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Interaction of ladder-shaped polyethers with transmembrane α-helix of glycophorin A as evidenced by saturation transfer difference NMR and surface plasmon resonance

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ABSTRACT

Ladder-shaped polyether (LSP) compounds are thought to interact with transmembrane α-helices, but direct evidence has scarcely obtained for these interactions. We adopted a transmembrane α-helix of glycophorin A, and quantitatively evaluated its interaction with LSPs such as yessotoxin (YTX), desulfated YTX and artificial LSPs, using surface plasmon resonance and saturation transfer difference NMR. As a result, dissociation constants (K_D) of YTX and desulfated YTX to a transmembrane domain peptide of glycophorin A were determined to be in the submillimolar range. Furthermore, in saturation transfer difference NMR, the signals at the polyene side chain and the angular methyl groups of YTX were significantly attenuated, which probably comprised an interacting interface of LSPs with a transmembrane α-helix. These results suggest that hydrophobic interaction plays an important role in molecular recognition of the α-helix peptide by LSPs.

Ladder-shaped polyether (LSP) compounds,[1] including brevetoxin B,[2] ciguatoxin,[3] and yessotoxin[4] (Fig. 1), are a group of unique products produced by dinoflagellates. LSPs possess a molecular stem composed of continuous trans-fused cyclic ethers. To date, more than 50 naturally occurring LSPs have been reported, most of which were found in association with food poisoning or massive fish kill. However, few mode-of-action studies at the molecular level have been carried out, chiefly due to the short supply of materials. Brevetoxins and ciguatoxins are a few exceptions in that their molecular target has been identified;[5] the toxins share a common binding site on the α subunit of voltage-sensitive sodium channels (VSSCs), with very high affinity. In addition, it has been recently reported that gambierol[6] selectively inhibits the action of voltage-gated potassium channels (VGPCs) in mouse taste cells[7] and *Xenopus laevis* oocytes.[8] Because VSSCs and VGPCs largely consist of transmembrane (TM) helices, membrane-integral α-helix peptides are considered to be the common interacting motifs of LSPs. For example, gambierol,[9] gambieric acid A,[10] and brevenal[11] show moderate affinity to VSSCs despite that VSSCs are not their target proteins. This broad specificity with low affinity can be accounted for by their structural features; the distance between the neighboring skeletal oxygen atoms on one side of the LSPs is roughly consistent with an average pitch of α-helices (ca. 5 Å).[12,13] Thus, extremely high affinity to VSSCs as shown by ciguatoxins may be achieved when an arrangement of polyether oxygen atoms exactly matches the sequence of amino acid residues in the helix. However, there has been little experimental evidence to support this hypothesis, probably owing to the lack of effective methods for assessing the interaction of LSPs with transmembrane proteins.

We previously developed an assay system for evaluating the interaction between LSPs and the α-helices of glycophorin A (GpA),[13] a heavily glycosylated membrane protein occurring in erythrocyte. GpA is known to form a dimer[14] in membrane environments mainly by interaction between α-helical TM domain (Fig. 3), where the GXXXG motif[15] (glycine zipper[16]) is considered to be essential for dimerization. We evaluated the activities of LSPs on the basis of dissociation of the GpA oligomers and dimers, which could be detected by SDS–PAGE.[13]

Consequently, the interaction between GpA and a tetracyclic LSP model compound[17] was specifically detected, indicating the utility of this method. In another study,[18] we further examined the interaction of GpA with synthetic LSP models, artificial ladder-shaped polyethers (ALPs), and disclosed that hydrophobic matching plays an essential role in recognition of transmembrane peptides by LSPs.[18] For further exploring the mode of action by LSPs, we sought the methodology that provided structural infor-

