Insertion of Bacteriorhodopsin Helix C Variants into Biological Membranes

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ABSTRACT: A peptide corresponding to bacteriorhodopsin (bR) helix C, later named pHLIP, inserts across lipid bilayers as a monomeric α-helix at acidic pH, but is an unstructured surface-bound monomer at neutral pH. As a result of such pH-responsiveness, pHLIP targets acidic tumors and has been used as a vehicle for imaging and drug-delivery cargoes. To gain insights about the insertion of bR helix C into biological membranes, we replaced two key aspartic residues that control the topological transition from the aqueous phase into a lipid bilayer. Here, we used an in vitro transcription–translation system to study the translocon-mediated insertion of helix C-derived segments into rough microsomes. Our data provide the first quantitative biological understanding of this effect. Interestingly, replacing the aspartic residues by glutamic residues does not significantly alters the insertion propensity, while replacement by alanines promotes a transmembrane orientation. These results are consistent with mutational data obtained in synthetic liposomes by manipulating pH conditions. Our findings support the notion that the translocon facilitates topogenesis under physiological pH conditions.

INTRODUCTION

Most membrane proteins comprise a series of membrane-spanning hydrophobic helices separated by polar loops of different lengths. The orientation of the TM helices with respect to the bilayer plane defines the topology of the membrane protein.1,2 The translocon participates in the genesis of membrane protein topology. However, membrane topology is believed to be ultimately determined by the interplay between the physical properties of protein and lipid molecules. The path followed by many simple membrane proteins to acquire their final topology can be satisfactorily conceptualized within the framework of the two-stage model.3 This model postulates that independent helices that completely span the membrane assembly to acquire the final three-dimensional structure. More complex membrane proteins, however, can adopt more intricate mechanisms frequently due to the presence of poorly hydrophobic transmembrane (TM) segments.4−9

The helix C of bacteriorhodopsin (bR) is a poorly hydrophobic TM domain, and the pH-dependent membrane interaction of this sequence inspired its use as a tumor-targeting peptide, dubbed as pHLIP (pH-Low Insertion Peptide).10,11 Helix C of bR contains several charged residues including two key aspartic residues (D85 and D96, see Figure 1) that participate in the proton permeation pathway.12 The hydrophobicity of pHLIP varies with the protonation of these groups, and so it depends on the pH of the environment.13 As a result, pHLIP exhibits at least two different types of membrane interaction. At neutral pH, the side chains of the acidic residues are negatively charged, decreasing the overall hydrophobicity of the peptide. At this pH, the peptide is water-soluble while it can bind to the membrane surface, with a low α-helical content that is modulated by the physical properties of the lipid bilayer and ionic conditions.14,15 On the other hand, at low pH, the acidic residues lose their negative charge with pK values near 6.16 The protonation of acidic side chains increases the hydrophobicity of the peptide, causing insertion across the lipid bilayer and establishment of a TM helix.11,17 Since pHLIP is marginally hydrophobic, and the topology can be controlled by the pH, this peptide has been used as a model system for studying membrane topological transitions of poorly hydrophobic TM segments. Here, we investigated the membrane insertion of helix C variants relevant to understand the transitions previously observed for pHLIP using an experimental system18−21 that allows accurate measurements of the apparent free energy (ΔGapp) of translocon-mediated insertion of TM helices into the endoplasmic reticulum (ER) membrane, which arguably is a more complex environment and measurement than those provided by other experimental assays based on model membranes.

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Figure 1. Cartoon representation of bacteriorhodopsin protein (PDB ID:1PY6). The helical residues (helix C) studied in this work are shown in yellow (from Trp80 to Val101, mature protein sequence). Positions of the hydrocarbon membrane boundaries (dotted lines) were obtained from the OPM database (https://opm.phar.umich.edu/). The red line indicates the extracellular membrane side, and the blue line indicates the cytoplasmic membrane side, shown by the horizontal dotted lines 35.5 Å apart. Aspartic 85 and 96 side chains are shown in sticks representation (red), giving distances between the carboxylic side chains and the interface of about 14.8 and 4.4 Å, respectively. Protein representation was rendered with Pymol Molecular Graphics System (v2.2.5).

RESULTS AND DISCUSSION

We determined the translocon-mediated insertion propensity of bR helix C variants into the ER membrane using a microsomal translocation assay. We worked with the wild-type (Wt) sequence and multiple variants of the residues that control the topological transition, D85 and D96. To this end, peptide sequences derived from Wt bR helix C were independently introduced into the large luminal P2 domain of the model protein leader peptidase (Lep) from Escherichia coli. The inserted sequences were limited to the putative TM region (Figure 2A top, residues W80-V101, Halobacterium salinarum bR, pdbID 1PY6) and were flanked by GPrepeat and GPtetrapeptides at both N- and C-termini to ‘insulate’ the tested sequences from the Lep carrier protein. Glycosylation sites were placed at both ends of the tested sequences to permit quantification of the insertion propensity. The different constructs were then expressed in vitro in the presence of ER-derived column-washed canine pancreas rough microsomes and radiolabeled amino acids. Because glycosylation takes place only in the interior (lumen) of the microsome, membrane-inserted sequences are monoglycosylated, whilst noninserted (translocated) sequences are double glycosylated (Figure 2A). Quantification of the fractions of monoglycosylated (f1g) and double glycosylated (f2g) molecules allows calculating the apparent equilibrium constant, app for the membrane insertion of a given sequence tested, app = f1g/f2g. The app value can be converted into the apparent free energy difference between the inserted and the noninserted state.

