

Solid-Phase Synthesis and Chemical Ligation of Transmembrane Segments of Rhodopsin

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Introduction

To investigate the conformational changes associated with photoactivation of rhodopsin [1], the GPCR of vision, we have embarked on a program of chemical synthesis of its transmembrane segments. By reconstituting such synthetic segments containing specific fluorescent and spin labels with expressed segments of rhodopsin, we hope to generate hybrid “split receptors” that are fully functionally for biophysical studies. For this reason, a number of TM fragments of rhodopsin were synthesized by various solid-phase protocols, and chemical ligation was used to link these fragments into larger fragments of rhodopsin.

Results and Discussion

The major anticipated obstacles in the chemical synthesis of membrane proteins are the large regions of highly hydrophobic amino acid residues and the difficulty in solubilizing the hydrophobic segments under conditions that are suitable for the chemical ligation [2]. Our synthetic approach has proven efficient for synthesizing hydrophobic transmembrane peptides typically yielding material with crude purity of 60–70% that could be purified to homogeneity with an overall yield of 8–10%.

Two kinds of polypeptides were synthesized for chemical ligation. One was the amino-terminal peptide with an active thioester at the carboxy end. Another was the carboxy-terminal peptide with the free amino group of cysteine in the N-terminus. Chemical synthesis of α -thioester polypeptide on a 3-mercaptopropionylamide-MBHA resin was achieved by using Boc amino acid derivatives employing *in situ* neutralization/HBTU activation protocols for Boc-SPPS. The α -thioester polypeptide was obtained after cleavage with hydrogen fluoride and purification with HPLC. The amino terminal peptide was made by Fmoc strategy with HBTU coupling reagent on SynnovaTGA resin. Two points should be considered:

1. During the synthesis of peptides with more than 50 amino acids, the deprotecting reagent(s) should be changed gradually from 25 to 40% piperidine/DMF. After 40 amino acids, it is better to add 2% DBU to the deprotecting solution;
2. In the transmembrane peptides, there are many hydrophobic and sterically hindered amino acids side chains, and this increases the coupling time for each amino acid. In order to reduce the total coupling time of the peptide, the pH of the reaction was adjusted to between 6 and 7. By keeping the reaction in this pH range, we obtained the peptide in higher yield with higher purity.

The major problem encountered in the purification and characterization of such transmembrane fragments was formation of insoluble gels in the ligation solvent. A number of strategies have been tried to solubilize or modify the solubility of these TM fragments. To date, there has been no generally successful approach to this problem. However, by adding five lysine residues to the amino terminal of the thioester peptide and two lysine residues to the carboxy-terminal of cysteine peptide, we have overcome this hurdle in several cases. The addition of these lysine residues to the peptides reduces their hydrophobicity and increases their solubility in the ligation solvent. In

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addition, once the two peptides are ligated together, the added lysines will not hinder the fragment's insertion into artificial membranes because the lysines will lie on the extracellular side of the membrane and will help orient the peptide in the membrane.

The following peptide fragments were synthesized:

Rhodopsin sequences P49, 140-188; P22, 167-188; P51, KK-140-188

Thioester sequence P33, 107-139; P38, KKKKK-107-139; P27, 140-166; P27, Msc-140-166.

Ligation of TM fragments was successful under the following conditions:

A. P22, 0.5 μ M and P38, 0.1 μ M in 50 μ L solvent (0.1 M) phosphate buffer containing 6 M guanidine and 1% S-phenol, pH 7.5. The reaction solution was heated to 60 °C and 15 μ L trifluoroethanol was added. At 72 h, the ratio of P38 to ligation product was 3 : 98. MALDI MS of the product gave MW = 6595.20; calculated 6596

B. P2 (Cys-Val-NH₂) 0.15 μ M and P38, 0.1 μ M in solvent 50 μ L (0.1 M phosphate buffer pH 7.5 with 6 M guanidine and 0.1% phenol). After 24 h, the ratio of product to P38 was 6 : 1. (MW = 4444.9; calculated 4444.5)

C. P51 Lys2-140-188, 0.15 μ M and P38, 0.1 μ M were dissolved in solvent 50 μ L (0.1 M phosphate buffer pH 7.7 with 6 M guanidine and 0.1% phenol). After 90 h, the ratio of product to P38 was 1 : 2. (MW = 9836; calculated 8832)

D. Finally, we report that the peptide P38 (lys5/107-139) and P51 (lys2/140-188) have been chemically ligated with positive results by MS.

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References

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