.

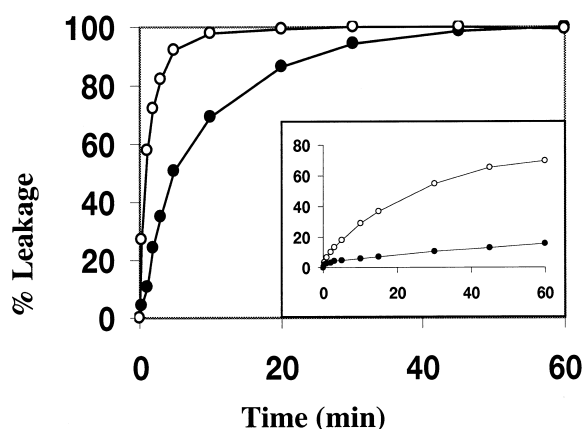


Fig. 2. g9p (1 nmol) added in respectively 4 µl (●) and 40 µl (○) TFE to CF-loaded DOPC at an L/P ratio of 25. (Inset) g9p (0.8 nmol) added in respectively 50% (v/v) (○) and 20% (v/v) TFE/water (●) to CF-loaded DOPC at an L/P ratio of 38.

of g9p under a phospholipid monolayer gave rise to a surface pressure increase, which reached a stable end level after 20–30 min. Injection of the solvent alone had a small effect on the surface pressure of 0.5–1.0 mN/m. The maximal pressure increase in DOPC is dependent on the amount of protein added in the subphase up to a saturating level. For the negatively charged DOPG similar results were found (data not shown). The saturating level was determined (data not shown) and for further experiments an injected amount of 11.6 nmol of protein was chosen, which is sufficient to saturate the monolayer and produce a stable end pressure within 30 min. Fig. 1 shows the dependence of the final pressure increase on the initial pressure with a DOPC monolayer. A higher initial surface pressure results in a smaller pressure increase, indicating a more restricted penetration. From this figure, a limiting surface pressure of about 35 mN/m could be derived. Measurements performed with DOPG monolayers, also shown in Fig. 1, resulted in the same limiting surface pressure of about 35 mN/m, although the slope of the curve was steeper.

### 3.2. Leakage of carboxyfluorescein

In addition to the insertion of g9p into phospholipid monolayers the effect of the g9p on bilayers was investigated. Fig. 2 shows the influence of the TFE/g9p ratio on the rate of leakage of CF-loaded 200

nm DOPC vesicles. The initial leakage rate is higher at a higher TFE/g9p ratio. No leakage was observed upon addition of TFE alone. Decreasing the percentage TFE (v/v) in the protein solution, before adding, also decreases the leakage rate as shown in Fig. 2 (inset).

### 3.3. Location of the antigenic site

The antigenic site of the g9p was predicted to be located at the C-terminal side (Fig. 3A). We found evidence for this, based on the expression and detection of two mutant proteins. In both mutant proteins the Cys<sub>16</sub> was changed to Ser. Additionally, one mutant protein also contained the Ser<sub>32</sub> to Cys mutation (C16S/S32C). Although both mutant proteins were present after overexpression, as shown for the C16S/S32C mutant protein by protein coloured SDS gel electrophoresis (Fig. 3B), only the protein with the C16S mutation is detectable by Western blotting (Fig. 3B). This indicates that changing Ser<sub>32</sub> to Cys completely abolishes antibody binding to the protein. This confirms the C-terminal location of the antigenic site.

