JMB

Positively and Negatively Charged Residues Have Different Effects on the Position in the Membrane of a Model Transmembrane Helix

Magnus Monné[1], IngMarie Nilsson[1,2], Marie Johansson[1], Niklas Elmhed[1] and Gunnar von Heijne[1*]

[1]*Department of Biochemistry Stockholm University, S-106 91 Stockholm, Sweden*

[2]*Department of Biosciences Karolinska Institute, NOVUM S-141 57 Huddinge, Sweden*

*Corresponding author

We have studied the effects of single charged residues on the position of a model transmembrane helix in the endoplasmic reticulum membrane using the glycosylation mapping technique. Asp and Glu residues cause a re-positioning of the C-terminal end of the transmembrane helix when placed in the one to two C-terminal turns but not when placed more centrally. Arg and Lys residues, in contrast, have little effect when placed in the two C-terminal turn but give rise to a more substantial shift in position when placed 9-11 residues from the helix end. We suggest that this difference between the effects of positively and negatively charged residues can be explained by the so-called snorkel effect, i.e. that the very long side-chains of Arg and Lys can reach up along the transmembrane helix to allow the terminal, charged moiety to reside in the lipid head-group region while the C^α of the residue is positioned well below the membrane/water interface.

© 1998 Academic Press

Keywords: membrane protein; protein structure; glycosylation; transmembrane helix; charged residues

Introduction

While it is generally accepted that the overall hydrophobicity provides the primary driving force for the integration of transmembrane α-helices (TMHs) into biological membranes (von Heijne, 1997), very little is known about the detailed molecular interactions that control the precise positioning of an isolated TMH in the bilayer. This is due both to the lack of high-resolution structural data and to the difficulties inherent to biophysical studies of highly hydrophobic peptides. A deeper understanding of this aspect of membrane protein structure might lead to improvements in our abilities to predict the tertiary structure of integral membrane proteins from their amino acid sequence, in particular since the approximate location of the TMHs can often be reliably inferred from sequence analysis (von Heijne, 1996).

In the accompanying paper (Nilsson *et al.*, 1998), we describe a new, highly accurate method for locating the ends of a TMH relative to the endoplasmic reticulum (ER) membrane. This so-called glycosylation mapping technique, in principle, allows any transmembrane segment to be positioned relative to a known reference TMH in the direction perpendicular to the membrane plane. As a first application of this method, the effects of a proline residue introduced in different positions in a model poly-Leu TMH were studied, and it was shown that proline residues disrupt the TMH when introduced in the first one to two N and C-terminal turns of the helix, but not when placed more centrally (Nilsson *et al.*, 1998).

Here, we have used the same methodology to study the effects of positively (Arg, Lys) and negatively (Asp, Glu) charged residues on a model TMH composed of 23 contiguous leucine residues and a C-terminal valine. We have also analyzed a new reference TMH from the phage M13 major coat protein whose conformation and position in a model membrane and in detergent micelles are known from NMR, ESR, and fluorescence quenching studies (Stopar *et al.*, 1996).

The glycosylation mapping results for the M13 coat protein and for Lys and Arg mutations in the poly-Leu TMH described here are entirely consistent with and extend the biophysical studies of the M13 coat protein: both sets of results show that basic residues do not have a strong effect on the location of the C-terminal end of the TMH relative to the membrane when located in the terminal one to two helical turns. In contrast, both Asp and Glu mutations cause a marked re-positioning of the poly-Leu TMH in the membrane when placed near the C-terminal end. Compared to the helix-disrupting effects seen with proline residues (Nilsson et al., 1998), the charged amino acid residues cause less drastic changes, suggesting that while proline residues may break the helical conformation of the transmembrane stretch, charged residues do not cause a break in the TMH but only affect its position relative to the membrane. The different effects seen with Arg and Lys on one hand, and Asp and Glu on the other, may be explained by the so-called snorkel effect (Gavel et al., 1988; Mishra et al., 1994; Segrest et al., 1990), i.e. that the long, aliphatic portion of the Arg and Lys side-chains may stretch along the surface of the helix, allowing the charged terminal group to reach into the lipid headgroup region even when the C^α atom is more than one helical turn below the membrane/water interface.

