Research article

Different conformations of nascent polypeptides during translocation across the ER membrane

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Abstract

Background: In eukaryotic cells, proteins are translocated across the ER membrane through a continuous ribosome-translocon channel. It is unclear to what extent proteins can fold already within the ribosome-translocon channel, and previous studies suggest that only a limited degree of folding (such as the formation of isolated \(\alpha\)-helices) may be possible within the ribosome.

Results: We have previously shown that the conformation of nascent polypeptide chains in transit through the ribosome-translocon complex can be probed by measuring the number of residues required to span the distance between the ribosomal P-site and the lumenally disposed active site of the oligosaccharyl transferase enzyme (J. Biol. Chem 271: 6241-6244). Using this approach, we now show that model segments composed of residues with strong helix-forming properties in water (Ala, Leu) have a more compact conformation in the ribosome-translocon channel than model segments composed of residues with weak helix-forming potential (Val, Pro).

Conclusions: The main conclusions from the work reported here are (i) that the propensity to form an extended or more compact (possibly \(\alpha\)-helical) conformation in the ribosome-translocon channel does not depend on whether or not the model segment has stop-transfer function, but rather seems to reflect the helical propensities of the amino acids as measured in an aqueous environment, and (ii) that stop-transfer sequences may adopt a helical structure and integrate into the ER membrane at different times relative to the time of glycan addition to nearby upstream glycosylation acceptor sites.

Background

Most eukaryotic secretory protein are translocated across the membrane of the endoplasmic reticulum (ER) through a continuous ribosome-translocon channel that is sealed to the cytoplasmic compartment and open towards the ER lumen [1]. Recent structural studies of ribosomes from Haloarcula marismortui suggest that the channel through the large ribosomal subunit is ~ 100 Å long and 10-20 Å wide [2], and the same appears to hold true also for yeast and rabbit ribosomes [3]. The internal diameter of the translocon channel present in dog pancreas microsomes has been estimated to be 40-60 Å [4]. It is unclear to what extent proteins can fold already within the ribosome-translocon channel; studies on ribosome-nascent chain complexes suggest that only a limited degree of folding (such as the formation of isolated \(\alpha\)-helices) may be possible within the ribosome [5,6,7].
In this paper, we report experiments where we have used truncated nascent chains trapped within the ribosome-translocon complex to measure the number of residues of nascent polypeptide (dP-O) required to span the distance between the ribosome P-site and the active site of the oligosaccharyl transferase (OST). By placing 18-residue long stretches of model sequences (e.g., L17V, A17V, V18, P18) in locations that are either within the ribosome or the translocon in the truncated nascent chain, we find that dP-O values tend to fall near either 65 or 70 residues, and we equate these two values with extended and helical conformations, respectively. We conclude that the L17V and A17V segments tend to form α-helices both in the ribosomal and translocon parts of the channel, while the V18, P18, and the transmembrane segment from glycoporphin A (GpA) have more extended conformations.

Since the GpA transmembrane segment (and, presumably, the V18 segment) is helical in the lipid-embedded form of the protein [8, 9], this suggests that a hydrophobic segment with low helix potential in water may enter the translocon as an extended stretch of polypeptide and will only fold into a helical conformation upon lateral exit from the translocation channel into the lipid bilayer. On the other hand, stretches in the nascent polypeptide with strong helix propensity but insufficient hydrophobicity to insert into the lipid bilayer (e.g., A17V) may form individually stable α-helices already in the ribosomal channel.