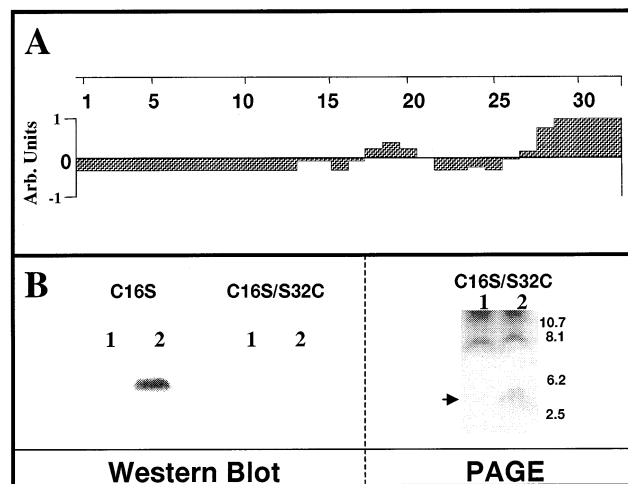


Fig. 3. (A) Antigenic index prediction for g9p, according to Jameson-Wolf [32]. (B) On the left side is a Western blot of the C16S and C16S/S32C mutated proteins before (lane 1) and after overexpression (lane 2). On the right side is a protein coloured SDS tricine gel. Lane 1 shows the situation before and lane 2 after overexpression of the mutant protein C16S/S32C. Marker protein positions are also indicated (kDa). The arrow indicates the position of the g9p.

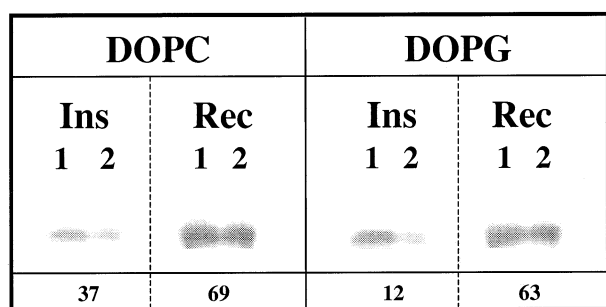


Fig. 4. Western blot of proteinase K digestion experiments of g9p incorporated in vesicles by reconstitution (Rec), or incorporated in vesicles by spontaneous insertion (Ins) after sucrose gradient purification. The left four lanes represent DOPC, the right four lanes represent DOPG. In each lipid system the left two lanes represent inserted (Ins) g9p, and the right two lanes represent reconstituted (Rec) g9p. The samples without proteinase K are marked by 1, the same samples after incubation with proteinase K are marked by 2. The figures at the bottom indicate the percentage intensity left at the spots after proteinase K digestion.

### 3.4. Proteinase K digestion experiments

The orientation of g9p in membrane vesicles after insertion was addressed by proteinase K digestion experiments. Cleavage of the C-terminal side, which contains the antigenic site, was followed by the disappearance of intensity on a Western blot. In these experiments vesicles with inserted g9p were compared with vesicles in which g9p was reconstituted. The experiment was performed with 200 nm vesicles consisting of DOPC or DOPG. After incubation, sucrose density centrifugation followed by Western blot analysis was performed. Comparing the initial blot intensities of the inserted and reconstituted system before digestion shows that the efficiency for insertion is low. No inserted protein, which is most likely aggregated, ended up at the bottom of the tube after centrifugation (data not shown). Fig. 4 shows the Western blot of the samples before and after addition of proteinase K to sucrose gradient purified g9p containing DOPC and DOPG vesicles. In comparing DOPC and DOPG, it can be seen that after reconstitution about 69% of the protein in DOPC and 63% of the protein in DOPG cannot be cleaved during the time of the experiment. After insertion of g9p in DOPG and DOPC respectively 12% and 37% of the initial amount of protein was left after digestion.

## 4. Discussion

### 4.1. Interaction with lipids

Monolayer measurements show that g9p has the ability to penetrate into lipid monolayers of DOPC and DOPG. Fig. 1 shows that upon addition of protein to a lipid monolayer the surface pressure increases. Protein molecules that are known to interact only with the phospholipid head group do not induce such an increase in surface pressure [27]. Therefore this increase can be interpreted as the result of protein insertion in the hydrophobic part of the monolayer. We found for monolayers of DOPC as well as DOPG a limiting surface pressure of about 35 mN/m. The limiting pressure value is indicative of the potency of the protein to penetrate into membranes. The measured limiting pressure for the g9p is in the range of pressures assumed for biological membranes, for which an 'equivalence pressure' of 25–35 mN/m has been estimated [28]. This indicates that the ability of the g9p to insert into lipid monolayers might as well occur *in vivo*. The limiting surface pressure is comparable with values found for other penetrating peptides, such as PhoE and M13 major coat protein signal sequences [29]. With either lipid, the limiting surface pressure is about 35 mN/m. Apparently, the insertion is mainly based on hydrophobic forces, and less on electrostatic attraction. Although these experiments show insertion into monolayers, they do not provide information on how much and what region of the protein inserts. Nevertheless, it can be concluded from these monolayer experiments that after binding to the membrane the protein spontaneously insert into the membrane.