Results

Glycosylation mapping

The glycosylation mapping technique is described in detail in the accompanying paper (Nilsson et al., 1998). Briefly, the lumenally oriented active site of the ER enzyme oligosaccharyl transferase (OST) is used as a fixed point of reference against which the position of a TMH in the ER membrane can be measured; in particular, point mutations in a TMH that affect its position in the membrane will change the ''minimal glycosylation distance'' (MGD), i.e. the number of residues in the nascent chain needed to bridge the distance between a given reference residue at the lumenal end of the TMH and the OST active site.

For the studies presented here, we used the well-characterized *Escherichia coli* inner membrane protein leader peptidase (Lep) which has two TMHs (H1 and H2) and N_{lum}/C_{lum} orientation when assembled into microsomal membranes (Nilsson & von Heijne, 1993). As shown in Figure 1(a), H2 was replaced with either the TMH from the phage M13 major coat protein, a hydrophobic stretch composed of 23 leucine residues and one valine residue flanked by four N-terminal lysine residues and a C-terminal Gln-Gln-Gln-Pro segment ($L_{23}V$), or various point mutants where individual residues in the $L_{23}V$ TMH were replaced by charged residues.

Consensus N-glycosylation sites were created at different positions downstream of the TMH. All constructs were expressed *in vitro* in the presence of dog pancreas microsomes, and MGD values were obtained by determining the number of residues between a given reference residue at the lumenal end of the TMH and the glycosylation acceptor Asn needed to get half-maximal glycosylation.

Calibration: the phage M13 coat protein

We have shown that the N-terminal TMH from the H-subunit of the photosynthetic reaction center can be used as a reference helix against which the membrane position of other TMHs can be determined (Nilsson et al., 1998). In order to extend our set of reference TMHs and, in particular, in order to determine the MGD for a TMH with known position in a bilayer and containing charged residues near its C-terminal end, we chose to study the TMH from the phage M13 major coat protein. This TMH has been shown by NMR to extend from approximately residue Ala25 to Phe45 (Almeida & Opella, 1997). Further studies in detergents and model lipid bilayers have confirmed this structure, and have located Ala25 and Thr46 in a somewhat hydrophobic environment close to the membrane/water interface (Spruijt et al., 1996; Stopar et al., 1996). As seen in Figure 1(b), this places the C^α atom of residue Lys40 well into the hydrophobic part of the bilayer.

A segment encompassing the M13 coat protein TMH (residues 19-48) was introduced into Lep in place of the H2 segment, and the MGD was determined as shown in Figure 1(a). The MGD value (counting from Thr46) is similar to that obtained previously for the reaction center H-subunit TMH (counting from Glu34) for which the position in the bilayer can be estimated from the known X-ray structure (Wallin et al., 1997), and also to the MGD value obtained for a model TMH of comparable length composed of 20 leucine residues and one valine (Nilsson et al., 1998). We thus conclude that the glycosylation mapping technique gives results that are consistent with data obtained by different biophysical approaches, and also places the C^α of Lys40 significantly below the membrane/water interface.

Effects of Arg and Lys residues on the membrane position of a poly-Leu transmembrane segment