We determined the membrane insertion efficiency by phosphor-imager scans of SDS-PAGE gels that measure the fractions of mono (f1g) and double (f2g) glycosylated proteins. As shown in Figure 2B, translation—glycosylation results reveal that helix C wild-type sequence does not insert efficiently (15.9% singly glycosylated, Table 1) into the biological membranes (Figure 2B, lanes 1–2). This result is in agreement with previous biophysical data, obtained with peptides assayed at neutral pH, expected to be similar to pH found in the microsomal environment. Replacement of Asp85 and Asp96 both independently and at the same time to glutamic residues do not alter significantly the insertion efficiency (Figure 2B, lanes 4–5, 10–11, and 19–20), although a slightly higher insertion efficiency was consistently observed for D96E mutant when compared to D85E (compare lanes 5 and 11 in Figure 2B; see also Table 1) in agreement to recent biophysical data.

Alanine replacement increased the insertion propensity for D8SA, D96A, and D85/96A variants (Figure 2B, lanes 7–8,
Protonation occurs at pK values for aspartate residues studied in this work. It was found that defects as well as explicit inclusion of protein helices in dynamics simulations have suggested that both large water energy cost of burying a charge in the membrane. Molecular thermodynamically complicated by the fact that the free energy of changing their ionization state may outweigh the free energy cost within the cell has a role in this process is still an open question. Our glycosylation results in microsomes agree with previous data obtained in reconstituted systems, where pHLIP was added to the exterior of liposomes. When conditions were selected to avoid pHLIP aggregation, by working at low micromolar concentrations, pHLIP spontaneously (and reversibly) inserted into the bilayer upon a pH drop. The careful selection of experimental conditions might play a critical role in the TM helix formation is still to be determined.

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**EXPERIMENTAL SECTION**

**Enzymes and Chemicals.** All enzymes used in this work, as well as a pGEMI-derived plasmid, TNT T7 Quick Coupled System and rabbit reticulocyte lysate, were obtained from Promega (Madison, WI). ER-derived rough microsomes from canine pancreas were obtained from trna Probes (College Station, TX). EasyTag EXPRESS³⁵S Protein Labeling Mix (containing [³⁵S]-l-methionine and [³⁵S]-l-cysteine) for in vitro radioactive labeling was purchased from Perkin Elmer (Waltham, MA). Proteinase K was purchased from Sigma-Aldrich (St Louis, MO). Restriction enzymes used for DNA manipulation were obtained from Roche Molecular Biochemicals (Basel, Switzerland). The DNA plasmid, RNA clean up, and PCR purification kits were from Thermo Fisher Scientific (Ulm, Germany). All oligonucleotides were purchased from Macrogen (Seoul, South Korea).

**DNA Manipulation.** Oligonucleotides encoding the pHLLIP variants were introduced into the p2 domain (between residues 226 and 253) of E. coli leader peptidase (Lep). Glycosylation acceptor sites (G1 and G2) were placed in positions 96–98 (Asn-Ser-Thr) and 277–279 (Asn-Ser-Thr), flanking the tested sequences. Pairs of complementary oligonucleotides (25 μM) encoding bR helix C sequence (residues 80 to 101) were initially annealed at 85 °C for 10 min in annealing buffer (20 mM Tris-HCl, 20 mM MgCl₂, 500 mM NaCl) followed by slow cooling to 30 °C. Subsequently, the two-annealed double-stranded oligonucleotides were mixed, incubated for 5 min at 65 °C, cooled down slowly to room temperature, purified, and treated with polynucleotide kinase for 30 min at 37 °C. The annealed oligonucleotides were ligated overnight with T4 DNA ligase (Promega) at 16 °C into purified pGEM-Lep plasmid digested with SpeI/KpnI restriction enzymes and treated with alkaline phosphatase (Promega). All bR helix C variants were obtained by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, California). All helix C-derived inserts were confirmed by sequencing the plasmid DNA at Macrogen Company (Seoul, South Korea).

**Translocon-Mediated Insertion into Microsomal Membranes.** Variants in pGEM1 were transcribed and translated in one step using the TNT SP6 Quick Coupled System (Promega, USA). The reaction mixtures contained 75 ng of DNA template, 0.5 μL of EasyTag (5 μC), and 0.25 μL of column-washed canine microsomes (trNA Probes, USA) and were incubated for 30 min at 30 °C. Translation products were subsequently ultracentrifuged (100 000g for 15 min) on a sucrose cushion and analyzed by SDS-PAGE. The protein bands were quantified using a Fuji FLA-3000 phosphoimager and Image Reader 8.1j software. As mentioned above, the membrane-insertion probability for a given pHLLIP-derived segment was calculated as the quotient of the intensity of the double glycosylated band divided by the summed intensities of the singly glycosylated and doubly glycosylated bands. For the protease K (PK) protection assay, 2 μL of protease K (2 mg/mL) was added to the sample, and the digestion reaction was incubated for 15 min on ice. Before SDS-PAGE analysis, the reaction was stopped by adding 2 mM phenylmethane-sulfonyl fluoride.

**Normalization of Glycosylation Data.** The fraction $f_{2g} = C_{2g}/(C_{1g} + C_{2g})$ (where C is the pixel-count for the band in question) of doubly glycosylated molecules varies from 0 for tested segments that are fully integrated with a transmembrane disposition within the microsomal membrane, to 0.86 for H-segments that are fully translocated across the membrane, reflecting the fact that a lumenally exposed glycosylation site is only modified in ~ 95% of the molecules. To correct this, the values used in the calculation of $\Delta G_{49}$ were normalized as in

$$f'_{2g} = \frac{(f'_{1g} - 0.14)}{0.86}; \quad f_{2g} = 1 - f'_{2g}$$

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**Notes**
The authors declare no competing financial interest.

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