Design of a Soluble Transmembrane Helix for Measurements of Water-Membrane Partitioning

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Use of model transmembrane helices and lipid bilayers is a tractable and straightforward approach to obtaining thermodynamic information on fundamental processes of membrane protein folding. The insertion of transmembrane helices from an aqueous phase into membranes, the initial step in the folding process, is especially difficult to investigate because of the insolubility of helices in the aqueous phase. We report here the design of a soluble transmembrane helix, $(KR)_5$-AALALAA-AALWLAA-AALALAA-C(NBD)-NH_2 (NBD, 7-nitrobenz-2-oxa-1,3-diazole), consisting of a transmembrane region $(AALALAA)_3$, a central guest residue (W), and an N-terminal charged tag $(KR)_5$. Circular dichroism and fluorescence spectroscopy revealed that the peptide dissolved in water as a monomer with the guest residue exposed to the solvent. After the addition of large unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, the peptide rapidly partitioned into the vesicles and assumed a transmembrane helix. The partitioning Gibbs free energy was estimated to be -34.2 kJ mol^{-1} at 25 °C. The Trp-to-Gly substitution reduced the partitioning by $\sim$1.6 kJ mol^{-1}. Thus, the transmembrane helix was found to be a useful template for thermodynamic measurements of the partitioning of amino acids from water to the hydrophobic core of membranes.

1. Introduction

Helical membrane proteins, which consist of single or multiple transmembrane helices connected with cytosolic and exoplasmic domains, play essential roles in cell membranes, for example, as channels, transporters, energy converters, and receptors. However, the folding fundamentals, especially thermodynamic stability, of membrane proteins in lipid bilayers are not well understood compared with those of soluble proteins because of experimental limitations. One difficulty is that membrane proteins are resistant to complete unfolding by heat or denaturants.[1,2] Furthermore, investigations of membrane proteins are generally hampered by their intractability due to insolubility in the aqueous phase.[3] Because the detergent micelles commonly used to purify and handle membrane proteins often perturb the native conformation,[4] the development of experimentally accessible systems without using detergents is essential for research on membrane proteins.

The folding of helical membrane proteins has been clearly conceptualized as a two-stage model,[5,6] in which the formation of transmembrane helices in lipid bilayers (stage I) and their self-association (stage II) are considered to be distinct thermodynamic processes, although some proteins are further modified (stage III) to adopt the native form.[7] On the basis of the model, experimental approaches using model transmembrane helices instead of native proteins have often been employed, because one can straightforwardly interpret the effects of amino acid mutations and lipid composition on the thermodynamics of helix−helix, helix−lipid, and lipid−lipid interactions.[3,8−13] Several groups have designed hydrophobic peptides to investigate helix−lipid interactions,[14−17] the stability of transmembrane helices,[18−21] the membrane insertion of helices,[22−29] the intermembrane partitioning of helices,[30,31] and helix−helix interactions.[32−36]

Elucidating the thermodynamics of the transmembrane insertion process (stage I), which involves initial binding and folding at the water−membrane interface followed by transmembrane insertion,[3] is particularly challenging because of the aggregation of hydrophobic helices in the aqueous phase. For example, the partition free energy of amino acids from water to the hydrophobic core of a membrane, from which one can predict the stability of the transmembrane state (hydrophobicity scale) has not been successfully determined yet. As alternatives, the water−lipid bilayer interface scale measured using short model peptides[37,38] and the water−octanol scale[38,39] have been reported. Recently, von Heijne and co-workers reported position-dependent hydrophobicity scales in biological membranes in which the protein channel translocon mediates the insertion of model transmembrane helices.[40,41] It is controversial how these biological scales are related to the biophysical scale determined in a simple system composed of water, bilayers, and helices.[40,42] Note that an appropriate comparison requires the discrimination of three possible states, that is, water-solubilized, interface-bound, and transmembrane helices, of which the former two are indistinguishable in the biological scale.[42]

We report here the synthesis and characterization of a soluble transmembrane helix (STMH) designed to measure the water-bilayer partitioning of helices and their membrane topology (interface-bound or transmembrane). The transmembrane domain is composed of three heptad repeats of the amino acid sequence AALALAA forming a stable transmembrane helix[35] in which the central Ala is substituted with a guest amino acid (Table 1). In this study, a hydrophobic and fluorescent Trp residue was introduced at the guest position. In addition, a Gly-substituted peptide was also synthesized to examine the effect of guest residue on the water-bilayer partitioning (STMH1 (G)). The positively charged tag sequence $(KR)_5$ was attached to the N-terminus to prevent aggregation in water.[43,44] The membrane binding and insertion of the peptide were monitored using the fluorescent probe 7-nitrobenz-2-oxa-1,3-diazole (NBD) attached