To further study the effects of positively charged residues on the membrane position of a TMH, we introduced both Arg and Lys residues in different positions in a model TMH ($L_{23}V$) composed of 23 leucine residues and one valine and flanked by four N-terminal lysine residues and a C-terminal Gln-Gln-Gln-Pro sequence, and analyzed the resulting Lep-derived constructs by glycosylation mapping. The effects of proline residues placed in a TMH of this composition are
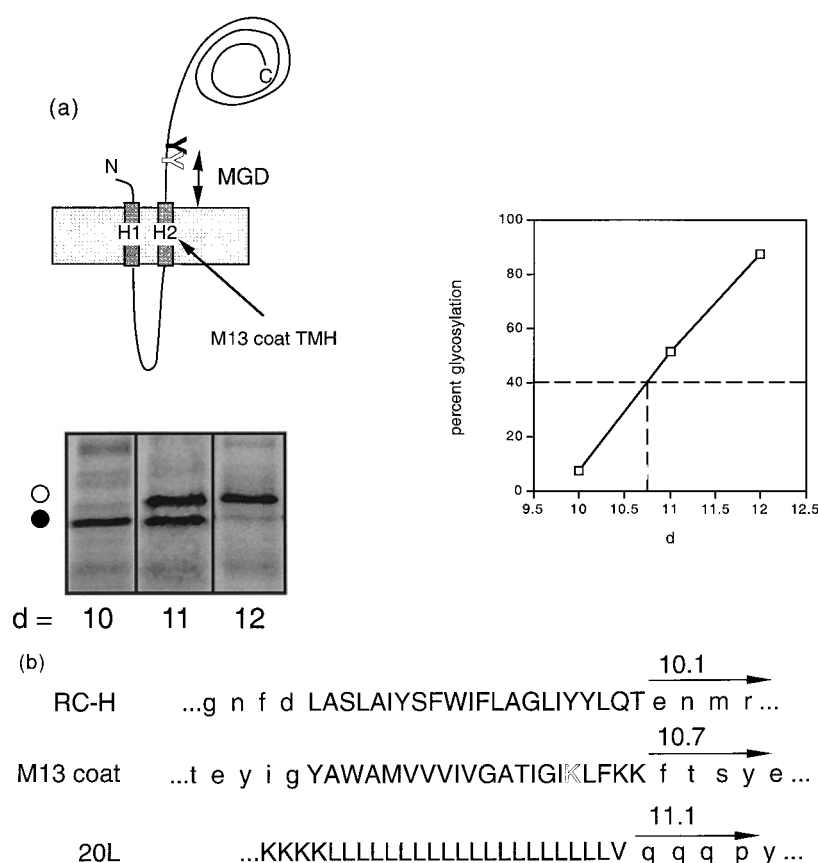
Figure 1. Determination of the MGD for the M13 major coat protein TMH. (a) Residues 19-48 from the M13 coat protein were inserted in place of the H2 TMH in Lep, and the MGD was determined by placing potential glycosylation acceptor sites (Asn-Ser-Thr) in various positions downstream of H2. Results are shown for three constructs where the acceptor Asn is $d = 10$, 11, and 12 residues away from Lys44 (bottom left); the nonglycosylated and glycosylated forms of the protein are indicated by a filled and open circle, respectively. The MGD value corresponds to half-maximal (i.e. 40%) glycosylation and is determined by interpolation (right). (b) Relative positions as determined by glycosylation mapping of the TMHs from the *R. sphaeroides* photosynthetic reaction center H-subunit (Nilsson *et al.*, 1998), M13 coat protein (with Lys40 depicted in outline font), and a model TMH composed of 20 leucine residues and one valine (Nilsson *et al.*, 1998). The MGD values are shown.

reported in the accompanying paper (Nilsson *et al.*, 1998).

The MGD values obtained for an extensive set of Arg and Lys mutations in the $L_{23}V$ TMH are summarized in Figure 2. Remarkably, the substitutions in position −1 (Gln − 1 → Arg and Gln − 1 → Lys) both give rise to an increase in MGD. Substitutions placed progressively further into the hydrophobic segment lead to a gradual decrease in MGD values with a minimum around position 10, followed by an increase; overall, the changes in MGD values are considerably smaller than for the proline mutations reported in the accompanying paper. Interestingly, there is a local oscillation superposi-
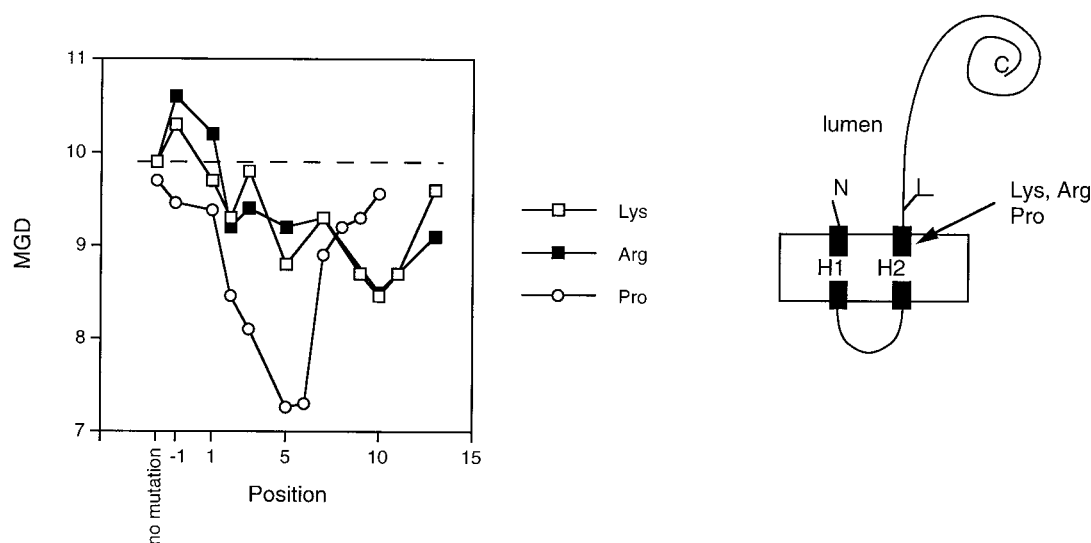

