Pergamon

Tetrahedron Letters, Vol. 38, No. 40, pp. 7071-7074, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)01651-1

Assembling of the Four Individual Helices Corresponding to the Transmembrane Segments (S4 in Repeat I-IV) of the Sodium Channel

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Abstract: A template having four cysteines with selectively detachable protecting groups was developed, which enables selective introduction of individual helices by chemical ligation between the free sulfhydryl group on the template and the chloroacetyl moiety of the helical peptide. Using the template, a protein having ion channel activity with rectification was obtained.

In the past decade, the de novo design of artificial proteins has achieved prominent success in creating functional molecules.[1)] The four-helix-bundle motif has been one of the main targets of the de novo design of artificial proteins because the helical structure is the most suitable as a protein backbone to maintain the spatial arrangement of the protein. Mutter's TASP (template-assembled synthetic proteins)[2)] strategy is one of the most promising approaches to manifest this motif, where helices are programmed to assemble by template molecules. Montal and his coworkers[3)] have applied Mutter's approach to tether transmembrane helices of ion channels on a peptide template, demonstrating that these helices are deeply involved in channel function. However, each structure constructed by Montal's group was a tetramer of an identical helical peptide corresponding to one of the transmembrane helices of ion channels.

Helical motifs of natural proteins are usually composed of multiple, heterogeneous helices, displaying highly complicated functions; development of a strategy to organize multiple protein components is important to acquire sophisticated functions. We have already developed two approaches to assemble the α-helical peptide units by selective inter-unit disulfide[4)] or thioether[5)] cross-linking. The most salient feature of these approaches is the feasibility of tethering helical pepitides composed of different amino acid sequences. However, these approaches are mainly for the construction of antiparallel helical proteins. Here we report a new and efficient approach to construct the parallel helical protein structure comprising helices with different amino acid sequences with the aid of a multi-component introducible template.

The structure of the template[6)] is shown in Fig.1. The template was designed based on a Mutter template which was expected to assume an antiparallel β-sheet structure to assist the assembling of helices, but the four Lys residues on the Mutter template were replaced by cysteines having selectively detachable protecting groups, *i.e.*, Cys(Trt), Cys(MBzl), Cys(Ad), and Cys(Acm) (Trt=trityl, MBzl=*p*-methoxybenzyl, Ad=adamantyl, Acm=acetamidomethyl). These alterations eventually enable selective introduction of helices by chemical ligation between the free sulfhydryl group on the template and the chloroacetyl moiety

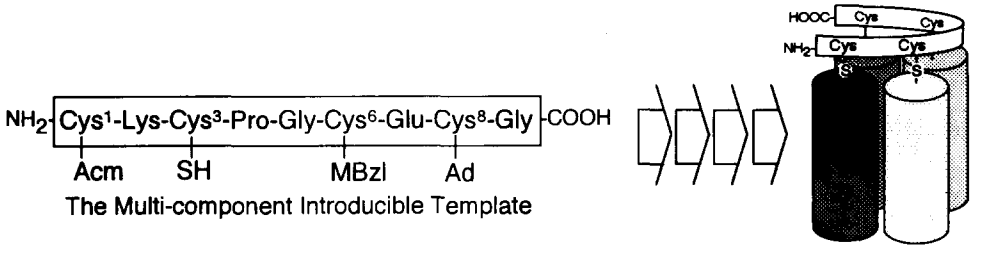

Fig. 1. Structure of the multi-component introducible template which enables to assemble four helices with different amino acid sequences.

of the helical peptide segment. Another property of proteins designed with our approach is that the helices are connected with the template at their N-terminal; whereas, in the case of the Mutter template, the helices are connected by their C-terminal on the template. This property could be important where the C-terminus of helices plays a crucial role in protein function.