Results
The glycosylation mapping assay
The number of residues required to span the distance between the ribosomal P-site and the active site of the oligosaccharyl transferase (OST) can be conveniently measured by a previously described "glycosylation mapping" assay [10]. Ribosome-nascent chain complexes attached to the ER translocon are generated by translation, in the presence of dog pancreas microsomes, of truncated mRNA molecules coding for a membrane protein (E. coli Lep) lacking a 3' stop-codon, Fig. 1A. A series of neighboring truncation points on the mRNA is chosen such that a unique Asn-Ser-Thr acceptor site for N-linked glycosylation is moved from a position ~60 codons to a position ~75 codons away from the 5' end of the mRNA, and the degree of glycosylation is measured for each truncated chain. As we have shown previously [10], glycosylation of the ribosome-attached nascent chain is generally observed when the glycosylation acceptor site for N-linked glycosylation is moved from a position ~60 codons to a position ~75 codons away from the 5' end of the mRNA, and the degree of glycosylation is measured for each truncated chain. As we have shown previously [10], glycosylation of the ribosome-attached nascent chain is generally observed when the glycosylation acceptor site is placed at a distance of ~65-75 residues away from the ribosomal P-site. As the maximum level of glycosylation typically observed for the truncated Lep constructs discussed below is ~60%, we define the "critical" number of residues (dP-O) required to span the distance between the P-site and the OST active site to be the chain length where 30% glycosylation is observed.
Ala- and Leu- but not Val- or Pro-based segments have a compact conformation both in the ribosome and the translocon

In the earlier study referred to above [10], we found that 65 residues from the C-terminal P2 domain of wild type Lep are required to span the distance between the P-site and the OST active site, i.e. \( d_{P-O} = 65 \). We further found that when a model hydrophobic stretch with the sequence ...QQQLVVKKK... was inserted 15 residues downstream of the glycosylation site (i.e., in a location that is largely inside the translocon when the nascent chain is long enough to allow glycosylation, Fig. 1C), \( d_{P-O} \) increased to 71 residues, suggesting a more compact, possibly \( \alpha \)-helical, conformation of the L\_17V segment compared to the corresponding stretch from the wildtype P2 domain. In a fully extended chain (~ 3.3 \( \AA \) per residue), approximately 11 residues need to be converted to an \( \alpha \)-helix (~ 1.5 \( \AA \) per residue) to account for the observed change in \( d_{P-O} \) of ~ 6 residues.

Here, we have extended these studies, both by analyzing a wider selection of model segments (V\_18, A\_17V, P\_18, and the transmembrane \( \alpha \)-helix from GpA) and by changing the position of the glycosylation site relative to the model segment such that the model segment is located within the ribosome rather than the translocon at chain lengths around the \( d_{P-O} \) value, Fig. 1B. Results for the two Val-based constructs with the hydrophobic stretch in the ribosome (upper panel) and in the translocon (lower panel) are shown in Fig. 2B, i.e., close to the value found for wild type Lep. The results for the GpA and P\_18 constructs are similar, Fig. 2B, with \( d_{P-O} \approx 65 \) residues. In contrast, the \( d_{P-O} \) values for the L\_17V and A\_17V segments are 70-71 residues when they are present in the ribosome, and somewhat larger (~ 73 residues) for L\_17V in the translocon, Fig. 2B. Assuming that an increase in \( d_{P-O} \) corresponds to a more compact conformation of the model segment, we conclude that the L\_17V and A\_17V segments have a compact, possibly helical, conformation when located in the ribosome-translocon channel, while the V\_18, P\_18, and GpA segments are more extended.

The differences in \( d_{P-O} \) values do not correlate with the ability of the model segments to insert into the ER membrane

To assess the ability of the model segments to insert into the ER membrane (i.e., their stop-transfer function), a second glycosylation acceptor site was added at the C-terminal end of the protein (see Methods). As shown in Fig. 3A, model segments with efficient stop-transfer function will only be glycosylated on the upstream acceptor site, while those lacking stop-transfer function will be glycosylated on both sites. Since acceptor sites located close to the C-terminus of a protein are only about 30% glycosylated even if efficiently translocated into the ER lumen [11], we expect a mixture of singly and doubly glycosylated molecules in the latter case.

As seen in Fig. 3B, the L\_17V and V\_18 constructs were glycosylated on only one site, while 19% and 27% of the A\_17V and P\_18 constructs were doubly glycosylated, respectively. Protease treatment of microsomes also demonstrated that only a small fragment (corresponding to the H2-V\_18 region) was protected in the V\_18 construct while a major part (corresponding to the H2-P2 region) of wild type Lep was protected inside the microsome, Fig. 3C. Thus, although the V\_18 and GpA (8) segments do not appear to fold into a compact conformation inside the ribosome-translocon channel, they are efficient stop-transfer sequences. In contrast, the A\_17V sequence adopts a compact (possibly \( \alpha \)-helical) conformation in the channel, but has little or no stop-transfer function. The L\_17V segment is both compact and an efficient stop-transfer sequence, while the P\_18 segment has an extended conformation (possibly forming a poly-proline II helix with a rise of 3.2 \( \AA \) per residue [12], very close to the rise of ~ 3.3 \( \AA \) per residue for a fully extended chain) and no stop-transfer function.