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TABLE 1: Sequences of Soluble Transmembrane Helices (STMHs)

abbreviation	sequence[a]
STMH1	KRKRKRKRKR-AALALAA-AAL**W**LAA-AALA LAA-C(NBD)-NH_2
STMH1-TMR	KRKRKRKRKR-AALALAA-AAL**W**LAA-AALA LAA-C(TMR)-NH_2
STMH1 (G)	KRKRKRKRKR-AALALAA-AAL**G**LAA-AALA LAA-C(NBD)-NH_2

[a] NBD or TMR was attached to the Cys side chain at the C-terminus. A guest residue (Trp or Gly, bold) was introduced at the center of the transmembrane region (underlined).

to the Cys side chain at the C-terminus. To examine the aggregation of the peptides using fluorescence resonance energy transfer (FRET), a tetramethylrhodamine (TMR)-labeled peptide was prepared (STMH1-TMR). The STMH1 peptide was found to dissolve in water as monomers and to rapidly partition into bilayers with a transmembrane topology after mixing with large unilamellar vesicles (LUVs) composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). The water-bilayer partition free energy of STMH1 was estimated by conducting titration experiments.

2. Experimental Section

Peptides. STMH1 peptides were synthesized by a standard 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid phase method using NovaSyn TGR resin (Novabiochem, Darmstadt, Germany) on a 0.1 mmol scale. For coupling, Fmoc amino acid (0.5 mmol), 1-hydroxybenzotriazole (0.5 mmol), and *N*-*N*′-diisopropylcarbodiimide (0.5 mmol) dissolved in *N*,*N*-dimethylformamide (DMF) were reacted for 2.5 h. The reaction was monitored using the ninhydrin test. Fmoc was removed by treatment with 20% piperidine in DMF for 20 min. The peptide was removed from the resin by treatment with TFA/ethaneditiol/m-cresol/thioanisole/H_2O = 8.75/2/1/1/1(v/v) for 4 h, precipitated by diethylether, and purified by reversed-phase HPLC. Fluorophores (NBD or TMR) were attached to the Cys side chain at the C-terminus by treatment with 10 equivalents of *N*-((2-(iodoacetoxy) ethyl)-*N*-methyl) amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD) or tetramethylrhodamine-5-maleimide (Invitrogen, Carlsbad, CA) in TFE containing 0.5% triethylamine under an argon atmosphere for 30 min. The purity of the synthesized peptides (higher than 95%) was determined by analytical HPLC and ion spray mass spectroscopy. The concentrations of the fluorophore-labeled peptides were determined on the basis of extinction coefficients in methanol (ε_{472} = 23 000 M^{-1} for NBD and ε_{541} = 95 000 M^{-1} for TMR). F5W-magainin 2 (GIGKWLHSAKKFGKAFVGEIMNS) and NBD-$(AALALAA)_3$ were obtained as reported in refs 45 and 35, respectively. Cys-IANBD was synthesized by mixing L-cysteine (Sigma-Aldrich, St. Louis, MO) with IANBD ester (5 equivalents) in TFE. Mellitin was obtained from Sigma-Aldrich (St. Louis, MO).

Other Materials. POPC was obtained from Avanti Polar Lipids (Alabaster, AL). Spectrograde chloroform and methanol were products of Nacalai Tesque (Kyoto, Japan). The lipid was dissolved in chloroform and its concentration was determined in triplicate by phosphorus analysis.[46] Poly-D-lysine hydrobromide (molecular weight 30 000−70 000) was purchased from Sigma-Aldrich. Ultrapure water (>18 MΩ cm) was generated using a Millipore Direct-Q system. All other chemicals were of special grade and obtained from Wako (Tokyo, Japan).

Coating of Cuvettes. To prevent the adsorption of the peptides, quartz cuvettes for circular dichroism (CD) and fluorescence measurements were coated with poly-D-lysine. The cuvettes were successively immersed into 60% nitric acid (overnight), chloroform/methanol (1/1, v/v) (5 min), and acetone (5 min). The cleaned cuvettes were filled with an aqueous solution of poly-D-lysine (0.5 mg mL^{-1}) and incubated for 10 min. The solution was then discarded and the cuvettes were dried with N_2 gas. Finally, the cuvettes were rinsed ten times with ultrapure water and dried.

CD Measurements. A peptide stock solution in 2,2,2-trifluoroethanol (TFE) was diluted with water to obtain aqueous solutions (0.2% TFE). CD spectra of peptides were measured at 25 °C on a Jasco J-820 apparatus using 1.0 or 0.1 cm path length quartz in the absence or the presence of vesicles, respectively. Sixteen to sixty-four scans were averaged for each sample. The averaged blank spectra were subtracted. The poly-D-lysine coating did not affect the CD spectra.

