ACS OMEGA

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Cite This: ACS Omega 2020, 5, 556−560

http://pubs.acs.org/journal/acsodf

Insertion of Bacteriorhodopsin Helix C Variants into Biological Membranes

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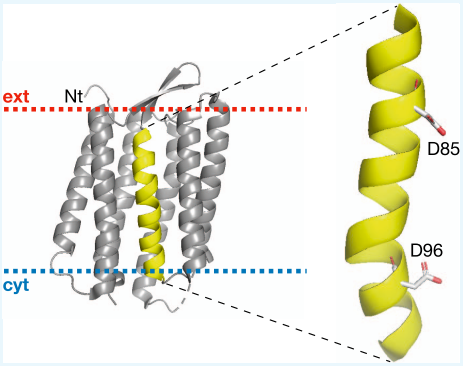
ABSTRACT: A peptide corresponding to bacteriorhodopsin (bR) helix C, later named pHLIP, inserts across lipid bilayers as a monomeric α-helix at acidic pH, but is an unstructured surface-bound monomer at neutral pH. As a result of such pH-responsiveness, pHLIP targets acidic tumors and has been used as 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 system[18−21] that allows accurate measurements of the apparent free energy (ΔG_{app}) 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.

Received: September 23, 2019
Accepted: December 4, 2019
Published: December 31, 2019

DOI: 10.1021/acsomega.9b03126
ACS Omega 2020, 5, 556−560

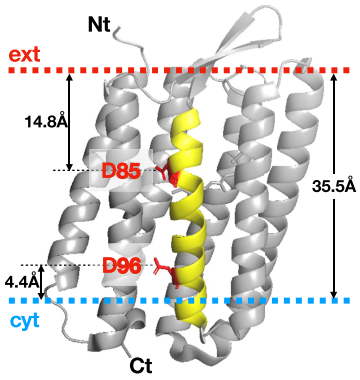


Figure 1. Cartoon representation of bacteriorhodopsin protein (PDB ID:1PY6). The helical residues (helix C) studied in this work are shown in yellow (from Trp80 to Val101, mature protein sequence). Positions of the hydrocarbon membrane boundaries (dotted lines) were obtained from the OPM database (https://opm.phar.umich.edu/):[42] red line indicates the extracellular membrane side, and the blue line indicates 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 Graphycs 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.[18,21] 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[22]) and were flanked by GGPG- and -GPGG tetrapeptides at both N- and C-termini to 'insulate' the tested sequences from the Lep carrier protein.[18] 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 (f_{1g}) and double glycosylated (f_{2g}) molecules allows calculating the apparent equilibrium constant, K_{app}, for the membrane insertion of a given sequence tested, $K_{app} = f_{1g}/f_{2g}$. The K_{app} value can be converted into the apparent free energy difference between the inserted and the noninserted state.[6] We determined the membrane insertion efficiency by phosphorimager scans of SDS-PAGE gels that measure the fractions of mono (f_{1g}) and double (f_{2g}) 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,[10] obtained with peptides assayed at neutral pH, expected to be similar to pH found in the microsomal

