Insertion and glycosylation of Pf3-derived membrane proteins in microsomes

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Abstract To get insight into the insertion mechanism of small newly synthesized single-spanning membrane proteins, Pf3 coat protein mutants were constructed with potential glycosylation sites in the N-terminus. Some of these proteins, when synthesized in vitro in the presence of microsomes, became efficiently glycosylated, proving that they insert into the membrane and translocate their N-terminus to the lumenal side. Such Pf3 constructs also insert efficiently into *Escherichia coli* vesicles and even in pure lipid vesicles, suggesting a common mechanism, which might be spontaneous. Glycosylation was sensitive to changes in the amino acid sequence of the N-terminus, suggesting that it depends on the structure of the protein and/or its positioning with respect to the lipid–water interface.

Key words: Membrane protein insertion; Glycosylation; Microsome; Pf3 coat protein

1. Introduction

The Pf3 major coat protein is the major constituent of the protective coat of the filamentous bacteriophage Pf3, which infects *Pseudomonas aeruginosa*. It is a small protein of only 44 amino acids, which is stored in the inner membrane of the bacterium during the infection cycle. In contrast to coat proteins of closely related phages such as M13, fd or Pf1 (for a review about filamentous phages, see [1]), it is synthesized without a leader sequence [2]. Its membrane insertion is independent of Sec proteins [3], but depends on the proton motive force, which translocates the negatively charged N-terminus across the membrane [4].

Due to its small size and simple topology, Pf3 coat protein has been extensively used as a model system for the investigation of membrane protein insertion in *Escherichia coli*, either fused to other proteins [3,5,6], or by itself [4,7]. Since certain Pf3 mutants are also able to insert into pure lipid vesicles with many different lipid compositions, different aspects of the lipid specificity of membrane protein insertion could be investigated [8,9]. In all these studies, protease accessibility experiments were used to deduce the extent of insertion and the topology of the protein.

An alternative tool to study membrane protein insertion and topology is the glycosylation mapping technique. This approach makes use of the fact that the active site of the enzyme oligosaccharyl transferase (OST) is located in the lumen of the endoplasmic reticulum (ER) and lies at a fixed distance above the membrane [10]. Potential glycosylation sites are engineered into a protein, which is then synthesized in vitro in the presence of microsomes. By determining which sites can become glycosylated, information about the topology of a protein is obtained (e.g. [11–14]). This technique has been used to determine the effects of different amino acid substitutions on the positioning of transmembrane helices in a model membrane protein [15,16].

The glycosylation mapping technique could be a valuable additional tool to study the topology and positioning of Pf3 coat proteins in membranes. Vice versa, since much information is available on its structural properties from studies on model membrane systems [17,18], Pf3 could be a suitable model protein to gain insight into the process of glycosylation. In this study we have synthesized Pf3 mutants with potential glycosylation sites in vitro in the presence of microsomes and subsequently we have analyzed whether and to what extent these mutants became glycosylated.

2. Materials and methods

2.1. Construction of mutants

A plasmid with the gene encoding the 3L-4N mutant ([7]; see Fig. 1) was used as a template for QuikChange site-directed mutagenesis (Stratagene). The AAA mutant, which has a less hydrophobic N-terminal tail, was created by using two partially overlapping primers in a single reaction, the 5′ primer containing the V8A and L12A mutations and the 3′ primer containing the V15A mutation. This is a modification of the protocol of Kirsch and Joly where two non-overlapping primers were used [19]. Subsequently, glycosylation sites were engineered in these two proteins, resulting in the 3L-4N(glyc18) and AAA(glyc18) mutants. These were constructed on the 3L-4N and AAA templates in a QuikChange reaction by replacing the second and third codons by base pairs encoding the glycosylation acceptor site N-S-T. All mutations were confirmed by DNA sequencing.

2.2. Translocation of mutants into microsomes

Translocations were performed essentially as described [8]. After transcription, the proteins were translated using an *E. coli* S-135 lysate, which was prepared as described [20]. Translation/translocation reactions included 5 μCi [^{35}S]methionine to produce radiolabeled proteins. Canine pancreatic microsomal membranes (Promega) were present during the translation, 3.0 or 3.6 μl per reaction and 7.0 or 6.4 μl IMV buffer (50 mM triethanolamine acetate, pH 7.5, 250 mM sucrose, 1 mM dithiothreitol) was added to adjust the total volume of the translocation mixture to 50 μl. After the translation/translocation reaction an aliquot of 5 or 10 μl was withdrawn to serve as a standard and the rest was centrifuged in a TLA100 rotor at 60 000 rpm for 30 min at 4°C in order to pellet the microsomes. After centrifugation, the supernatant was removed and the pellet was resuspended into 40 μl

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IMV buffer. The samples were subsequently trichloroacetic acid (TCA)-precipitated.