Vesicle Preparation. Large unilamellar vesicles (LUVs) were prepared by an extrusion method as described elsewhere.[35,47] Briefly, a lipid or a lipid−peptide mixed film, after drying under vacuum overnight, was hydrated with ultrapure water and vortex-mixed to produce multilamellar vesicles. The suspension was subjected to 10 freeze−thaw cycles and then extruded through polycarbonate filters (0.1 μm pore size filters) 31 times.

Fluorescence Measurements. Fluorescence spectra were recorded on a Shimadzu RF-5300 spectrofluorometer at 25 °C. Tryptophan and NBD were excited at 280 and 470 nm with a 1.5 nm slit, and observed at 250−500 nm and 430−650 nm with a 20 nm slit, respectively, unless otherwise noted. The intensity of the blank was subtracted. A peptide stock solution (0.1 mM) in TFE was diluted with water to obtain 0.1−2 μM solutions (containing 0.1−2% TFE). In membrane partitioning experiments, LUVs composed of POPC were added to the peptide solution. The time course of membrane partitioning of the peptides was monitored by measuring the increase in NBD fluorescence at 530 nm. The membrane topology of peptides was determined using dithionite, because the addition of dithionite to LUVs chemically quenches the NBD groups in the outer leaflets of the bilayers.[30] NBD fluorescence was monitored after the addition of 20 μL of 1 M sodium dithionite/1 M Tris (pH 10.0) to the sample (2 mL).

3. Results

Solubilized State in Water. First, we preliminarily examined an aqueous phase appropriate for solubilization of the STMH peptides. Two types of Tris buffers (10 mM Tris/150 mM NaCl/1 mM EDTA (pH7.4) and 1 mM Tris/0.1 mM EDTA (pH7.4)) apparently solubilized the STMH peptides but not as monomers, because membrane partitioning of the peptides after addition of LUVs was not observed or was very slow (typically several hours are needed for the equilibration, data not shown). Because the addition of even 1 mM of electrolytes (which is required for efficient buffering) induced aggregation of the peptide (Supporting Information, Figure S1A), we used pure water as an aqueous phase (the minimal Tris concentration that did not induce the aggregation was 0.05 mM as shown in Supporting Information, Figure S1C). We diluted the peptide stock solution in TFE (∼0.1 mM) into water because the concentrated aqueous solution of STMH was not stable and therefore not suitable for storage. CD spectroscopy revealed that STMH1 predominantly assumed random structures in water at 2 μM (Figure 1, spectrum1). The spectrum was identical to that obtained at a lower peptide concentration of 0.8 μM (data not shown). Similar CD spectra have been reported for hydrophobic peptides that dissolve in the aqueous phase as monomers

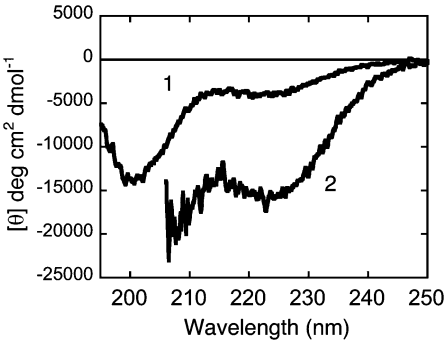

Figure 1. CD spectra of STMH1 in water (1) and in the presence of vesicles (2) at 25 °C. A TFE stock solution of STMH 1 was diluted into water (final concentration of 2 μM in 0.2% TFE) to measure the spectrum in water. For measurement in vesicles, STMH1 (5 μM) was mixed with POPC LUVs (20 mM) to obtain a lipid/peptide ratio of 4000 (containing 0.2% TFE). The blank spectrum (vesicles) has been subtracted. Because of substantial light scattering from the vesicles, the spectrum could only be measured from 250−205 nm.

[TMX3[26] and pHLIP[28]]. To examine the microenvironments around the guest amino acid and the C-terminus, emission spectra of Trp and NBD in STMH1 were measured. The emission maximum of Trp fluorescence (~350 nm) was similar to that of acetyl-Trp-amide, indicating aqueous exposure of the guest residue in the middle of the sequence (Figure 2A). The slight blue-shifted emission of Trp in STMH1 compared with that of acetyl-Trp-amide indicates a difference in the local environment around the fluorescent moieties between the polypeptide and the amino acid analog. Indeed, the Trp residue in the soluble, random-coil peptide F5W-magainin 2[48] exhibited an emission almost identical to that of Trp in STMH1 (Figure 2A). The NBD group of STMH1 also exhibited a similar but marginally blue-shifted emission compared with that of Cys-NBD, indicating the aqueous exposure of the C-terminus (Figure 2C).