Poly-Val and poly-Leu TMH segments behave differently during integration into the ER membrane

Given that all stop-transfer sequences, once integrated into the membrane, are expected to form transmembrane \( \alpha \)-helices [13], the extended conformation of the V\_18 and GpA model segments in the translocon channel was somewhat surprising. To study this further, we determined the "minimal glycosylation distance" (MGD) for the full-length V\_18 and A\_17V constructs, and also for a construct where the third Val residue from the N-terminal end of the V\_18 stretch had been changed to Pro.

The MGD value is defined as the minimum number of residues required to bridge the distance between the luminal end of a hydrophobic transmembrane segment in a membrane protein and the OST active site [14], Fig. 4A. MGD measurements can be used to roughly position the luminal end of a transmembrane segment relative to the ER membrane by comparison to MGD values for transmembrane helices where the position relative to the lipid bilayer has been derived from various biophysical experiments [15, 16]. In our previous studies, we have mainly measured MGD values for poly-Leu based sequences. As an example, the glycosylation profile for the L\_17V construct is shown in Fig. 4B, yielding an MGD value of 15.7 residues [14].

Since, as shown above, the A\_17V construct is efficiently translocated into the lumen of the microsomes, it is not expected to have a "minimal glycosylation distance". In-
deed, all \( \alpha_{V} \)-based glycosylation mutants tested are 60%-80% glycosylated, Fig. 4B. Interestingly, the \( V_{18} \) construct, which, like the \( L_{17}V \) construct, forms a transmembrane segment, nevertheless has a glycosylation profile that is clearly distinct from that of the \( L_{17}V \) construct: an initial drop from ~ 80% to ~ 40% glycosylation at roughly the same glycosylation distance as \( L_{17}V \) (~ 15.5 residues) is followed by a plateau, and background levels of glycosylation are approached only at a glycosylation distance of ~ 10.5 residues. One possible interpretation is that there are two populations of \( V_{18} \) molecules at the time when the glycan moiety is added to the growing nascent chain: one that has a similar disposition relative to the OST active site as the \( L_{17}V \) molecules (i.e., presumably \( \alpha \)-helical and membrane-integrated with an MGD value of ~ 15.5 residues) and one with a significantly smaller MGD value (~ 10.5 residues).

To test this idea further, we also analyzed a construct where the third Val residue in the \( V_{18} \) segment was changed to Pro. We have previously shown that the introduction of a Pro residue in corresponding positions in a \( L_{23}V \) transmembrane segment leads to a reduction in the MGD value of about 2.5 residues, presumably as a result of a break in the poly-Leu \( \alpha \)-helix caused by the Pro residue [14]. Indeed, the initial drop in the glycosylation profile for the \( V_{18}(P^{3}) \) construct was ~ 2 residues, Fig. 4B, while the shift in the location of the second drop was only ~ 1 residue. This is consistent with the possibility that \( V_{18} \) molecules with MGD ~ 15.5 residues indeed have already formed a transmembrane \( \alpha \)-helix at the time of glycosylation, whereas the remaining ones have not. More detailed kinetic studies will be needed to further substantiate this idea.

**Discussion**

In this paper, we have analyzed the conformation of model polypeptide segments as they traverse the ribosome-translocon channel during translocation across the ER membrane. The study extends previous data, from
which we concluded that a segment from the globular P2 domain of the *E. coli* leader peptidase (Lep) protein most likely traverses the ribosome-translocon channel as an extended chain, whereas a model L_{17}V segment placed in the P2 domain adopts a more compact, possibly α-helical, conformation when placed in the translocon part of the channel [10].