2.3. Endo H treatment

For endoglycosidase H (Endo H) treatment, the microsomal pellet was resuspended into 50 μl of reaction buffer (100 mM sodium acetate pH 5.5, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 2% (v/v) Triton X-100 and 10 μg/ml each of the protease inhibitors chymostatin, leupeptin, antipain and pepstatin). To be able to analyze the proteins in the microsomal pellet, 5 μl of this mixture was directly precipitated to serve as a standard. The rest of the mixture was divided into two portions, to which either 1 μl of H_2O or 1 μl of Endo H (Boehringer) was added. Subsequently these mixtures were incubated at 37°C for 1 h and then TCA-precipitated.

Proteins were visualized using Tricine–SDS–PAGE according to [21] followed by autoradiography and quantitation on a PhosphoImager (Molecular Dynamics).

3. Results and discussion

We first investigated whether Pf3 mutants are able to insert into ER membranes and subsequently can become glycosylated. The 3L-4N mutant already contains a potential glycosylation site in the N-terminal tail, namely N-V-T (see Fig. 1). However, the asparagine of this site is located only 12 residues before the start of the hydrophobic segment of the protein. This might be too close to the membrane, since it was previously found for leader peptidase that the acceptor site has to be 14–15 residues away from the N-terminal end of the transmembrane segment in order to be glycosylated [10]. Therefore we constructed the 3L-4N(glyc18) mutant, in which an extra glycosylation site is engineered at 18 residues from the hydrophobic segment, which should be sufficiently distant from the membrane.

The 3L-4N(glyc18) mutant was produced in an in vitro transcription/translation reaction, making use of an *E. coli* lysate. Canine pancreatic microsomes were present during the translation, to allow the protein that is produced to insert into these membranes and to become glycosylated. As can be seen in Fig. 2, this translation reaction produced two labeled protein bands (lane 1). The lower one, which runs at the same position on the gel as other Pf3 mutants in previous studies, represents the 3L-4N(glyc18) protein [8,9]. The upper band could represent the glycosylated form of the protein. When the microsomes were pelleted by centrifugation, some of the 3L-4N(glyc18) protein was found in the supernatant (lane 2), but most of it (50–60%) was associated with the microsomes (lane 3). The higher molecular weight form of the protein was found to be exclusively associated with the microsomes, where it constitutes 35% of the total protein (for the quantified glycosylation efficiencies see Fig. 4). To determine whether this is indeed glycosylated protein, the pellet was divided into two, one half of which was treated with Endo H, an enzyme which deglycosylates proteins. The other half of the pellet was mock-treated. After treatment with Endo H for 1 h at 37°C, the upper band has disappeared and the lower band has increased in intensity (compare lanes 4 and 5), while in the mock-treated sample, the upper band is still present. The total amount of labeled protein remains the same with or without Endo H treatment (82 or 85% of originally pelleted protein respectively). From this experiment, we conclude that the upper band is the glycosylated form of the protein. Since the active site of OST resides in the lumen of the ER, this demonstrates that a substantial proportion of the proteins has inserted into the microsomal membrane with their N-terminus translocated into the lumen. This demonstrates that the N-terminus indeed reaches the *trans*-side of the membrane in the in vitro system. This observation supports the interpretation of previous in vitro experiments, where protease protection assays were used to get insight into the translocation of the N-terminus into *E. coli* or lipid vesicles [4,7–9].

The finding that the 3L-4N(glyc18) mutant inserts efficiently into microsomal membranes and *E. coli* inner membrane vesicles (data not shown) suggests a common insertion pathway. The observation that such a protein also inserts efficiently into synthetic large unilamellar vesicles [8,9] proves that membrane proteins are not required for insertion. This leads to the suggestion that the insertion in microsomal membranes is spontaneous, although it cannot be excluded that membrane proteins are involved. The finding that the protein becomes glycosylated suggests, but does not prove, that glycosylation can take place outside the context of the translocation pathway.