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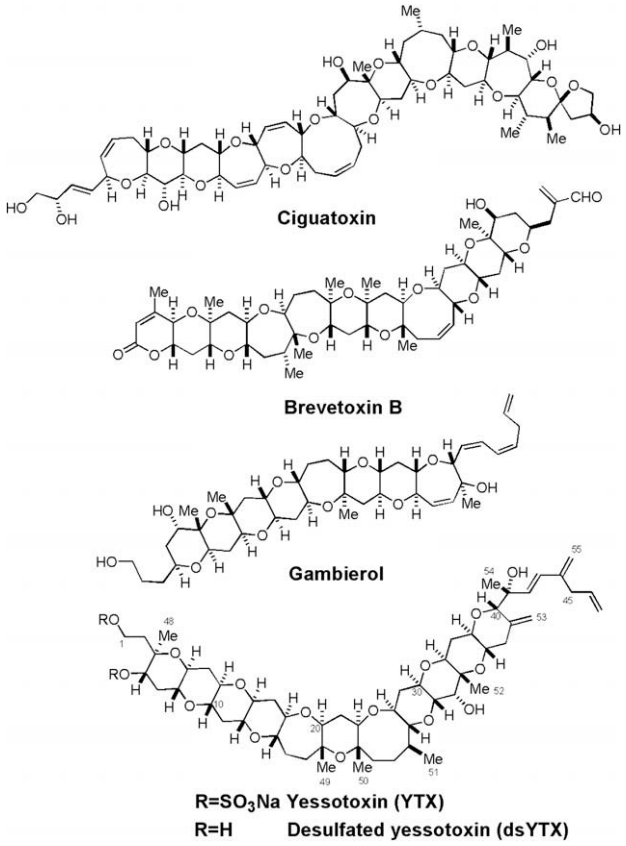

Figure 1. Structures of brevetoxin B, ciguatoxin, gembierol, and yessotoxins.

mation on mechanisms underlying their molecular recognition. In this study, we report the structure basis of the interaction between LSPs and transmembrane α-helices evidenced by saturation transfer difference (STD)-NMR.[19] Their affinities evaluated by surface plasmon resonance (SPR)[20] are also discussed.

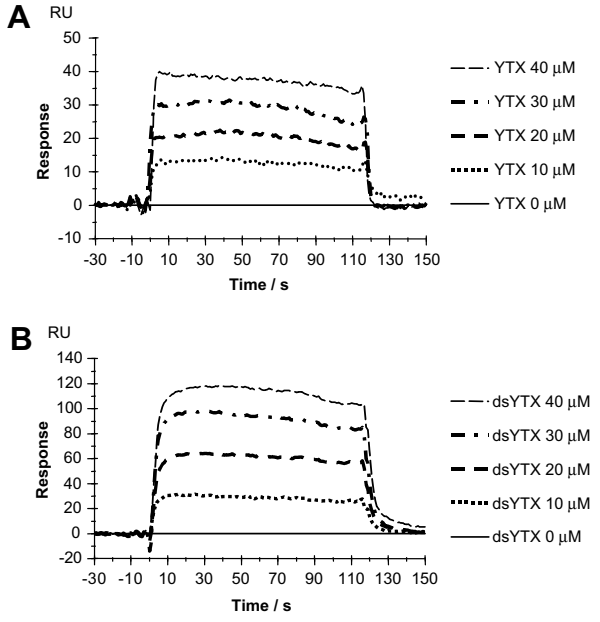

Figure 2. SPR sensorgrams of LSPs binding to GpA–TM. (A) YTX was injected in 0–40 μM; (B) ds-YTX was injected in 0–40 μM (containing 1% DMSO and 400 μM Tween 20). Four traces in either (A) or (B) corresponds 40, 30, 20, and 10 μM from the top.

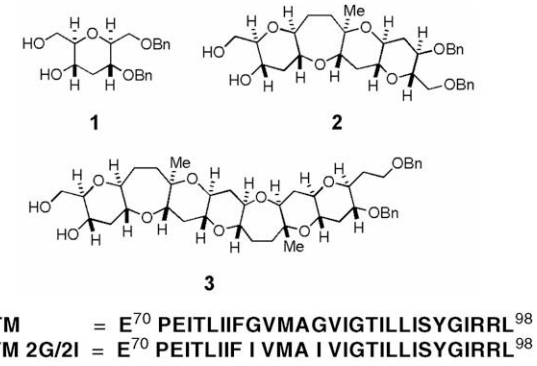

Figure 3. Structures of monocyclic ether **1**, tetracyclic, and heptacyclic artificial ladder-shaped polyethers (**2** and **3**) with those of synthetic peptides corresponding to the transmembrane region of GpA.

