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Proline localized to the interaction interface can mediate self-association of transmembrane domains ☆

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ABSTRACT

Assembly of transmembrane domains (TMDs) is a critical step in the function of membrane proteins. In recent years, the role of specific amino acids in TMD–TMD interactions has been better characterized, with more emphasis on polar and aromatic residues. Despite the high abundance of proline residues in TMDs, contribution of proline to TMD–TMD association has not been intensively studied. Here, we evaluated statistically the frequency of appearance, and experimentally the contribution of proline, compared to other hydrophobic amino acids (Gly, Ala, Val, Leu, Ile, and Met), with regard to TMD–TMD self-assembly. Our model system is the assembly motif (^{22}QxxS^{25}) found previously in TMDs of the *Escherichia coli* aspartate receptor (Tar-1). Statistically, our data revealed that all different motifs, except PxxS (P/S), have frequencies similar to their theoretical random expectancy within a database of 41916 sequences of TMDs, while PxxS motif is underrepresented. Experimentally, using the ToxR assembly system, the SDS-gel running pattern of biotin-conjugated TMD peptides, and FRET experiments between fluorescence-labeled peptides, we found that only the P/S motif preserves the dimerization ability of wild-type Tar-1 TMD. Although proline is known as a helix breaker in solution, Circular Dichroism spectroscopy revealed that the secondary structure of the P/S and the wild-type peptides are similar. All together, these data suggest that proline can stabilize TM self-assembly when localized to the interaction interface of a transmembrane oligomer. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

1. Introduction

Membrane receptor proteins play a significant role in mediating signal transduction across the membrane bilayer. This vital information is usually transferred as a result of conformational changes of the protein. Oligomerization is one of the most documented examples of such a change [1,2]. Receptor oligomerization is mainly mediated by the extracellular or intracellular domains. However, considerable data have been accumulated concerning the causal involvement of the transmembrane domains (TMD) in this process as well [3–7]. In contrast to the soluble regions of membrane proteins, our knowledge of the factors that control protein–protein interaction and recognition of the membrane-embedded domains is still limited.

In recent years, the role of specific residues in TMD–TMD interactions has been better characterized and various patterns of polar and hydrophobic amino acid motifs have been proposed [8–13]. However, despite the presence and the involvement of proline in the function of membrane proteins [14–18], its direct contribution to helix–helix interaction within the membrane, compared with other hydrophobic amino acids, has not been intensively studied. Structurally, proline is unique among the 20 amino acids because its side chain cycles back to the backbone amide, thus lacking the proton necessary for hydrogen bond formation, therefore inducing a kink in the protein backbone structure. In water-soluble proteins and peptides, proline is generally considered a helix breaker. A few antimicrobial peptides are known to contain conserved proline residues that confirm structural flexibility to the peptides and allow them to get only partially inserted into the membrane environment. In membrane embedded proteins, however, the structure induced by proline can vary from breaks to merely kinks of the helix and is determined by the structural context of the TMD and the specific position of the proline residue [11,19–23].

Proline is widely distributed in the putative TMDs of many integral membrane proteins such as the low-density lipoprotein receptor, the insulin receptor, and many transport proteins [14,15,24]. It has been suggested that proline in TMDs of transport proteins serves as a "switch" between the different conformations adopted by the protein in different steps of its transport cycle by cis-trans isomerization of proline [16,19,25,26]. Interestingly, analysis of TMD sequences from Human Gene Mutation Database reveals that mutations of proline have one of the highest phenotypic propensities [27]. This suggests

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that proline located within the TMD plays a functional or structural role in the activity of membrane proteins.

The first indication of the involvement of proline in TMD self-assembly was reported in a study that examined patterns that stabilize helical oligomers in a Glycophorin A randomized library [28]. Excluding glycine from the design of this library resulted in enrichment of proline together with serine and threonine in motifs that exhibit strong helix association. Additional evidence that supports the assumption that proline may be involved in TMD association is the finding that proline has the second highest packing value in membrane proteins following glycine [21], which indicates that it is often tightly packed in the structures of natural proteins.

To explore the contribution of proline to TMD associations, compared to other hydrophobic amino acids, we first statistically evaluated the appearance of ZxxS motifs (where Z = Gly, Ala, Leu, Ile, Val, Mat, or Pro) in a database that contains 41,916 bacterial TMD with lengths ranging between 15 and 30 amino acids. A similar motif (QxxS) has been shown to form a homodimer through by forming hydrogen bonds within the *Escherichia coli* aspartate receptor TMD (Tar-1) [29]. In this study we also used several complementary methods including (i) the ToxR assembly system, which can detect protein–protein interactions within the *E. coli* membrane environment; (ii) the SDS-gel running pattern of biotin-conjugated TMD peptides; (iii) the FRET experiment between fluorescence-labeled peptides; and (iv) analysis of the frequencies of occurrence of the ZxxS motifs in a bacterial TMD database where Z is proline or one of the other hydrophobic amino acids (Gly, Ala, Val, Leu, Ile, and Met). In addition, the secondary structure of a synthetic WT TMD was compared to that of the proline mutant. The results are discussed with regard to the contribution of proline to TMD self-assembly.