Figure 2. MGD values for Lys and Arg mutations in the $L_{23}V$ TMH. Data for Pro mutations (Nilsson *et al.*, 1998) are included for comparison. The broken line indicates the MGD value for the non-mutated $L_{23}V$ TMH. Positions are counted in a C-to-N-terminal direction from the C-terminal end of the $L_{23}V$ segment; position 1 is the C-terminal Val residue (cf. Figure 1(b)).

tioned on the overall trend for both the Arg and Lys-series, with local maxima around positions −1, 3 and 7 and minima around position 2, 5 and 10.

Our interpretation of these results is based on the so-called snorkel model (Segrest *et al.*, 1990), which has also been invoked to explain the membrane burial of the C^α of Lys40 in the M13 coat protein (Stopar *et al.*, 1996). The side-chains of Arg and Lys are both very long, about 6 Å. The Arg C^δ carries a bulky, positively charged guanido group, while Lys has a terminal amino group on C^ε. According to the snorkel model, the long aliphatic part between the C^α and the charged moieties at the tip of the side-chains in the $Gln^{-1} \rightarrow$ Arg and $Gln^{-1} \rightarrow$ Lys mutants partition into the hydrophobic core of the membrane, essentially extending the hydrophobic segment by one residue and thus increasing the MGD.

The relatively minor drop in MGD values seen for the other Arg and Lys mutations suggests that the aliphatic part of the respective side-chains reach up along the surface of the helix, allowing the basic guanido and amino groups to interact with the acidic phospholipid head groups while most of the C-terminal hydrophobic residues in the TMH are still buried in the membrane. Since the Arg and Lys side-chains are long enough to reach five to six residues along the helix (Stopar *et al.*, 1996), a rough estimate is that the mutations in position 10 should position the TMH such that at most four terminal hydrophobic residues are pushed out of the membrane. Given that each residue adds 1.5 Å to the length of the helix and that each residue advances a fully extended chain by 3.3 Å, the expected drop in the MGD value is approximately $4 \times 1.5/3.3 = 1.8$ residues, close to the observed drop of 1.4 residues.

The small oscillations in the Arg and Lys-plots are in phase with each other, suggesting that they reflect a common property. Since we do not have data for all positions it is difficult to draw strong conclusions, but the oscillations seem to be compatible with a helical repeat. Further studies will be necessary to understand the source of these oscillations.

Since the drop in MGD values is much less than for the Pro-substitutions reported in the accompanying paper, it seems that the helical structure of the entire hydrophobic stretch is maintained in the Arg and Lys mutants, and that the charged residues cause a change in the position of the TMH relative to the membrane but not in overall conformation. In the mutants with Arg or Lys residues in position 13, the charged residue is about halfway through the membrane and the charged side-chains might start to interact with the lipid head groups on the other side of the membrane.

