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Studies on the Insolubility of a Transmembrane Peptide from Signal Peptide Peptidase

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Polytopic helical integral membrane proteins represent an important class of proteins involved in a diverse array of cellular processes. Very little is known about their structures or how they function on a molecular level due to the inherent difficulties in producing and handling them in their functional forms.[1] Chemical protein synthesis (CPS) potentially offers an alternative route to the production of integral membrane proteins in quantities sufficient for biophysical studies. CPS also allows for complete chemical control over the covalent structure of the protein, which can often greatly assist in functional studies of a protein through precise placement of unique reactive/chemical groups and probes.

We chose signal peptide peptidase (SPP) as an initial target with which to develop synthetic methods for polytopic helical integral membrane proteins. SPP is a member of the intramembrane-cleaving proteases (I-CLiPs), a novel family of membrane proteases thought to cleave peptide bonds within the plane of the membrane.[2] The most famous in this family are the presenilins, which have been implicated in Alzheimer's disease.[3] SPP is 377 amino acids in length and is predicted to span the membrane seven times and is, therefore, typical of a large number of polytopic helical membrane proteins, such as the G protein-coupled receptors.

Our synthetic strategy is outlined in Figure 1. Stepwise solid phase peptide synthesis (SPPS) is generally limited to peptides of about 50 amino acids in length; longer polypeptides must be constructed through the ligation of smaller building blocks.[4] The building blocks we envision consist of one transmembrane (TM) helix with portions of the loops on the N- and C-termini. These peptides contain an α-thioester functionality ($^{\alpha}$COSR) on the C-terminus to allow ligation to another peptide with an N-terminal cysteine through native chemical ligation,[4] which could be performed either in a lipid bilayer[5] or in solution.

We first chose to synthesize the putative fourth transmembrane domain of signal peptide peptidase as a test case for the other TM segments because it contains part of the putative catalytic apparatus for intramembrane peptide bond cleavage.[6] Because the exact location of the TM domain is unknown, we included four residues on the N-terminus and five residues on the C-terminus beyond what is predicted as transmembrane in SWISS-PROT. The 30 residue peptide was made by stepwise SPPS[7] and cleaved/deprotected by HF. Upon attempting to dissolve the crude products in 1:1 ACN/H_2O + 0.1% TFA, the resin became chunky and a cloudy precipitate formed that clogged the filter. Neat TFA was used to dissolve the peptide. Under standard reversed-phase HPLC (RP-HPLC) conditions, the peptide did not elute from a C4 column. MALDI-MS of the crude product showed that we had made the right peptide (Supporting Information). Various combinations[8−10] of temperature, columns, and eluents were tried, none of which gave satisfactory results. The peptide was soluble in strong organic acids, such as TFA, or strong polar aprotic organic solvents, such as DMF and DMSO, but was largely insoluble in acetonitrile and protic solvents, such as methanol or water.

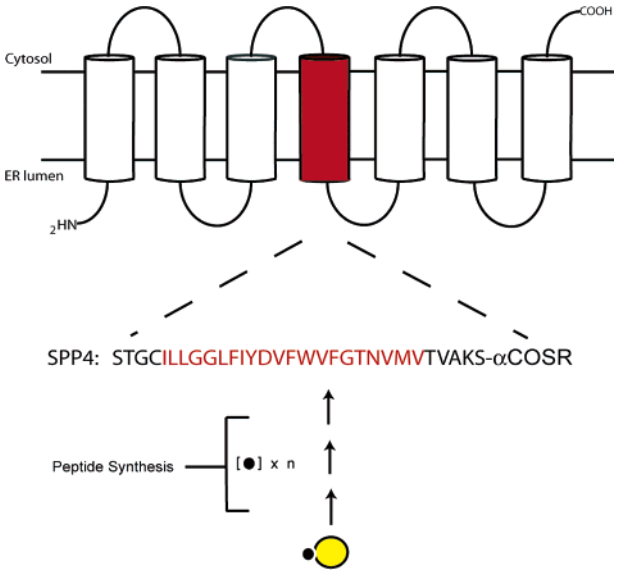

Figure 1. Model peptide from SPP. The predicted TM region is shown in red.

We hypothesized that, in the case of TM peptides, the difficulty in the handling and purification is due to their ability to form helices that present a mostly hydrophobic interaction surface. This causes a strong interaction with the hydrophobic reversed-phase and also causes aggregation. We therefore sought to disrupt any helix formation in order to disfavor the aggregated state and thus increase the hydrogen-bonding interactions of the backbone with water, thereby increasing the solubility of the peptide. To disrupt helix formation, we introduced modifications within the putative TM region of the peptide that are known to disfavor helix formation. These modifications were conservative with regard to the overall hydrophobicity of the peptide sequence.[11] Peptides were synthesized with 1 and 3 proline substitutions (SPP4-1Pro and SPP4-3Pro), 1 and 3 D-amino acid substitutions (SPP4-1D and SPP4-3D), 3 glycine substitutions (SPP4-3Gly), and 3 *N*-methyl amino acid substitutions (SPP4-3NMe). (For peptides made, their masses, and CD spectra, see Supporting Information.) The modifications in the *N*-methyl peptide were shifted by one amino acid toward the C-terminus because these *N*-methyl amino acids were commercially available.