2. Materials and methods

2.1. Construction of the ToxR chimeras

A NheI-BamHI TM-DNA cassette encoding 16 residues of the Tar-1 WT TMD (^{13}MVLGVFALL**Q**LI**S**GSL^{28}) was inserted between the ToxR transcription activator and the *E. coli* maltose binding protein (MalE) within the ToxR-MalE plasmid. Point-mutations of Tar-1 were done in Gln at positions 22 (Table 1). The sequences of all the constructs were confirmed by DNA sequencing. The nomenclature of the TMDs represents the two amino acids located in the positions of the original polar residues of the QxxS motif.

2.2. In vivo detection of homo-dimerization of TMD domains within the membrane

The ToxR transcription activator can be successfully used to assess weak protein–protein interactions within the *E. coli* membrane. A Tar-1 TMD encoding the DNA cassette was grafted between the ToxR transcription activator and the maltose binding protein in the ToxR-MalE plasmid. The plasmid was then transformed into *E. coli* FHK12 cells that contain β-galactosidase, under the control of a *ctx* promoter. Dimerization of the TMDs, in this system, results in association of the ToxR transcription activator, which then becomes active and is able to bind the *ctx* promoter [30]. Quantification of the level of homo-dimerization was done by measuring the activity of the β-galactosidase reporter gene and by normalizing it to the cell protein content (OD_{590}) (miller units). The baseline activity of a negative control ToxR'A_{16}, which remains a monomer, was subtracted from all the results. The transformed cells were grown in the presence of chloramphenicol for 18 h at 37 °C. β-galactosidase activities were quantified in crude cell lysates after adding *o*-nitrophenylgalactosidase and by monitoring the reaction at 405 nm for 20 min, at intervals of 30 s at 28 °C by a Molecular Devices kinetic reader [30,31]. Specific β-galactosidase activities were calculated from the V_{max} of the reaction.

Table 1
Sequences of the TM domain that were inserted between the ToxR transcription activator and the maltose-binding protein in the ToxR-MalE plasmid.

TM Domain	Sequence[a,b,c,d]
Tar-1 WT	^{13}MVLGVFALL**Q**LI**S**GSL^{28}
Tar-1 A/S	^{13}MVLGVFALL**A**LI**S**GSL^{28}
Tar-1 G/S	^{13}MVLGVFALL**G**LI**S**GSL^{28}
Tar-1 L/S	^{13}MVLGVFALL**L**LI**S**GSL^{28}
Tar-1 V/S	^{13}MVLGVFALL**V**LI**S**GSL^{28}
Tar-1 I/S	^{13}MVLGVFALL**I**LI**S**GSL^{28}
Tar-1 M/S	^{13}MVLGVFALL**M**LI**S**GSL^{28}
Tar-1 P/S	^{13}MVLGVFALL**P**LI**S**GSL^{28}

[a] Amino acids are numbered according to their position in the WT protein (swissprot p07017).
[b] The amino acids in the positions of the dimerization motif are in bold.
[c] Mutations in the Tar-1 TMD are bold and underlined.
[d] The nomenclature of the TMDs represents the two amino acids replacing the original polar residues glutamine and serine of the WT sequence at positions 22 and 25, respectively.

2.3. ToxR-TM-MalE chimera protein expression levels

Western blot analyses were performed for any mutant tested. Aliquots of 10 μl FHK12 cells, each with a different plasmid or in the presence of a different peptide, were mixed with a sample buffer, boiled for 5 min, separated on 12% SDS-PAGE, and then transferred to the nitrocellulose membrane. The primary antibody used was anti-Maltose binding protein. The detection was done with a "Phototope-HRP Western Blot Detection System" from Cell Signaling Technology.

2.4. Maltose complementation assay

Membrane insertion and correct orientation were examined as previously described [32]. Briefly, PD28 cells, transformed with the different plasmids, were cultured overnight. The cells were then washed twice with PBS and used to inoculate M9 minimal medium including 0.4% maltose at a 200-fold dilution. The growth of the cells was measured at different time points by a spectrophotometer at 650 nm.

2.5. Peptide synthesis and purification

Peptides were synthesized by the Fmoc solid-phase method on a Rink amide MBHA resin. The peptides were cleaved from the resin by trifluoroacetic acid (TFA) and were purified by RP-HPLC on a C_4 reverse phase Bio-Rad semi-preparative column (250 × 10 mm, 300 Å pore size, 5 μm particle size). The purified peptides were shown to be homogeneous (>95%) by analytical HPLC. The peptides' compositions were confirmed by electrospray mass-spectrometry. Lysine residues were added to the N- and C-termini of the peptides to confer water solubility to the hydrophobic TMDs [33,34]. It was previously shown that hydrophobic peptides conjugated to lysines tags were correctly oligomerized and inserted into the membrane [33–35].

2.6. Circular Dichroism (CD) Spectroscopy

The CD spectra of the peptides were measured in an Aviv 202 spectropolarimeter. The spectra were scanned with a thermostated quartz optical cell with a path length of 1 mm. Each spectrum was recorded at 1-nm intervals with an average time of 10 s, at a wavelength range of 260 to 190 nm. The peptides were scanned at a 100 μM concentration in 1% LPC micelles. Fractional helicities [36,37] were calculated as follows:

$$\frac{[\theta]_{222}-[\theta]^{0}_{222}}{[\theta]^{100}_{222}-[\theta]^{0}_{222}}, \quad (1)$$