Effects of Asp and Glu residues on the membrane position of a poly-Leu transmembrane segment

To be able to compare the effects of positively and negatively charged residues, we made a set of corresponding Asp and Glu mutations in the $L_{23}V$ TMH. As seen in Figure 3, the relation between MGD value and position for the these mutations differ from both the Arg and Lys results reported above and the Pro results reported in the accompanying paper. Compared to the positively charged residues, Asp and Glu have a stronger effect in the first one to two turns of the TMH and have almost no effect when placed further into the hydrophobic stretch. Pro mutations affect the MGD values in approximately the same positions as do Asp and
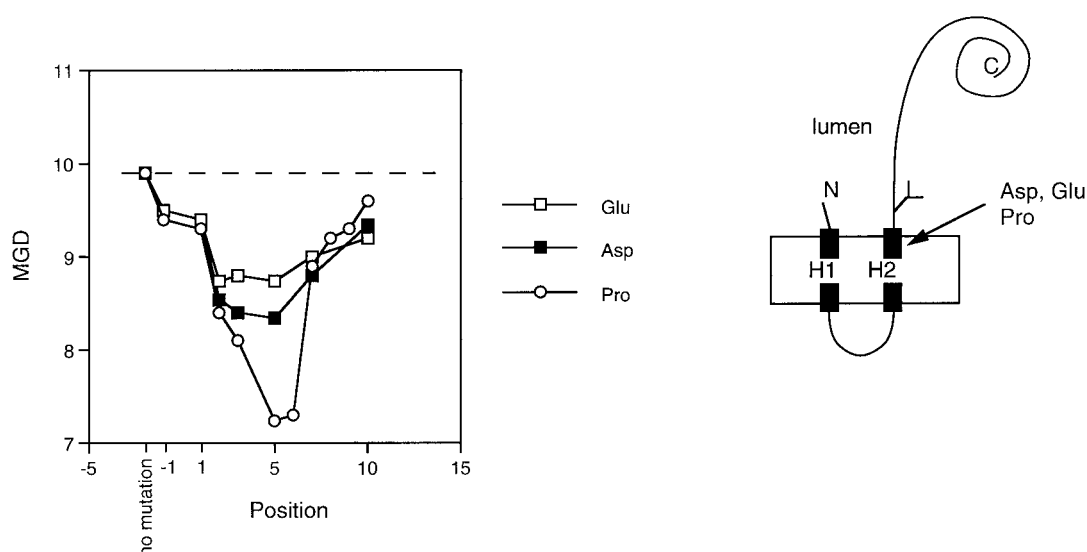

Figure 3. MGD values for Asp and Glu mutations in the $L_{23}V$ TMH. Data for Pro mutations (Nilsson *et al.*, 1998) are included for comparison. The broken line indicates the MGD value for the non-mutated $L_{23}V$ TMH. Positions are counted in a C-to-N-terminal direction from the C-terminal end of the $L_{23}V$ segment; position 1 is the C-terminal Val residue (c.f. Figure 1(b)).

Glu, but give rise to a much larger drop. It is also apparent that the Asp mutations give somewhat lower MGD values than the Glu mutations.

The side-chains of Asp and Glu are short, and thus cannot "snorkel" in the same way as Arg and Lys. In addition, there might be some repulsion between the negatively charged residues and negatively charged phospholipid head groups. Indeed, both Asp and Glu cause a drop in MGD by about 1.5 residues already in position 2, with the effect of Asp being somewhat stronger than that of Glu, possibly because the Glu side-chain is one carbon longer than the Asp side-chain. The MGD is almost constant between the positions 2 and 5, and then increases. Since the local pH at the membrane/water interface is lower than in bulk solution (Heberle *et al.*, 1994), protonation and burial of acidic side-chains in the membrane interior should not be energetically very costly, and the approximately constant MGD values seen for mutations in positions 2-5 could possibly reflect an equilibrium between helices with an unprotonated acidic residue protruding from the membrane and helices with a protonated, buried acidic residue.