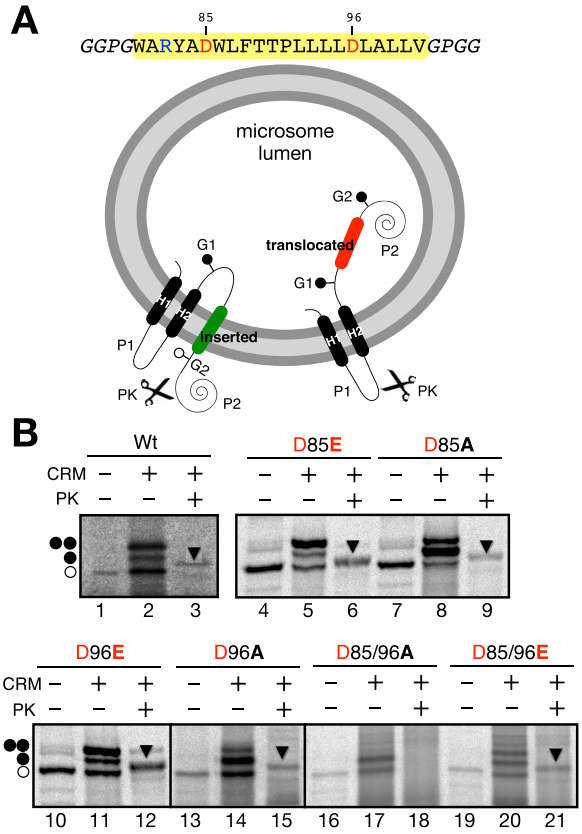


Figure 2. Translocon-mediated insertion of bR helix C variants into microsomal membranes using Lep as a model protein. (A) The bR helix C wild-type sequence (residues W80-V101, yellow box) flanked by the insulating tetrapeptides (italics) is shown on top (acidic and basic residues are shown in red and blue, respectively). Schematic representation of the Lep constructs. The tested sequence under investigation (colored) was introduced into the C-terminal P2 domain of Lep, flanked by two designed glycosylation acceptor sites (G1 and G2). Recognition of the tested sequence as a TM domain by the translocon machinery results in the modification of only the G1 acceptor site located in the luminal side of the microsome, preventing G2 glycosylation (left). The Lep chimera will be doubly glycosylated when the sequence being tested is translocated into the lumen of the microsomes (right). (B) In vitro translation in the presence of column-washed rough microsomes (CRMs) of the different Lep constructs. Constructs containing bR helix C Wt (residues 80−101; lanes 1−3), D85E variant (lanes 4 to 6), D96A variant (lanes 7−9), D96E variant (lanes 10−12), D96A variant (lanes 13−15), and double mutants D85/96A and D85/96E variants (lanes 16−18 and 19−21, respectively) were translated in the presence (+) and absence (−) of CRMs and proteinase K (PK). Bands of nonglycosylated proteins are indicated by a white dot; mono and double glycosylated proteins are indicated by one and two black dots, respectively. The protected doubly glycosylated H2/helix C/P2 fragments are indicated by an arrowhead. Each gel is representative of at least three independent experiments.

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.[23]

Alanine replacement increased the insertion propensity for D85A, D96A, and D85/96A variants (Figure 2B, lanes 7−8,

DOI: 10.1021/acsomega.9b03126
ACS Omega 2020, 5, 556−560

Table 1. Apparent Free Energy Difference (ΔG_{app}) and Insertion Percentage (Ins^{Exp}) Calculated for bR Helix C Variants[a]

Variant	Sequence	ΔG^{Pred}_{app}	ΔG^{Exp}_{app}	Ins^{Exp} (%)
Wt	WARYADWLFTTPLLLLDLALLV	0.46	1.00	15.9±6.9
D85E	WARYAEWLFTTPLLLLDLALLV	0.39	0.88	18.3±7.6
D85A	WARYAAWLFTTPLLLLDLALLV	-0.99	-0.20	58.3±14
D96E	WARYADWLFTTPLLLLELALLV	0.39	0.58	27.6±6.9
D96A	WARYADWLFTTPLLLLALALLV	-0.98	-0.34	63.7±10.2
D85/96E	WARYAEWLFTTPLLLLELALLV	0.31	0.16	43.3±9.3
D85/96A	WARYAAWLFTTPLLLLALALLV	-2.32	-2.01	96.6±4.5

[a]Prediction of ΔG for membrane insertion (ΔG_{app}^{pred}) was based on the ΔG prediction server (http://dgpred.cbr.su.se/), and experimentally determined values (ΔG_{app}^{exp}) were obtained from the in vitro glycosylation assay (in kcal/mol). The normalization of glycosylation data was calculated as done previously.[18,19] A negative value (shown in green) of ΔG_{app} indicates that the sequence is integrated into the membrane, a positive value (shown in red) of ΔG_{app} is indicates a non-TM disposition. The percentage of molecules inserted in the membrane is given in the right column (average and standard deviation of at least three independent experiments).

13−14, and 16−17, respectively). These results were confirmed by proteinase K (PK) treatment. Digestion with PK degrades membrane protein regions located exclusively outside the microsome, while lumenally exposed or membrane-embedded domains are protected. As expected, Lep chimeras harboring Wt, D85E, D85A, D96E, D96A, and D85/96E sequences were partially resistant to protease treatment due to the luminal location of the large C-terminal P2 domain (Figure 2B, lanes 3, 6, 9, 12, 15, 21, arrowhead). However, Lep constructs harboring the D85/96A variant was totally sensitive to PK digestion (Figure 2B, lane 18). Although alanine is the second most prevalent residue in transmembrane helices,[24] it is poised on the threshold between hydrophobic and hydrophilic residues in the translocon-mediated assay.[18,25] Thus, the higher insertion efficiency observed for this later sequence is probably due to the absence of the aspartic (or glutamic) acidic side-chain, more than to the presence of the methyl side-chain groups of the two alanine residues. The distribution of aspartic residues along the hydrophobic core of the membrane is thermodynamically complicated by the fact that the free energy for changing their ionization state may outweigh the free energy cost of burying a charge in the membrane. Molecular dynamics simulations have suggested that both large water defects[26] as well as explicit inclusion of protein helices mimicking biological membrane composition,[27] support the partition of charged residues into the hydrocarbon core.