STMH1 appears to be monomeric in water. A linear concentration dependence of NBD fluorescence indicates no self-quenching due to peptide aggregation in the concentration range 0−2 μM (Figure 2D). FRET between NBD (donor) and TMR (acceptor)-labeled peptides further supported this conclusion. The Förster distance (distance between chromophores at which the donor fluorescence is reduced to 50% by energy transfer) for this pair is ~50 Å.[30,49] The Trp residue, which has the excitation wavelengths of <310 nm, does not participate in the FRET. Figure 3A shows the fluorescence spectra of 0.1 μM STMH1 in the absence and presence of 0.1 μM STMH1-TMR after the subtraction of the directly excited TMR fluorescence. A subtle decrease in NBD fluorescence in the presence of STMH1-TMR (7.8 ± 1.1%, $n = 3$) can be attributed to inner filter effects, because the water-soluble acceptor TMR-magainin 2 induced a similar decrease in donor fluorescence (Figure 3B, 8.1 ± 2.3%, $n = 3$). In contrast, a significant decrease in NBD florescence (~25%) concomitant with an enhanced TMR emission was observed in a buffer (1 mM Tris, pH 7.4) 2 min after dilution from the stock solution, indicating self-aggregation in the presence of salt (Supporting Information, Figure S2).

Transmembrane Helix State in Membrane. STMH1 assumed an α-helical conformation in the presence of POPC LUVs (lipid/peptide = 4000), as evidenced by a CD spectrum with double minima at 222 and 208 nm, although the latter is less clear because of light scattering (Figure 1, spectrum 2). Under the experimental conditions, peptides were almost completely (>99%) bound to the membranes (see "Water−Membrane Partition Free Energy"). We confirmed that the light scattering

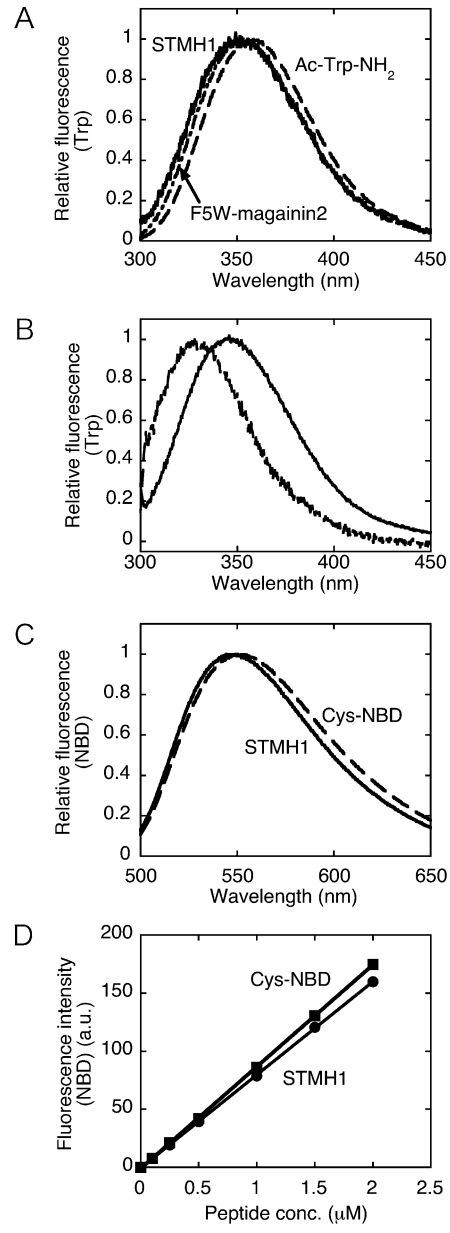

Figure 2. Microenvironments of the central Trp and the C-terminal NBD in STMH1 (25 °C). (A) Normalized Trp emission spectra of STMH1 (solid line), Ac-Trp-NH_2 (broken line), and F5W-magainin 2 (dash-dot line) at a concentration of 0.5 μM (1% TFE). (B) Normalized Trp emission spectra of STMH1 (0.3 μM) in water (solid line) and in the presence of 1.2 mM POPC LUVs (broken line). (C) Normalized NBD emission spectra (0.5 μM) of STMH1 (solid line) and Cys-NBD (broken line). (D) Concentration-dependence of NBD fluorescence for STMH1 (541 nm, circles) and Cys-NBD (543 nm, squares).

did not distort the spectrum (Supporting Information, Figure S3). The helicity (f_H) was estimated from the molar ellipticity at 222 nm ($[\theta]_{222} \sim -17\,000$) to be ~48% using the equation, $f_H = (-[\theta]_{222} - 2340)/30\,300 \times 100$.[50] Note that this overall helicity includes that of the $(RK)_5$ tag, which comprises approximately one-third of the peptide sequence. Assuming a random structure for the tag, the helicity of the transmembrane region of the bound peptide was estimated to be high (~70%).