We first examined the interaction of LSPs with a transmembrane α-helix of GpA by SPR. For the SPR experiments (BIACORE X), a synthetic peptide corresponding to the transmembrane domain (EPEITLIIFGVMAGVIGTILLISYGIRRL, GpA–TM)[21] was immobilized via an amide bond onto the CM5 sensorchip, which possesses carboxymethyl groups covalently attached to dextran.[20] As LSP models, we selected synthetic compounds **1**, **2**, **3**, and natural YTX (Fig. 3)[18] since YTX was sufficiently obtained from the laboratory cultures of the dinoflagellate *Protoceratium reticulatum*[13,22] and ALPs had been synthesized in the previous studies.[17,18] Desulfated YTX (ds-YTX) was prepared by hydrolysis of sulfate esters by a reported method.[13] These LSPs have been shown to dissociate GpA dimers into monomers in the SDS–PAGE experiments.[13,17,18] Their interaction with GpA–TM was analyzed under the following conditions; a running buffer HBS (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA) containing 1% DMSO and 400 μM Tween 20, a flow rate 10 μL/min, and temperature 25 °C. Higher concentration of Tween 20 was used to mimic biomembrane environments by covering the hydrophobic surface of the transmembrane peptide with the detergent.

As a result, dose-dependent changes in sensorgrams were observed for all the analytes tested (Fig. 2, data not shown for ALPs). The equilibrium dissociation constant (K_D) was determined using BIAevaluation software with a steady state affinity model based on stoichiometric interaction (Table 1). The affinity of YTX to GpA–TM is higher than that of ds-YTX although this stabilization effect for the YTX–GpA–TM complex may be derived from the electrostatic interaction between two sulfate esters of YTX and basic amino acids in GpA–TM (e.g., R96 and R97). On the other hand, affinities of tetracyclic and heptacyclic ALPs (**2** and **3**), a part of which have been published previously,[18] were comparable to or higher than that of YTX, while monocyclic ether **1** showed virtually no interaction. These results are roughly consistent with those of SDS–PAGE.[18] The higher affinity of **3** suggests that the shape of LSP molecules influences the proximity to transmembrane pep-

Table 1
Dissociation constant (μM) of LSPs against GpA–TM

LSP	GpA–TM	GpA–TM (2G/2I)
YTX	120	85
ds-YTX	270	99
1	2400*	860
2	240*	150
3	48*	7.7

* The values are cited from a previous study.[18]

tides since the molecular stem of ds-YTX is bent at the 7/6/8 tricycle, whereas **3** having rather extended conformation.

The SPR responses at each concentration in Figure 2 differed between YTX and ds-YTX, where ds-YTX always gave rise to a higher response. This apparent difference in the quantity of binding sites can be accounted by the hydrophilicity of YTX; the theoretical saturation quantity of YTX binding to GpA–TM is around 2000 RU, and thus the less than 5% of the sites are occupied by YTX even at the higher concentrations. Under these conditions, hydrophilic YTX can not penetrate into the interior of GpA–TM-coupled dextran which is densely covered with detergent while hydrophobic ds-YTX can enter deep into the interior. In addition, the poor water solubility of ALPs may affect their effective concentrations in a mobile phase. Thus, the SPR responses can not be compared among the LSPs tested. The present SPR data allow us to approximately compare the affinity of LSPs to the transmembrane peptides, whereas making it difficult to estimate the quantity of their binding sites on the peptides.