Relative hydrophilicity was determined by analytical RP-HPLC.[12,13] The SPP4-1D and SPP4-1Pro peptides, as well as SPP4-3Gly, showed insolubility in 1:1 ACN/H_2O + 0.1% TFA similar to that of the SPP4-WT and did not elute as well-defined peaks from the reversed-phase column (data not shown). However, SPP4-3Pro, SPP4-3D, and SPP4-3NMe were soluble in 1:1 ACN/H_2O + 0.1% TFA and eluted as well-defined peaks from a C4 column

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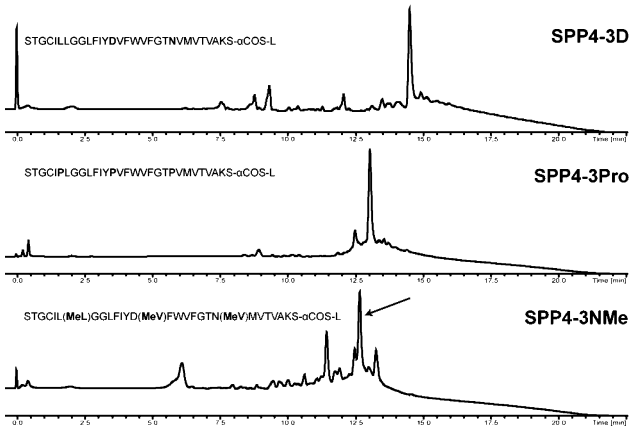

Figure 2. RP-HPLC of crude SPP4 analogues designed to disfavor secondary structure formation. The arrow indicates the target product in the SPP4-3NMe peptide. Peptides were loaded from 1:1 acetonitrile/water + 0.1% TFA and eluted from a C4 column with a gradient of 10−75% acetonitrile at 40 °C.

using a standard acetonitrile gradient (Figure 2). Out of all the analogues synthesized, the peptide with methyl groups on the backbone was the most hydrophilic.[14] SPP4-3D showed better solubility than the WT or SPP4-3Gly peptides, but was less hydrophilic than the SPP4-3NMe and SPP4-3Pro peptides.

Two of these modifications, D-amino acids and *N*-methyl amino acids, did not change the chemical nature of the side chains in SPP4, while the proline mutations introduced conservative changes in the overall hydrophobicity of the side chains according to standard hydrophobicity scales. One might expect that introducing methyl groups on the backbone would, in fact, act to *increase* the overall hydrophobicity of the peptide, yet exactly the opposite was seen—SPP4-3NMe was the most *hydrophilic* peptide of the analogues created. As N-substituted groups in peptides are known to strongly disrupt secondary structure formation, this observation suggests that secondary structure formation itself contributes a large driving force for aggregation in SPP4. SPP4-3Gly showed the worst solubility characteristics after the WT peptide, a result that is consistent with our hypothesis given the fact that glycines commonly occur within TM helices and are even essential for some TM helix−helix interactions.[15,16] The main driving force in TM helix formation is the requirement to shield polar backbone groups from the hydrophobic environment of the membrane.[17] This overcomes any entropic penalty introduced by glycine.

Previous studies have suggested that helix formation can account for a majority of the binding energy of a peptide to the reversed-phase,[18] and that D-amino acid replacements in amphipathic helices can reduce their retention times on RP-HPLC presumably by disrupting helix formation.[19,20] These studies, taken together with our results, suggest that formation of a TM helix increases the overall hydrophobicity of a peptide and creates a more favorable interaction with the reversed-phase during liquid chromatography. In contrast to amphipathic peptides, where the strong binding interaction with the reversed-phase through the hydrophobic face of the helix is balanced with the hydrophilicity of the opposite face, helical TM peptides cannot present a hydrophilic face to the mobile phase and, therefore, do not show the standard elution properties characteristic of most peptides.

Proposed methods for purifying hydrophobic peptides have largely focused on modifications to standard RP-HPLC[21−23] but have not proven general for all TM helices. An alternative strategy promoted by Deber et al. is to modify the peptide to increase its aqueous solubility, which renders it easier to handle and purify. In this strategy, hydrophilic groups are placed on the ends of the TM peptide to increase its apparent hydrophilicity while maintaining the structural properties of the transmembrane region.[24] This type of modification allows one to study the properties of a single TM helix, but it is not suitable for chemical protein synthesis because the modifications to the peptide are permanent and would interfere with the function of the protein.

As a general method to improve the solubility of TM helices, we reasoned that by disrupting helix formation we could disfavor the aggregated state and therefore increase the *effective* solubility of the peptide, leading to improved elution on RP-HPLC. Except for SPP4-3Gly, all of the peptides with three helix-breaking residues in the TM region showed markedly improved solubility in aqueous solvents and elution from the reversed-phase column. Reversible backbone modification of the TM peptide to disrupt helix formation during segment assembly may lead to improved ease of polytopic helical membrane protein chemical synthesis. We are currently working on such a strategy.

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Supporting Information Available: MALDI-MS spectrum of crude SPP4-WT, a table of synthesized SPP4 peptide analogues and their CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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