The Trp fluorescence indicated a transmembrane orientation of the helix. The spectrum blue shifted to 325 nm in the presence of vesicles (lipid/peptide = 4000) (Figure 2B), indicating its burial in the hydrocarbon core region.[21] The absence of any spectral distortion at the high lipid-to-peptide ratio was con-

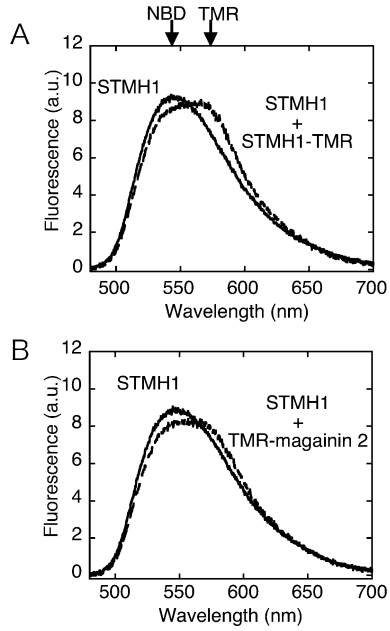

Figure 3. Aggregated state of STMH1. FRET between NBD- and TMR-labeled peptides were determined at a concentration of each peptide of 0.1 μM (1% TFE) at 25 °C. NBD was excited at 460 nm to minimize the direct excitation of TMR. (A) NBD fluorescence of STMH1 in the absence (solid line) and presence (broken line) of STMH1-TMR after subtracting the contribution of the directly excited TMR. (B) Control experiment using the water-soluble acceptor TMR-magainin 2. The peak positions of NBD and TMR fluorescence are indicated by the arrows.

firmed (Supporting Information, Figure S4). Consistent with the transmembrane orientation of the peptide, an increase and a blue shift (545 to 530 nm) of NBD fluorescence was observed upon binding to vesicles (Figure 5A), suggesting that the C-terminus of the helix is located at the water−membrane interface.[33] The topology of STMH1 in the membrane was examined by adding dithionite (membrane-impermeable quencher for NBD) to the vesicles (Figure 4). As a control, when NBD-$(AALALAA)_3$-NH_2 had been incorporated in bilayers in the vesicle preparation step, a decrease in NBD fluorescence to ~50% was observed, demonstrating no preference in membrane topology (Figure 4A).[30] In contrast, STMH1 partitioned into the vesicles from the external aqueous phase exhibited no decrease in NBD fluorescence after addition of the quencher (Figure 4B), indicating that STMH1 assumed a unique transmembrane topology with the C-terminus positioned inside of the vesicles because the charged tag moiety could not cross the lipid bilayers. To further confirm this topology, we demonstrated that the NBD site becomes accessible to dithionite from the inside of the vesicle by using the pore-forming peptide melittin, which allows dithionite ions to penetrate the vesicle without completely destroying the bilayer (Figure 4C). Immediately after addition of melittin, a rapid and complete quenching was observed, confirming that the C-terminus of STMH1 is not buried in the hydrocarbon core of bilayers. STMH1 (G) also assumed a transmembrane topology in the presence of the vesicles (Figure 4D). From these results, we concluded that STMH peptides assume transmembrane helical structures with the C-terminus positioned inside of the vesicles.

Water-Membrane Partition Free Energy. Partitioning of STMH1 to bilayers was monitored based on an increase of NBD fluorescence (Figure 5A). After the addition of POPC LUVs to the peptide solution, an initial rapid phase was observed for several seconds, followed by a slow phase