In order to examine the applicability of our approach to construct artificial functional proteins, a four-helix-bundle protein, the sequence of which is derived from the S4 segment in the repeat I to IV of the *Electrophorus electricus* sodium channel[7] was constructed. The sequence is assumed to be involved in the voltage sensor function, one of the most important functions for regulation of the opening of the channel. Four α–helical peptide segments, named **[NaI]**, **[NaII]**, **[NaIII]**, **[NaIV]** that correspond to the S4 region in the repeat I to IV, respectively, were designed as shown in Fig. 2.[8] On the N-terminus of the sequences, chloroacetyl-γ-aminobutyric acid (GABA) was placed as a linker. The C-terminus amino acid was chosen as Tyr for facilitating purification by its UV absorption at 275nm, and the C-terminus was amidated to stabilize the helical structure.[9] In order to avoid the steric hindrance on the introduction of the helices, the helical segments were successively introduced to the sulfhydryl group on the template from the center to the end, namely in the order of $Cys^3 \rightarrow Cys^6 \rightarrow Cys^8 \rightarrow Cys^1$, respectively (Fig. 3). Incorporation of **[NaII]** onto the template was conducted by the reaction of the free SH group of Cys^3 with the chloroacetyl moiety of **[NaII]**. The product was then treated with 1 M $(CH_3)_3SiBr$-thioanisole/TFA[10] to remove the MBzl group from Cys^6. After introduction of **[NaIII]**, the obtained peptide was treated with 1 M CF_3SO_3H-thioanisole/TFA[11] to remove the Ad group from Cys^8. **[NaIV]** was introduced and the Acm group removed from Cys^1 by AgOTf/TFA treatment.[12] Finally, introduction of **[NaI]** afforded the desired protein **4**. The protein was highly pure according to HPLC analysis on a C_4 column. The estimated molecular weight [M/Z: 12596.8 $(M+Na)^+$] from time of flight mass spectrometry (TOFMS) was in good agreement with the theoretical average mass (12596.2). The CD spectrum of the protein in MeOH suggested that the protein has an helical structure in the membrane [$[\theta]_{222}$: -13,100 deg·cm^2/dmol (14 μM protein / MeOH)].

The ion channel activity of the protein **4** was examined electrophysiologically using the "tip-dip" method.[13] Figure 4(a) illustrates the single channel current recordings obtained for the protein **4** when the membrane potential was held at +100 mV, where two different channel states with conductances of 490 pS (Fig. 4 (a)-(i)) and 140 pS (Fig. 4 (a)-(ii)) were observed.[14,15] Either state showed discrete, square events that display transitions between two current levels corresponding to the closed and open states in lipid bilayers. Increase in the conductance at the positive transmembrane potential should reflect the conformational change in the peptides in the S4 region resulting in voltage dependence of the sodium

[NaI] Cl-CH_2CO-NH$(CH_2)_3$CO-RNVSALRTFRVLRALKTITIFPGY-NH_2
[NaII] Cl-CH_2CO-NH$(CH_2)_3$CO-QGMSVLRSLRLLRIFKLAKSWPGY-NH_2
[NaIII] Cl-CH_2CO-NH$(CH_2)_3$CO-GAIKNLRTIRALRPLRALSRFEGY-NH_2
[NaIV] Cl-CH_2CO-NH$(CH_2)_3$CO-RVIRLARIARVLRLIRAAKGIRGY-NH_2

spacer S4

Fig. 2. Structure of the peptide segments. Each amino acid is expressed by one-letter codes.