The transfer of charged amino acids into the low dielectric environment of the membrane core is energetically unfavorable.[28] The ensuing loss in the conformational stability of membrane proteins is frequently compensated by gains in functional capabilities, such as catalytic or transport activities and binding of cofactors. Furthermore, charged residues can form intramembranous salt bridges that provide specificity and stability for the lateral association between transmembrane helices.[5,20,29,30] We speculate that acidic residue protonation might significantly contribute to TM insertion and can take place at the translocon environment. A recent solid-state NMR study determined the individual membrane pK_a values for the aspartate residues studied in this work.[16] It was found that protonation occurs at pK_a values of 5.82 ± 0.08 (Asp85) and 6.08 ± 0.08 (Asp96). Such values are much higher than the typical value for aspartate in solution (pK_a ∼4[16,31,32]). The higher pK_a values likely result from dehydration at the bilayer integration stage, leading to a decrease in the local dielectric constant.[33] In fact, London and co-workers have studied polyleucine peptides containing a single aspartic acid at the TM domain, also finding that they exhibited pK_a values ranging between 5.6 and 6.7, with higher values found for the more deeply buried aspartic acids.[34] Although a pure membrane-mimetic system might be a convenient depiction for many membrane properties, it might have thoughtful shortcomings, in particular, for describing the insertion of charged residues using peptide-based assays. Previous simulations of models mimicking biological membranes suggested that the actual insertion cost for a particularly charged residue appears to depend significantly on protein contents and on the presence of the translocon itself in the simulated membranes.[27] Whether the protonation of the aspartate residues occurs once the TM segment is located within the translocon[35] or even at an earlier stage inside the ribosome exit tunnel as recently reported for TM helix formation[36] is still to be determined.

Our glycosylation results in microsomes agree with previous data obtained in reconstituted systems, where pHLIP was added to the exterior of liposomes.[37] 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 similar role than the presence of the translocon: to prevent aggregation and misfolding events. Whether or not the different lipid composition in each endomembrane compartment within the cell has a role in this process is still an open question. Our data support that the large body of kinetic and thermodynamic information obtained in such reconstituted systems might be biologically relevant, and paved the way to study further pHLIP variants with potential biomedical applications.[38] However, our findings pointed out that the TM disposition of helices in the folded structure (Figure 1) do not always correspond to the efficient insertion of the corresponding isolated helices into biological membranes. Beyond the conceptual framework involving the mechanism of membrane insertion, the availability of quantitative experimental data on the contribution of aspartic residues to ΔG_{app} can facilitate the refinement of membrane protein insertion[39] and folding[40] prediction methods based on free-energy calculations.

DOI: 10.1021/acsomega.9b03126
ACS Omega 2020, 5, 556−560

EXPERIMENTAL SECTION

Enzymes and Chemicals. All enzymes used in this work, as well as a pGEM1-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 EXPRESS^{35}S Protein Labeling Mix (containing [^{35}S]-L-methionine and [^{35}S]-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 pHLIP 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 m Tris-HCl, 20 mM $MgCl_2$, 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/*Kpn*I restriction enzymes and treated with alkaline phosphatase (Promega).[20] 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_i), 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 (100 000*g* 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 pHLIP-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 proteinase K (PK) protection assay, 2 μL of proteinase 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 phenylmethanesulfonyl fluoride.[41]

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 ΔG_{app} were normalized as in[18]

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

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by Grants BFU2016-79487-P from the Spanish Ministry of Economy and Competitiveness (MINECO, ERDF supported) and PROMETEU/2019/065 from Generalitat Valenciana (to I.M.), and from the National Institutes of Health grant GM120642 (to F.N.B.). M.B-P. was a recipient of a postdoctoral fellowship from the Generalitat Valenciana (APOSTD 2016).

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DOI: 10.1021/acsomega.9b03126
ACS Omega 2020, 5, 556−560