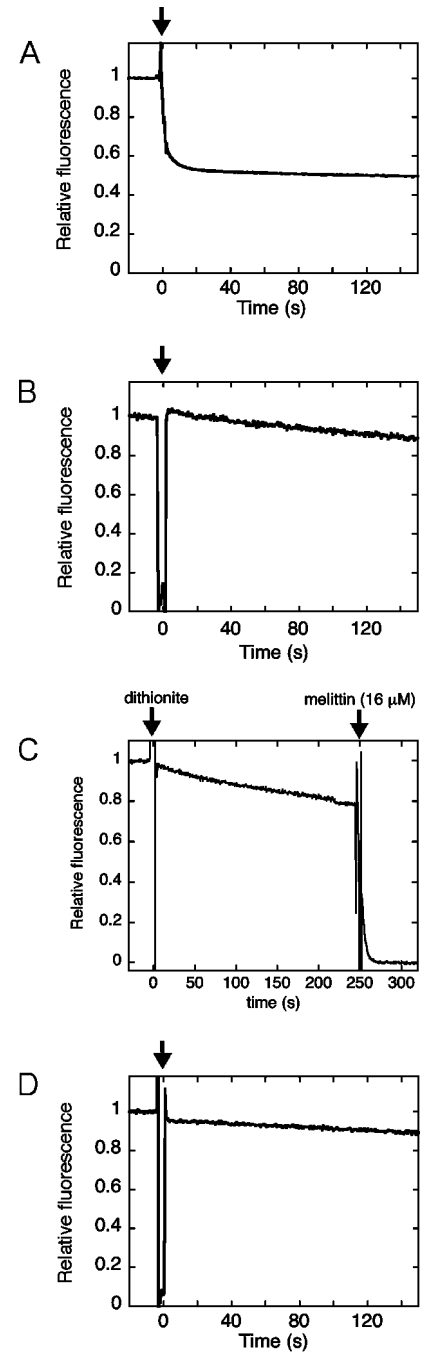

Figure 4. Detection of peptide insertion topology by addition of a membrane impermeable quencher for NBD. Sodium dithionite was added at the time indicated by the arrow at a final concentration of 10 mM, while fluorescence intensity at 530 nm was monitored at 25 °C. (A) Quenching of NBD-$(AALALAA)_3$-NH_2 incorporated into POPC LUVs (buffer: 10 mM Tris-HCl/150 mM NaCl/1 mM EDTA (pH7.4)). The peptide was mixed with the lipid in an organic solvent before preparation of vesicles. The concentrations of the peptide and the lipid were 0.5 and 100 μM, respectively. (B) Quenching of STMH1 inserted into preformed POPC LUVs (L/P = 32 000). (C) Quenching of NBD positioned at the inside of vesicles upon membrane permeabilization with melittin. STMH1 incubated with POPC LUVs (L/P = 32 000) was mixed with sodium dithionite (t = 0 s) and subsequently with mellitin (t = 250 s). (D) Quenching of STMH1 (G) inserted into preformed POPC LUVs (L/P = 32 000).

equilibrated in 30 s to 20 min, depending on the lipid/peptide ratio (1000−32 000) (Figure 5B). After equilibration, fluorescence spectra were measured (Figure 5A), which were used to calculate the membrane partitioning (Figure 6). Stepwise

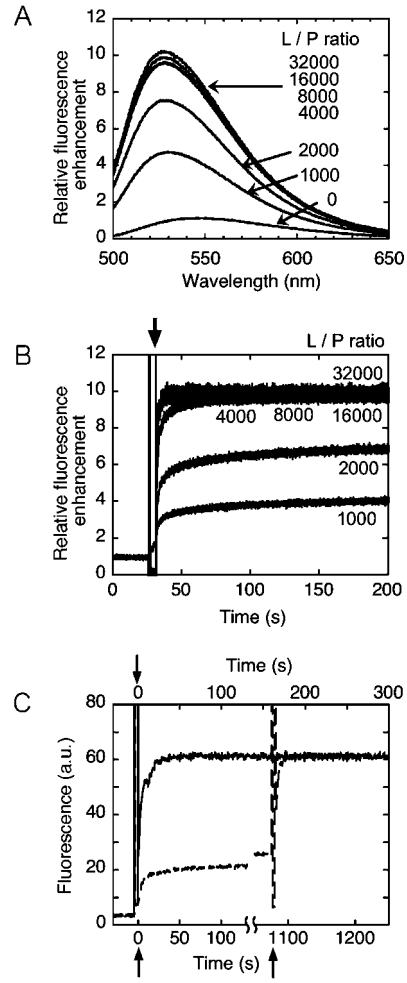

Figure 5. Membrane partitioning of STMH1 at 25 °C. POPC LUVs were added to a STMH1 solution (50 nM, 0.1% TFE) at different lipid/peptide ratios. (A) Emission spectra after equilibration (lipid/peptide = 0, 1000, 2000, 4000, 8000, 16 000, and 32 000, lower to upper). (B) Time courses of fluorescence enhancement measured at 530 nm. LUVs were added at $t = 0$ (arrow). (C) Reversibility of partitioning. LUVs were added in two steps at lipid/peptide ratios of 1000 ($t = 0$) and 16 000 ($t = 1050$) (broken line, lower axis) or added in one step at a ratio of 16 000 ($t = 0$, solid line, upper axis).

additions of vesicles increased fluorescence to the same levels as was seen with only a single addition of peptides to vesicles (Figure 5C), consistent with reversible partitioning of the peptides. The reversibility was also directly confirmed by diluting the vesicles containing the peptides into the aqueous phase (Supporting Information Figure S5). The peptides were either externally added to preformed vesicles (Figure S5A) or incorporated during vesicle formation (Figure S5B). In both cases, dissociation of the peptides into aqueous phase was observed following dilution, although only half of the peptide population exchanged in the latter case.