The 3L-4N(glyc18) mutant is only glycosylated once, most likely at the newly introduced glycosylation site 18 residues away from the transmembrane segment, and not at the intrinsic glycosylation site at 12 residues. Indeed, when a similar translocation experiment was performed with the 3L-4N mutant (Fig. 3A), the glycosylation efficiency was only 5% or less (Fig. 4), although in principle N-*V*-T and N-*S*-T sites can both be glycosylated efficiently [22]. This suggests that a 12-residue distance between the acceptor site and the transmembrane segment is too small for efficient glycosylation.

The major coat proteins of filamentous bacteriophages like Pf3, M13, fd and Pf1 all have an N-terminus with an amphipathic nature [18,23–25]. Thus the N-termini of the Pf3 mutants probably possess specific structural properties and a high affinity for the interface, which may influence the glycosylation process. To investigate this possibility, we constructed the AAA mutant (Fig. 1) in which three hydrophobic residues (two valines and a leucine) were changed to alanine residues. When this AAA mutant is translated in the presence of microsomes, about 20% of the protein becomes glycosylated (Figs. 3B and 4). This demonstrates that, in contrast to the 3L-4N mutant, in the AAA mutant the glycosylation site at 12 resi-

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                    18......12.............
3L-4N               M QSVITNVTGQLTAVQANITTIGGAILLLIVLAAVVLGINWINAQLLHPVQLF

3L-4N(glyc18)       MNSTVITNVTGQLTAVQANITTIGGAILLLIVLAAVVLGINWINAQLLHPVQLF

AAA                 M QSVITNATGQATAAQANITTIGGAILLLIVLAAVVLGINWINAQLLHPVQLF

AAA(glyc18)         MNSTVITNATGQATAAQANITTIGGAILLLIVLAAVVLGINWINAQLLHPVQLF
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Fig. 1. Amino acid sequences of the Pf3 mutants used in this study. The glycosylation acceptor sites are underlined. The putative hydrophobic transmembrane segment is shown in italics. Amino acid substitutions are shown in bold. The numbers above the sequence indicate the distance from the asparagine in the glycosylation site to the first amino acid of the hydrophobic segment.

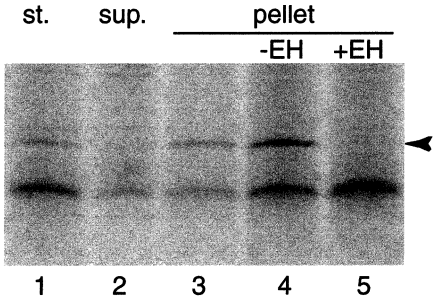

Fig. 2. Translocation of the 3L-4N(glyc18) mutant in microsomes. The protein was translated in the presence of microsomes and a 10% standard fraction (st.) was removed. The rest was centrifuged and divided into supernatant (sup.) and pellet fractions. Of the pellet, 10% was directly precipitated and the rest was incubated at 37°C for 1 h without (−EH) or with (+EH) Endo H. The arrowhead indicates the position of the glycosylated protein.

dues from the hydrophobic segment can be reached by OST. When the extra glycosylation site is introduced, resulting in the AAA(glyc18) mutant (Fig. 1), some of the protein (around 5%) even becomes doubly glycosylated (Fig. 3B). The fact that most of the protein is not doubly glycosylated is perhaps due to steric hindrance.

The results clearly show that the 3L-4N mutant is glycosylated significantly less efficiently than the other mutants used in this study (Fig. 4), which either have a glycosylation site further from the transmembrane segment or a less hydrophobic N-terminus. Therefore glycosylation is indeed quite sensitive to changes in the N-terminus of Pf3. Since OST glycosylates N-*A*-T sites with similar efficiency as N-*V*-T sites [22], the differences in glycosylation behavior between the 3L-4N and the AAA mutants suggest that the position of the glycosylation site at the N-terminus changes with respect to the lipid–water interface as a result of the amino acid substitutions.

There are several possible explanations for this difference in glycosylation behavior. First, substitution of two valines and one leucine for alanines could make the conformation of the N-terminus more extended, and thus affect the positioning of the glycosylation site with respect to the lipid–water interface. However, this possibility is unlikely since the α-helical propensities of these amino acids differ only slightly from each other, both in aqueous buffer and in non-polar solvents [26]. Second, since the N-terminus is less hydrophobic in the AAA mutant than in the 3L-4N mutant, its interfacial affinity is reduced, which could cause the glycosylation sites to be located further away from the surface of the membrane. Third, the transmembrane segments of these mutants are bordered by both hydrophobic and polar amino acids, but not by charged residues. Therefore, there may be some flexibility in the exact positioning of this segment. It is possible that the difference in hydrophobicity between the N-terminus of the 3L-4N and the AAA mutant affects the precise positioning of their transmembrane segments, thereby causing a different positioning of the glycosylation sites with respect to the membrane.