To verify the difference between Pro and Asp mutations, a mutant with Asp in position 5 and Pro in position 4 was also made. For this mutant, MGD = 5.8 (data not shown); a value comparable to that observed for a mutant in the $L_{23}V$ TMH with proline residues in positons 3 and 6 which has MGD = 5.1 (Nilsson *et al.*, 1998). The drastic drop in the MGD value from 8.3 for the Asp5 mutant to 5.8 for the Pro4Asp5 mutant strongly suggests that, in contrast to Pro, Asp and Glu do not break the helical conformation of the TMH but only affect its position in the membrane.

Discussion

Together with the results presented in the accompanying paper (Nilsson *et al.*, 1998), the study reported here demonstrates that glycosylation mapping is a sensitive technique for detecting structural and positional changes in a TMH. An obvious advantage is that the experiments are performed under *in vivo*-like conditions; the price one pays is that the complexity of the experimental system makes direct structural interpretations of the data difficult. However, we have shown here that the glycosylation mapping results for two different TMHs where the position in the membrane can be derived from either X-ray crystallography or NMR coupled with spin-labeling and fluorescence quenching experiments are entirely consistent with the biophysical results.

So far, we have used the glycosylation mapping technique to analyze the effects on conformation and membrane position of Pro, Arg, Lys, Asp, and Glu mutations in the context of a model poly-Leu TMH. Pro residues appear to both shorten and reposition the TMH when placed in the most C-terminal five to six positions (Nilsson *et al.*, 1998), Arg

and Lys residues do not shorten the TMH and have only minor effects on its position in the membrane (Figure 2), and Asp and Glu residues also do not shorten the TMH but have a more pronounced effect on its position when placed in the most C-terminal turn (Figure 3).

These effects can be readily explained by the known conformational properties of the respective residues. Pro residues do not fit sterically into the α-helical conformation and cannot donate a hydrogen bond to its $i - 4$ neighbor in the helix (Barlow & Thornton, 1988; MacArthur & Thornton, 1991; von Heijne, 1991), and hence can only be forced into a TMH if the gain in free energy resulting from burial of apolar residues on both sides of the Pro residue offsets the cost of the lost hydrogen bonds. Thus, Pro has a very strong effect on the MGD values when placed in the terminal one to two turns of the TMH, and most likely both terminates and re-positions the helix (Figure 4).

Arg and Lys both have very long and flexible side-chains with an appreciable apolar segment connecting to the terminal charged moiety. According to the snorkel model (Segrest *et al.*, 1990) this imparts an amphiphilic character to these residues, allowing them to bury a substantial fraction of their non-polar surface area in the membrane while still exposing the charged moiety to surrounding water. This model has been invoked to explain the observation that the C^{α} of Lys40 in the M13 coat protein TMH is buried well below the membrane/water interface (Stopar *et al.*, 1996), and we find that the behavior of the Arg and Lys mutants in the poly-Leu model TMH are also consistent with the snorkel model.
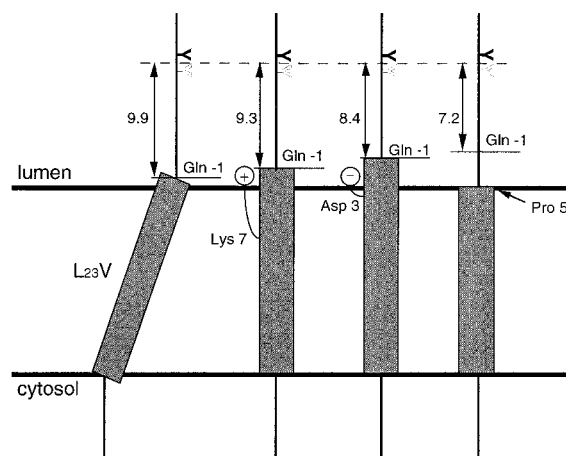

Figure 4. Model for the re-positioning of a TMH caused by different kinds of mutations. The lysine side-chain can reach into the lipid headgroup region even when the C^{α} is five to six residues below the membrane/water interface. This is not possible for the much shorter Asp side-chain. Pro, finally, terminates the helix, converting the downstream residues from a helical, membrane embedded state to an extended, lumenally exposed state. Lysine residues flanking the cytosolic end of the TMHs are not shown, but presumably can also snorkel out of the lipid bilayer.