We examined whether the water−membrane partition free energy of STMH1 could be estimated by titration of the vesicles. Figure 6 shows the relative enhancement of NBD fluorescence, F, at 530 nm as a function of the lipid concentration [L] at 25 °C (Figure 6A) and 5 °C (Figure 6B). We anticipated that the membrane partitioning of the peptide would become weaker at lower temperature if hydrophobic effects in water are the dominant force for bilayer insertion. The data were fitted using an equation assuming a simple water−membrane partitioning of the peptides in which there are only two populated states (monomeric peptide in water

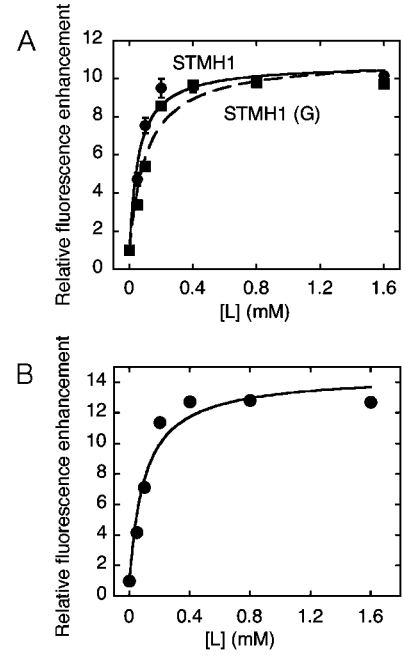

Figure 6. Estimation of the partition coefficients of STMH peptides. Fluorescence intensities at 530 nm ($n = 3$) at (A) 25 °C for STMH1 (circles) and STMH1 (G) (squares) and (B) 5 °C for STMH1 are plotted as a function of lipid concentration. The curves are the best-fit theoretical curves using eq 1 with (A) $K = (9.7 \pm 2.2) \times 10^5$ and $f_L = (2.2 \pm 0.1) \times 10^5$ ($r = 0.987$) for STMH1 (solid line) and $K = (5.2 \pm 1.4) \times 10^5$ and $f_L = (2.2 \pm 0.1) \times 10^5$ ($r = 0.980$) for STMH1 (G) (broken line) and (B) $K = (5.4 \pm 1.6) \times 10^5$ and $f_L = (2.9 \pm 0.2) \times 10^5$ ($r = 0.978$).

and membrane-inserted peptide) exist with a partition coefficient K.[51]

$$F = \frac{C[\mathrm{W}]f_w + C[\mathrm{L}]Kf_l}{[\mathrm{L}]K + [\mathrm{W}]} \quad (1)$$

[W], C, f_L, and f_W indicate the water concentration (=55.6 M), the total peptide concentration, the fluorescence yield in membranes, and the fluorescence yield in water, respectively. Only K and f_L are adjustable parameters. Note that the analysis is only valid for experimental conditions in which K is independent of the lipid/peptide ratio (infinite dilution states). Membrane partitioning studies for an antimicrobial peptide (indolicidin) and a peptapeptide (Ac-WLWLL) suggest that the assumption of constant K is appropriate for lipid/peptide ratio >700 and >100, respectively.[51] For STMH1, although the data at 25 °C could be approximately fitted by eq 1 with a K value of $(9.7 \pm 2.2) \times 10^5$ (correlation coefficient $r = 0.987$, $\Delta G = -34.2 \pm 0.6$ kJ mol^{-1}), a systematic deviation from the fitting curve was clearly observed at 5 °C ($r = 0.978$). To enhance sensitivity, similar analyses using F at 547 nm (a fixed wavelength to one side of the maximum) were also carried out.[52] However, this did not improve the fitting ($r = 0.986$ and 0.978 at 25 and 5 °C, respectively). Electrostatic repulsions between positively charged tags in the vesicles appear to hamper partitioning of multiple peptides into vesicles and underestimate the true membrane partitioning of the transmembrane region (see below).

Membrane partitioning of STMH1 (G) was also examined. The F values were lower than those of STMH1 at all lipid concentrations, indicating a weaker partitioning of STMH1 (G).

The K and ΔG values were estimated to be $(5.2 \pm 1.4) \times 10^5$ and -32.6 ± 0.7 kJ mol^{-1}, respectively (Figure 6A, $r = 0.980$).