We then examined the importance of transmembrane glycine residues, which play an essential role in dimerization of GpA. In this glycine zipper motif, weak hydrogen bonds between the Cα–H of glycine and a carbonyl oxygen (Cα–H···O)[23] are thought to stabilize the dimeric structure. The Cα–H of glycine may, therefore, act as a hydrogen bond donor toward ether oxygen atoms of LSPs when LSPs dissociate GpA dimers into monomers.[13,17,18] Thus, to examine whether LSPs specifically bind to the glycine zipper motif, a GpA–TM mutant where both of Gly79 and Gly83 were replaced with Ile (GpA-TM2G/2I) was synthesized by the solid-phase method, following a protocol for GpA-TM.[21] The interactions with LSPs were analyzed by SPR in the same manner (Table 1). GpA-TM2G/2I bound to all the LSPs with higher affinity than the wild type GpA-TM. Moreover, heptacyclic ALP **3** bound to the mutant helix with one order of magnitude higher affinity than YTX and ds-YTX. These results suggest that LSPs generally recognize hydrophobic α-helices rather than a glycine zipper motif.

For elucidating the mode of molecular recognition more precisely, we moved on to NMR experiments. Saturation transfer difference (STD)-NMR is based on transfer of ^{1}H-saturation from a target protein to a ligand by intermolecular spin diffusion.[19] Selective saturation of a protein is effected by irradiating protein signals

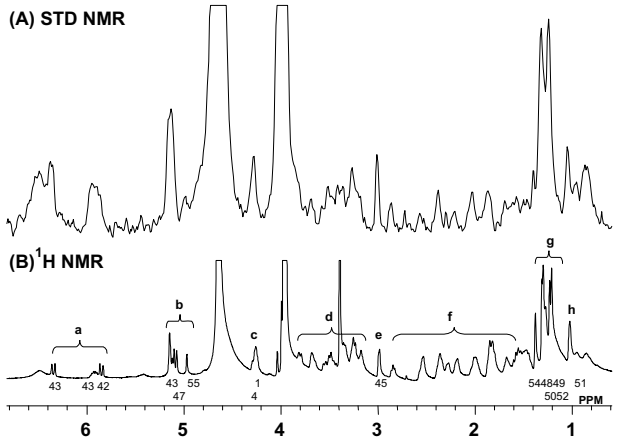

Figure 4. (A) Saturation transfer difference (STD)-NMR spectrum of YTX (3 mM) and GpA (70 μM, CF_3CD_2OD–D_2O, 3:7) upon irradiation at −0.4 ppm; (B) Normal ^{1}H NMR spectrum of YTX (3 mM, CF_3CD_2OD–D_2O, 3:7). Signals are categorized into eight groups; (a) olefinic side chain region for C42, C43, and C46; (b) olefinic side chain region for C47, C53, and C55; (c) α position of sulfate esters; (d) α position of oxygen atoms; (e) allylic C45 of side chain; (f) β and γ position of oxygen atoms; (g) angular methyl groups; and (h) C51 methyl group.

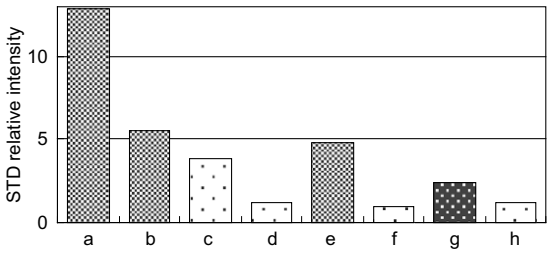

Figure 5. Diagram for the STD relative intensities (see text for details) of the individual groups of ^{1}H NMR signals of YTX. a–h denote groups of ^{1}H NMR signals, see the legend of Fig. 4 for their assignments.

that are anisotropically shifted and/or broadened to appear outside of the spectral window of small ligands. Since the protons of a small ligand residing in close proximity to the target protein receive much larger saturation transfer effects, the binding interface between a ligand and its target protein can be determined from the relative attenuation in signal intensities. The STD-NMR spectrum of YTX (3 mM) in the presence of GpA (Sigma–Aldrich, 70 μM) and the normal ^{1}H NMR spectrum of YTX (3 mM) were shown in Figure 4A and B, respectively. The STD spectrum evidently depicted the binding of YTX to GpA since clear signals derived from YTX, which were not detected in the absence of GpA, are observed in the STD differential spectrum.