Asp and Glu, finally, have short side-chains with little amphiphilic character. Indeed, their effect on the MGD values for the poly-Leu TMH is very different from that seen for Arg and Lys, and our interpretation is that Asp and Glu do not break the helical conformation of the TMH but effectively shorten the hydrophobic segment, causing a re-positioning. When placed more than one turn into the helix, they have only minor effects on the MGD values, suggesting that they become proto-nated and buried within the membrane.

In summary, the glycosylation mapping tech-nique has made it possible to obtain a first set of data on the effects of various point mutations on the conformation and membrane position of a model TMH. We anticipate that the same approach can be used to measure, e.g. differences in hydro-phobicity between different residues, the possibili-ties for salt-bridge formation within a TMH, and a host of other residue-dependent structural charac-teristics of single transmembrane helices.

Materials and Methods

Enzymes and chemicals

Unless otherwise stated, all enzymes were from Pro-mega. Ribonucleotides, deoxyribonucleotides, dideoxyri-bonucleotides, and the cap analog m7G(5′)ppp(5′)G, T7 DNA polymerase, and [^{35}S]Met were from Amersham-Pharmacia (Uppsala, Sweden). Plasmid pGEM1, DTT, BSA, RNasin and rabbit reticulocyte lysate were from Promega. Spermidine and PMSF were from Sigma. Oli-gonucleotides were from Kebo Lab (Stockholm, Sweden) and DNA Technology (Copenhagen, Denmark).

DNA manipulations

For cloning into and expression from the pGEM1 plas-mid, the 5′ end of the *lep* gene was modified, first, by the introduction of an *Xba*I site and, second, by changing the context 5′ to the initiator ATG codon to a ''Kozak con-sensus'' sequence (Kozak, 1989). Thus, the 5′ region of the gene was modified to: ...ATAACCC<u>TCTAGA</u>GC-CACC<u>ATG</u>GCGAAT... (*Xba*I site and initiator codon underlined). Replacement of the H2 region in Lep was performed by first introducing *Bcl*I and *Nde*I restriction sites in codons 59 and 80 flanking the H2 region and then replacing the *Bcl*I-*Nde*I fragment by the appropriate double-stranded oligonucleotides. Residues 59-81 in H2 were replaced by residues Thr19-Ser48 of M13 major coat protein and by the poly-Leu sequence PGLIKKKKL$_{23}$VQQQP. Site-specific mutagenesis used to add *Bcl*I and *Nde*I restriction sites at the 3′ and 5′ ends of H2 in Lep, to insert charged residues into the poly-Leu stretch and to introduce Asn-Thr-Ser acceptor sites for *N*-linked glycosylation was performed according to the method of Kunkel (Geisselsoder *et al.*, 1987; Kunkel, 1985). Glycosylation acceptor sites were designed as described (Nilsson *et al.*, 1994), i.e. by replacing three appropriately positioned codons downstream of H2 with codons for the acceptor tri-peptide Asn-Ser-Thr. In the glycosylation constructs Asn84-Ser85-Thr86 and Asn88-Ser89-Thr90 (numbering corresponding to the Lep wild-type sequence), the flanking Pro residues were changed to Gln since they were found to reduce the efficiency of

glycosylation, cf. Gavel & von Heijne (1990). To make glycosylation sites in positions $d = 5$ and 6 downstream of H2, one or two Gln residues were deleted from the flanking QQQP stretch in the $d = 7$ construct. All mutants were confirmed by DNA sequencing of plasmid or single-stranded M13 DNA using T7 DNA polymerase.

Expression *in vitro*

Synthesis of mRNA from pGEM1 by SP6 RNA poly-merase and translation in reticulocyte lysate in the pre-sence and absence of dog pancreas microsomes was performed as described (Liljeström & Garoff, 1991). Translocation of polypeptide segments of the M13 major coat protein constructs to the lumenal side of the micro-somes was assayed by resistance to exogenously added proteinase K (Nilsson & von Heijne, 1993; data not shown). Proteins were analyzed by SDS-PAGE and gels were quantified on a Fuji BAS1000 phosphoimager using the MacBAS 2.1 software. The extent of glycosylation of a given mutant was calculated as the quotient between the intensity of the glycosylated band divided by the summed intensities of the glycosylated and non-glycosy-lated bands. In general, the glycosylation efficiency var-ies by no more than ±5% between different experiments, and the precision in the MGD determinations is ±0.2 residue.