4. Discussion

The basic requirements for a model peptide for examining the water−bilayer partitioning of helices (and guest amino acids) are (1) dissolution into the aqueous phase as a monomer and exposure of the guest residue to the solvent, (2) rapid and reversible partitioning to membranes, and (3) ability to form a transmembrane helix without interhelical interactions. Such an ideal system has not been established yet in spite of a number of attempts.[22,24,25,27] Although the pHLIP peptide apparently fulfills the above criteria,[23,28] an accurate estimate of insertion energy is hampered by the pH-dependence of the membrane insertion.[23] Numerous tagged peptides, which are slightly water-soluble when only water is present but that insert almost completely into a membrane upon addition of the membrane, have been reported.[43,44] Although there are no ΔG measurements previously reported, such peptides are promising candidates for the ideal experimental system, because they adapt transmembrane conformations within bilayers and they are also soluble in aqueous solution. In this study, we examined the detailed properties of the hydrophobic peptides (STMH1s) tagged with charged residues in water and bilayers.

STMH1 in Water. The N-terminal tagging with the charged residues $(KR)_5$ resulted in the hydrophobic peptide (AALALAA AALWLAA AALALAA) being solubilized into the aqueous phase. The absence of a concentration-dependence of NBD fluorescence yield (Figure 2D) and CD spectra indicate (1) no self-quenching due to aggregation, (2) invariant polarity around the central and the C-terminal residues, and (3) no structural change, at least up to 2 μM. FRET experiments further supported that the peptide existed as a monomer (Figure 3). Even though the peptide may partially assume a helical structure, the local environment around the guest residue was found to be similar to that of a random coil peptide, guaranteeing an adequate estimation of the water-membrane partitioning.

STMH1 in Bilayers. Consistent with the monomeric solubilization of the peptides, a rapid partitioning of STMH1 to bilayers was observed (Figure 5B). STMH in the membranes assumed a helical conformation (Figure 1) and a transmembrane topology (Figures 2B, 4B,C), although surface-bound helices might form temporarily during the insertion process.[30] The peptide corresponding to the transmembrane region $(AALALAA)_3$ is known to assume stable transmembrane helices in POPC (Yano and Matsuzaki, unpublished data) and in various phosphatidylcholine bilayers with different hydrophobic thicknesses.[35] STMH1 helices exist as monomers in POPC bilayers. We have reported that the helices self-associate in bilayers preferentially in an antiparallel orientation, driven by electrostatic attraction between the helix macrodipoles.[35] In contrast, STMH1 in this study, which inserts into bilayers with the C-terminus positioned inside of the vesicles, resists self-association because of electric repulsions between parallel helix macrodipoles, in addition to repulsions between positively charged tag moieties. Although parallel associations have been observed for $(AALALAA)_3$ at high concentrations (>0.5 mol %), the concentrations of STMH1 used in this study (<0.1 mol %) were much lower than that threshold.

Repulsions between positively charged tags could inhibit the partitioning of multiple helices into LUVs and result in systematic error in the fitting assuming a simple water−membrane partitioning (Figure 6). At least ~20 peptide molecules appeared to be incorporated into LUVs, assuming a lipid number of 90 000 per LUV. To obtain an ideal partitioning in the infinite dilution regime ($L/P > 90\,000$), a transmembrane sequence with less hydrophobicity will be needed. Nevertheless, it is valuable to compare the measured partition energies with those obtained from other methods. The energy difference for membrane partitioning between Trp and Gly residues ($\Delta\Delta G_{Gly\rightarrow Trp}$) has been controversial. The $\Delta\Delta G_{Gly\rightarrow Trp}$ values from the water−octanol scale (-13.5 kJ mol^{-1})[38] and biological scale (-1.8 kJ mol^{-1})[40] are inconsistent. Our result ($\Delta\Delta G_{Gly\rightarrow Trp} = -1.6$ kJ mol^{-1}) is close to that of the biological scale. In this report, the method is illustrated for a guest residue in the center of the TM. In future work, it could be extended to other positions in the helix closer to the interface and could also test if some of these showed vectorial insertion.

An ideal peptide template to estimate a scale of the biophysical hydrophobicity of amino acids should be soluble as a monomer in the aqueous phase and should reversibly partition into lipid bilayers as a TM helix. Despite extensive studies, such a template has not yet been reported. In this study, we succeeded in designing STMHs by combining the less hydrophobic TM sequence with the charged tag, although the applicable experimental conditions are limited. Our peptides are promising templates for truly ideal STMHs.

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Supporting Information Available: NBD emission spectra and aggregated state of STMH1 in 1 mM Tris-HCl (Figures S1 and S2, respectively), effects of a high concentration of LUVs on CD and fluorescence spectra (Figures S3 and S4, respectively), and dissociation of STMH1 from the bilayers to the aqueous phase upon dilution of the vesicles (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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