In the case of the AAA mutant, glycosylation could take place while the Asn of the acceptor site was located 12 residues away from the start of the hydrophobic transmembrane segment. Previous studies using leader peptidase as a model protein indicated a sharp threshold for glycosylation of N-terminal sites at a distance of 14–15 residues from the following transmembrane segment [10]. An important difference between the two proteins is that the transmembrane segment of leader peptidase in these experiments was bordered on the N-terminal side by a negatively charged Glu and therefore has a much more clearly defined boundary than the Pf3 mutants [10]. The positioning of the transmembrane segment could therefore be much more flexible in the Pf3 mutants, allowing sites closer to the membrane to become glycosylated. Another difference is that glycosylation of leader peptidase was independent of the sequence context, whereas glycosylation of Pf3 was very sensitive to the amino acid sequence of

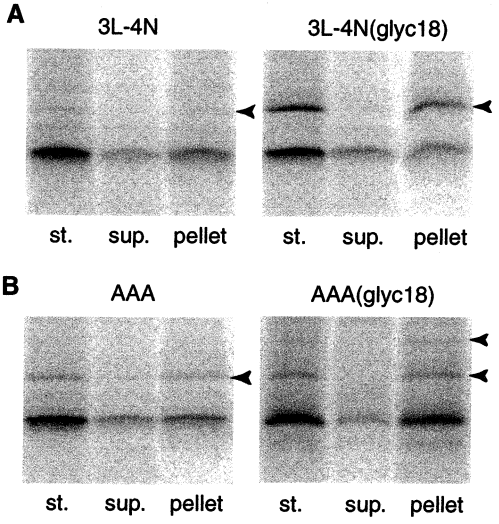

Fig. 3. Translocation of (A) the 3L-4N and 3L-4N(glyc18) mutants and (B) the AAA and AAA(glyc18) mutants into microsomes. Translation was performed in the presence of microsomes and a third of this reaction was put on gel as a standard (st.). The microsomes were pelleted by centrifugation and both supernatant (sup.) and pellet fractions are shown. The arrowheads indicate the positions of singly and doubly glycosylated forms of the protein.

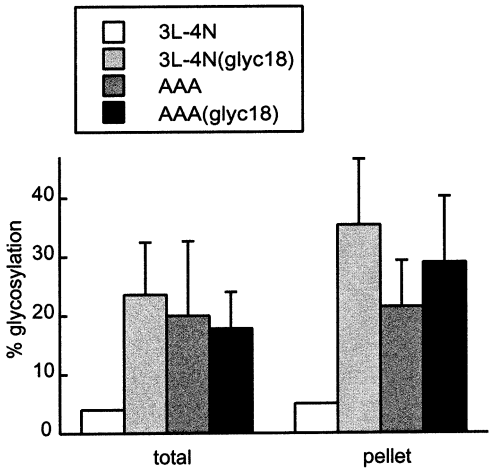

Fig. 4. Glycosylation efficiencies of the different mutants. Translocations into microsomes were performed and the glycosylation efficiencies in the total reaction mixture and microsomal pellets were determined. The average values and, where appropriate, standard deviations are shown (3L-4N: $n=1$, 3L-4N(glyc18): $n=8$, AAA: $n=2$, AAA(glyc18): $n=4$). For the AAA(glyc18) mutant, singly and doubly glycosylated forms were combined.

the N-terminus, most likely because the polypeptide chain of leader peptidase is in a flexible extended conformation [10].

Finally, the different results obtained with the Pf3 mutants on the one hand and leader peptidase on the other hand could also be caused by different translocation mechanisms, since the large protein leader peptidase inserts co-translationally into the ER membrane in a SRP- and Sec-dependent manner [10,27,28]. In contrast, as argued above, glycosylation of Pf3 might take place outside the context of the translocation pathway. In addition, the Pf3 proteins are very small and most likely insert post-translationally, since about 30–40 amino acids will be covered by the ribosome [29–31] and thus the transmembrane segment will not emerge before translation is terminated. Therefore, it is very likely that translocation of the Pf3 mutants takes place independently of the ribosome.

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