In the next step, we attempted to specify the binding sites of YTX to GpA. In order to compare the STD effects for each parts of YTX, the relative attenuation ratios were first determined by dividing the signal intensity in the STD spectrum by that of the normal spectrum (Fig. 5). Since all ^{1}H signals of YTX were somewhat attenuated, the STD effects were further standardized as STD relative intensity as shown in Figure 5 by the value for β and γ positions of ether oxygen atoms (Fig. 4f) which was taken as 1.0. Although signal broadening and short relaxation time prevented the one-by-one assignments of ^{1}H resonances of YTX, we could categorize those into eight groups (Fig. 4). The largest STD effects were observed for the olefinic protons of the polyene side chain (signals a, b, and e), and a prominent effect was detected for the other sulfate terminal portion (signal c). These observations indicate that YTX binds to GpA with both the hydrophobic polyene side chain and the anionic sulfate esters, which may be accountable by the hydrophobic matching as reported previously,[18] and by electrostatic interaction with interfacial cationic residues in GpA such as R^{96} and R^{97}. Among the signals of the polyether stem, angular methyl groups (g) gave rise to a higher value, suggesting that hydrophobic interaction also occurs with the molecular stem of YTX; the smaller effects in the α positions of ether (d) that are substituted at the same position as angular methyls may be due to the fact that these methine protons are less protruding than the methyls and thus slightly far from the protein interface. Taken together with the results in the GpA–TM mutation experiments, it was suggested that the angular methyl groups and olefinic moiety of YTX contact directly with hydrophobic residues of GpA. Interpretation of these data leads us to the conclusion that hydrophobic interactions play an important role for LSPs to bind to transmembrane α-helices under our experimental conditions. Although the glycine zipper is considered to be a key residue for dimerization of GpA, other hydrophobic residues are known to be involved in stabilizing the dimeric form.[24] Thus, our observations do not rule out the possibility that ether oxygen atoms of LSPs form multiple hydrogen bonds with transmembrane helices. Moreover, GpA may take a dimeric form at the concentration of the NMR experiment, thus concealing the glycine zipper and other interfacial portions from YTX. Our attempts to record STD-NMR for ALPs were not

successful mainly due to their poor solubility in proper solvents for α-helix formation of GpA.

Recently, X-ray crystal analysis by Tsumoto et al.[25] has disclosed that both π-electron-derived hydrophobic interactions and hydrogen bonds were essential for forming stable complexes between ciguatoxin and the antigen recognition site of its antibodies. Since the GpA–TM used in this study contains only two aromatic residues, π-electrons may not play a major role. However, other interactions shown in the X-ray study should shed light on the molecular mechanism of action by LSPs. Another plausible attractive force acting in hydrophobic membrane interior may be dipole–dipole interaction between weakly polarized functional groups, which would be a clue to understanding the molecular recognition between LSPs and TM α-helices.

In conclusion, we quantitatively compared the affinities between ladder-shaped polyethers (LSPs) and synthetic transmembrane (TM) peptides of glycophorin A (GpA) using SPR. By comparing K_D values, GpA-TM2G/2I was found to bind to LSPs with higher affinity than wild type (GpA–TM). Furthermore, STD-NMR experiments suggested that the hydrophobic polyene side chain and angular methyl groups have directly contacted to the GpA. These results further imply that hydrophobic matching is important for LSPs to bind to transmembrane α-helices.[18] Moreover, the marked enhancement of affinity between LSP and transmembrane peptide by glycine–isoleucine mutations indicates that properly designed peptides should show extremely higher affinity to LSPs as is the case with ciguatoxin and sodium channel domains. Further studies on the molecular recognition of other proteins/peptides by LSPs are now in progress in our laboratory.

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