The main conclusions from the work reported here are (i) that the propensity to form an extended or more compact (possibly α-helical) conformation in the ribosome-translocon channel does not depend on whether or not the model segment has stop-transfer function, but rather seems to reflect the helical propensities of the amino acids as measured in an aqueous environment, and (ii) that stop-transfer sequences may adopt a helical structure and integrate into the ER membrane at different times relative to the time of glycan addition to nearby upstream glycosylation acceptor sites.

Conclusion (i) follows from the observation that the A_{17}V and L_{17}V model stretches - which are composed of residues that are strong helix-formers in an aqueous environment [17] - both adopt a compact conformation in the ribosome-translocon channel (Fig. 2), whereas the V_{18} and GpA model segments do not. Val has a low helix potential in water but readily forms helices in micellar and vesicular media [9, 17], and the GpA segment also includes a number of residues with low helix potential in water (Gly, Val, Ile, Thr). The A_{17}V segment does not have stop-transfer function, as previously found for similar constructs composed of up to 19 Ala residues [18], while L_{17}V and V_{18} do (Fig. 3).

The known dimensions of the ribosomal channel (minimum diameter 10-20 Å [2, 3]) are consistent with the formation of an α-helix, but would not allow bigger structures to form. The walls of the ribosomal channel are mostly composed of RNA and have a hydrophilic character, suggesting that they do not interact strongly with hydrophobic segments in the nascent polypeptide chain. Our results are also consistent with a recent study where it was found that the N-termini of ribosome-attached nascent proteins become accessible to antibodies at different chain-lengths depending on the protein [5], also suggesting different degrees of structure formation within the ribosome channel. The reported size of the channel in the mammalian ER translocon is considerably larger (40-60 Å diameter [4]), and would seem to allow the formation of larger folded structures; we see no evidence for this, however.
The second conclusion, that stop-transfer sequences may adopt a helical structure and integrate into the ER membrane at different times relative to the time of glycan addition to nearby acceptor sites, is tentative and based on the finding that the L17V stop-transfer segment (which has a compact structure in the translocon channel) has a well-defined MGD value of ~ 15 residues, while molecules containing the V18 stop-transfer segment (which has an extended conformation in the translocon channel) appear to be characterized by two distinct MGD values (~ 15 and ~ 10 residues), Fig. 4. By comparison to the L17V result and based on the observation that a V → P mutation near the N-terminal end of the V18 segment causes a rather large drop in the larger of the two MGD values (as would be expected if a transmembrane α-helix was broken by the Pro residue), we suggest that the V18 segment in the population with MGD ~ 15.5 residues is helical and membrane-integrated whereas the population with MGD ~ 10.5 residues is not. It is thus possible that we have been able to catch the V18 molecules in the process of converting from an extended conformation in the translocon channel to a helical, membrane-embedded state. This interpretation is broadly consistent with the results of a recent study [19], where it was shown that more hydrophobic transmembrane segments move from the translocon channel into the lipid bilayer more easily (and thus presumably more rapidly) than less hydrophobic segments.

In summary, it appears that our approach using engineered glycosylation sites, truncated nascent chains, and different model polypeptide sequences makes it possible to characterize the conformational propensities of different polypeptide segments during translocation across and integration into the ER membrane. Our results also point to the necessity to carry out a careful analysis of the glycosylation profile when MGD values are used to infer the position of a transmembrane segment relative to the ER membrane.

**Materials and Methods**

**Enzymes and chemicals**

Unless otherwise stated, all enzymes as well as plasmid pGEM1, The RiboMAX SP6 RNA polymerase system, and rabbit reticulocyte lysate were from Promega (Madison, WI) or New England Biolabs (Boston, MA). T7 DNA polymerase, Taq polymerase, [35S]-Met, [14C]-methylated marker proteins, ribonucleotides, deoxyribonucleotides, dideoxynucleotides, and the cap analog m7G(5')ppp(5')G were from Amersham-Pharmacia (Uppsala, Sweden). The PCR purification and RNeasy RNA clean up kits were from Qiagen (Hilden, FRG). The PCR mutagenesis kit was from Stratagene (La Jolla, CA). Proteinase K was from Boehringer Mannheim GmbH (Mannheim, Germany). PMSF (phenylmethylsulfonyl fluoride) was from ICN Biochemicals Inc (Aurora, Ohio). Puromycin was from Sigma (St. Louis, Missouri). Oligonucleotides were from Kebo Lab and Cybergene (Stockholm, Sweden).