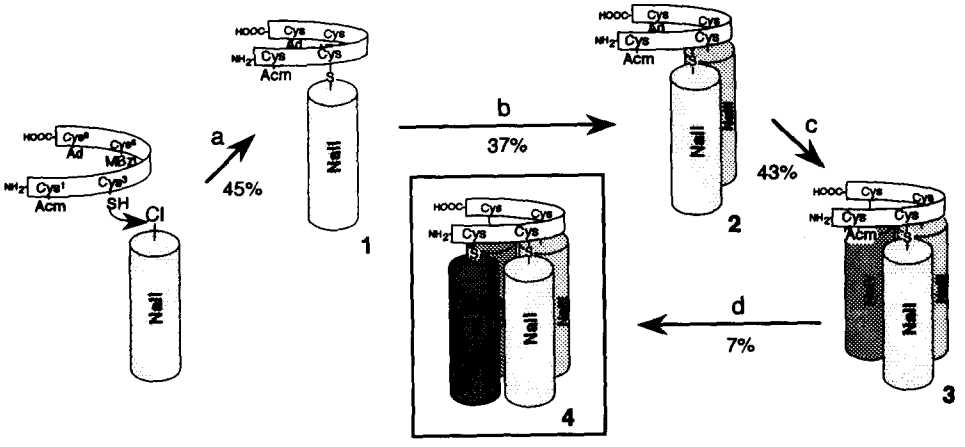

Fig. 3. Construction of the four-helix-bundle protein **4** comprising the peptides corresponding to the voltage sensor region (S4) of the sodium channel. (a) Introduction of **[NaII]** (in 6 M GnHCl-0.1 M Tris buffer, pH 8.0) (r.t., 16 h); (b) 1 M $(CH_3)_3$SiBr-thioanisole/TFA treatment (0°C, 2 h) followed by introduction of **[NaIII]**; (c) 1 M CF_3SO_3H-thioanisole/TFA treatment (0°C, 1.5 h) followed by introduction of **[NaIV]**; (d) AgOTf (100 eq)/TFA treatment (0°C, 1.5 h) followed by introduction of **[NaI]**.

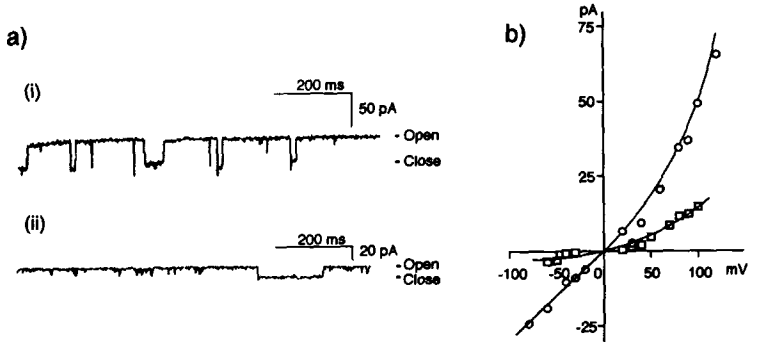

Fig. 4. (a) Single channel current recordings of the four-helix-bundle protein **4**. (b) The current-voltage (I-V) relationship of the channel. The current expressed by the open circles corresponds to the state (i), and that expressed by the open square to the state (ii). Lipid: diphytanoylphosphatidylcholine; buffer: 500 mM NaCl-10 mM MOPS (pH 7.2); protein concentration: 40 nM. The voltage polarity corresponds to the side of the working electrode (inside the patch pipette), the reference electrode in the bath to which the protein is added (cis side) being grounded.

channel, which indicated that this region is highly involved in the voltage sensing function of the channel.

In conclusion, we have succeeded in assembling four individual helices corresponding to the S4 segments of the sodium channel on our multi-component introducible template to obtain a highly homogenous protein. The protein showed ion channel activity with rectification in a lipid membrane, which characteristic should reflect the voltage sensing function of the sodium channel. We believe our approach using the multi-component introducible template has a great potential for the creation of artificial functional proteins.[16]

Acknowledgments. This work was supported by Grant-in-Aids for Scientific Research from the Ministry of Education, Science and Culture of Japan. This work was also supported by Uehara Memorial Foundation, Takeda Science Foundation, and Nissan Science Foundation.

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15. The channel activity of a 22-amino acid residue peptide corresponding to the S4 segment of the repeat IV has been reported by Tosteson (Tosteson, M. et al. *Proc. Natl. Acad. Sci., USA* **1989**, *86*, 707-710).
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(*Received in Japan* 7 *July* 1997; *revised* 4 *August* 1997; *accepted* 8 *August* 1997)