Since the monolayer is only half the thickness of the bilayer and therefore cannot represent the actual insertion process, we also studied the interaction with bilayers.

Information on protein penetration into bilayers was obtained from CF leakage experiments (Fig. 2). Although the mechanism by which leakage is induced is unknown, from Fig. 2 it can be seen that the initial leakage rate increases with increasing TFE/protein ratios. Because TFE has a solubilising effect on the protein, this suggests that smaller protein aggregates and monomers are more effective for leakage as compared to large protein aggregates. These

experiments show that g9p is able to interact with bilayers similar as in the monolayer experiments. This finding also agrees with the insertion experiments in Fig. 4, which show that the gene 9 protein is able to integrate with the membrane.

#### 4.2. Orientation after insertion

The finding that the antigenic site is located at the C-terminal end enabled us to specifically demonstrate cleavage in the C-terminal region with the help of antibodies. For the reconstituted g9p the protein is expected to be randomly oriented, so that 50% of the C-termini is predicted on the outside of the vesicle. This fairly well agrees with the 37% reduction of the spot intensity, observed for DOPG, on the Western blot. Thus, roughly half of the C-termini is accessible for proteinase K. For inserted protein, however, a unique orientation is expected if the N-terminal side penetrates the membrane, leaving the C-terminus out. In this case, all antigenic sites can be cleaved, leaving no intensity on blot. If it inserts in the opposite way, no antigenic site will be cleaved off, resulting in no reduction of the intensity on blot. The band on the Western blot of inserted g9p in DOPG disappears almost completely, leaving only 12% of the initial intensity after incubation with proteinase K (Fig. 4). This indicates that almost all C-terminal ends were accessible and therefore located outside the vesicle. The band positions of reconstituted g9p before and after cleavage are the same. Therefore, the size of the g9p that retains the C-terminus equals the size of the uncleaved protein. This indicates that the N-terminal region is unaffected by proteinase K, since a size reduction in molecular weight is expected if (part of) the N-terminal would be clipped off. Therefore, it is concluded that the N-terminus is inserted into the membrane. Comparison of the reconstituted with the inserted g9p leads to the conclusion that the protein inserts its N-terminal part into the DOPG membrane, whereas the C-terminal region is exposed to the aqueous vesicle exterior. In the case of DOPC vesicles, the difference between insertion and reconstitution is less apparent, since after insertion not all antigenic sites can be cleaved, leaving a clear band (37%). This effect is probably due to steric hindrance as a result of vesicle aggregation. This finding is supported by the observation

that addition of only a low molar percentage of DOPG, thereby introducing vesicle repulsion, resulted in a much better digestion (data not shown).

In conclusion we believe that the g9p inserts with a specific orientation in phospholipid vesicles, namely with the N-terminus inserted in the membrane bilayer and the C-terminus remaining at the exterior. Insertion into monolayers occurs at pressures that are of biological significance, thereby allowing extrapolating to the *in vivo* situation in the *E. coli* inner membrane. *In vivo* this would mean that the C-terminus retains in the cytoplasm. This orientation agrees with what would be expected based on the finding that the N-terminal formyl group is protected in the *E. coli* membrane [14]. Furthermore, the topology of the protein is in agreement with the 'positive inside rule' [30,31] and the role of the protein during phage assembly [10].

#### Acknowledgements

This research was supported by the Life Sciences Foundation (SLW) with financial aid of the Netherlands Organisation for Scientific Research (NWO).

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