**DNA manipulations**

Site-specific mutagenesis was performed according to the method of Kunkel [20, 21] or by PCR. All mutants were confirmed by sequencing of plasmid DNA. All cloning steps were done according to standard procedures.

**Construction of full-length and truncated Lep glycosylation mutants**

Insertion of the model sequences QQQL17-VKKKK, QQQA17-VKKKK, QQQV18-KKKK, and QQQP18-KKKK and
the transmembrane helix from glycoporphin A MITLIIF-GVMAGVITILLISSYG1KKKH (GpA-derived residues underlined) into the P2 domain of Lep was performed by introducing BclI and NdeI restriction sites in lep codons 214 and 220, respectively. Double-stranded oligonucleotides coding for the model sequences were then cloned between the BclI and NdeI restriction sites. Site-specific mutagenesis was used to introduce an Asn-Ser-Thr glycosylation acceptor site in lep codons 200 and 178 and to introduce the restriction enzyme cleavage sites. For cloning into and expression from the pGEM1 plasmid, the 5' end of the lep gene was modified by introducing a Kozak consensus sequence [22] for enhanced translation and an XbaI site for cloning: ...ATAACCCCTAGAGCCACCAT-GCCGAATATG...(XbaI site and initiator codon underlined). Mutants of Lep were cloned into pGEM1 behind the SP6 promoter as an XbaI-Smal fragment.

Expression in vitro

Templates for in vitro transcription of truncated mRNA with or without a 3' stop codon were prepared using PCR to amplify fragments from pGEM1 plasmids containing the relevant Lep constructs. The 5' primer was the same for all PCR reactions and had the sequence 5'-TTCGTC-CAACCAAACCGACTC-3'. This primer is situated 210 bases upstream of the translational start, and all amplified fragments thus contained the SP6 transcriptional promoter from pGEM1. The 3' primers were designed according to the desired C-terminal end of the truncated protein and either contained no stop codon or a TAG stop codon. All primers were designed to have approximately the same annealing temperature. PCR amplification was performed with a total of 30 cycles using an annealing temperature of 52 °C. The amplified DNA products were purified using the Qiagen PCR purification kit as described in the manufacturers protocol and verified on a 1.2 % agarose gel.

To introduce a C-terminal glycosylation site in the V

18 and P

18 constructs (Fig. 3B), the templates were amplified as above but with a 3' primer encoding the C-terminal sequence ...PTGLRLSNSTGIH(stop) corresponding to the C-terminal end of Lep but with the underlined residues changed to encode a glycosylation acceptor site.

MGD values (Fig. 4) were measured as described in [14] for the L

179V and V

18 constructs, and also for a V

18-derived construct where the third Val from the N-terminal end was replaced by Pro. Briefly, full-length constructs with the Asn residue in an Asn-Ser-Thr glycosylation acceptor site placed 9-18 residues upstream of the respective hydrophobic segments were translated in vitro in the presence of dog pancreas microsomes, and the MGD value was determined from a plot of the efficiency of glycosylation versus the position of the glycosylation site as shown in Fig. 4.

Translation in reticulocyte lysate in the presence of dog pancreas microsomes was performed as described in [23] at 30 °C for 1 h (when generating full-length, properly terminated chains) or at 22 °C for 30 min. (when generating truncated ribosome-nascent chain complexes). Proteins were analyzed by SDS-PAGE and gels were quantitated on a Fuji BAS1000 phosphoimager using the MacBAS 2.31 software. The extent of glycosylation of a given mutant was calculated as the quotient between the intensities of the glycosylated bands divided by the summed intensities of the glycosylated and non-glycosylated bands, or as the quotient between the intensities of the doubly glycosylated bands divided by the summed intensities of the singly and doubly glycosylated bands (for the experiment in Fig. 3B).

Translocation of polypeptides to the luminal side of the microsomes was assayed by resistance to exogenously added protease K and by analysis of constructs with two glycosylation acceptor sites flanking the model polypeptide segments as in [24].

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