References

Almeida, F. C. L. & Opella, S. J. (1997). fd coat protein structure in membrane environments: Structural dynamics of the loop between the hydrophobic trans-membrane helix and the amphipathic in-plane helix. *J. Mol. Biol.* **270**, 481–495.

Barlow, D. J. & Thornton, J. M. (1988). Helix geometry in proteins. *J. Mol. Biol.* **201**, 601–619.

Gavel, Y. & von Heijne, G. (1990). Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites – Implications for protein engineering. *Protein Eng.* **3**, 433–442.

Gavel, Y., Nilsson, L. & von Heijne, G. (1988). Mitochon-drial targeting sequences. Why 'non-amphiphilic' peptides may still be amphiphilic. *FEBS Letters,* **235**, 173–177.

Geisselsoder, J., Witney, F. & Yuckenberg, P. (1987). Effi-cient site-directed *in vitro* mutagenesis. *BioTechni-ques,* **5**, 786–791.

Heberle, J., Riesle, J., Thiedemann, G., Oesterhelt, D. & Dencher, N. A. (1994). Proton migration along the membrane surface and retarded surface to bulk transfer. *Nature,* **370**, 379–82.

Kozak, M. (1989). Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems. *Mol. Cell Biol.* **9**, 5073–5080.

Kunkel, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA,* **82**, 488–492.

Liljeström, P. & Garoff, H. (1991). Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. *J. Virol.* **65**, 147–154.

MacArthur, M. W. & Thornton, J. M. (1991). Influence of proline residues on protein conformation. *J. Mol. Biol.* **218**, 397–412.

Mishra, V. K., Palgunachari, M. N., Segrest, J. P. & Anantharamaiah, G. M. (1994). Interaction of synthetic peptide analogs of the class A amphipathic helix with lipids. *J. Biol. Chem.* **269**, 7185–7191.

Nilsson, I. & von Heijne, G. (1993). Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J. Biol. Chem.* **268**, 5798–5801.

Nilsson, I., Whitley, P. & von Heijne, G. (1994). The C-terminal ends of internal signal and signal-anchor sequences are positioned differently in the ER translocase. *J. Cell Biol.* **126**, 1127–1132.

Nilsson, I., Sääf, A., Whitley, P., Gafvelin, G., Waller, C. & von Heijne, G. (1998). Proline-induced disruption of a transmembrane α-helix in its natural environment. *J. Mol. Biol.* **284**, 1165–1175.

Segrest, J. P., De Loof, H., Dohlman, J. G., Brouilette, C. G. & Anantharamaiah, G. M. (1990). Amphipathic helix motif: classes and properties. *Proteins: Struct. Funct. Genet.* **8**, 103–117.

Spruijt, R., Wolfs, C., Verver, J. & Hemminga, M. (1996). Accessibility and environment probing using cysteine residues introduced along the putative transmembrane domain of the major coat protein of bacteriophage M13. *Biochemistry,* **35**, 10383–10391.

Stopar, D., Spruijt, R. B., Wolfs, C. & Hemminga, M. A. (1996). Local dynamics of the M13 major coat protein in different membrane-mimicking systems. *Biochemistry,* **35**, 15467–15473.

von Heijne, G. (1991). Proline kinks in transmembrane α-helices. *J. Mol. Biol.* **218**, 499–503.

von Heijne, G. (1996). Principles of membrane protein assembly and structure. *Prog. Biophys. Mol. Biol.* **66**, 113–139.

von Heijne, G. (1997). Getting greasy: how transmembrane polypeptide segments integrate into the lipid bilayer. *Mol. Microbiol.* **24**, 249–253.

Wallin, E., Tsukihara, T., Yoshikawa, S., von Heijne, G. & Elofsson, A. (1997). Architecture of helix bundle membrane proteins: An analysis of cytochrome *c* oxidase from bovine mitochondria. *Protein Sci.* **6**, 808–815.

Edited by F. Cohen