Heterodimer Formation between the Antimicrobial Peptides Magainin 2 and PGLa in Lipid Bilayers: A Cross-Linking Study†

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ABSTRACT: The antimicrobial peptides magainin 2 and PGLa, isolated from the skin of the African clawed frog *Xenopus laevis*, show marked synergism [Westerhoff, H. V., Zasloff, M., Rosner, J. L., Hendler, R. W., de Waal, A., Vaz Gomes, A., Jongsma, A. P. M., Riethorst, A., and Juretić, D. (1995) *Eur. J. Biochem. 228*, 257−264]. We suggested previously that these peptides form a potent heterodimer composed of either parallel or antiparallel helices in membranes [Matsuzaki, K., Mitani, Y., Akada, K., Murase, O., Yoneyama, S., Zasloff, M., and Miyajima, K. (1998) *Biochemistry 37*, 15144−15153]. To detect the putative heterodimer by chemical cross-linking, analogues of magainin 2 and PGLa with a Cys residue at either terminus were synthesized. These cross-linking experiments suggested that both peptides form a parallel heterodimer in membranes composed of phosphatidylglycerol/phosphatidylcholine but not in either buffer or a helix-promoting 2,2,2-trifluoroethanol/buffer mixture. The isolated parallel heterodimers exhibited an order of magnitude higher membrane permeabilization activity compared with the monomeric species, indicating that the observed synergism is due to heterodimer formation.

There is accumulating evidence that membrane-acting antimicrobial peptides play important roles in innate immunity (*1*, *2*). Many of these peptides form polycationic amphipathic secondary structures (α-helices and *β*-sheets) that can bind to and permeabilize negatively charged bacterial membranes. Broad antimicrobial spectra and highly selective toxicity as well as difficult resistance development make these compounds promising candidates as novel antibiotics for clinical use. Magainin 2 (*3*) and PGLa (*4*) isolated from the skin of the African clawed frog *Xenopus laevis* (Table 1) are representative peptides of this class. Our group has proposed the following mechanisms for membrane permeabilization induced by these peptides (*5*−*7*). The peptide forms an amphipathic helix in lipid bilayers, which essentially lies parallel to the membrane surface (*8*), imposing positive curvature strain on the membrane (*9*). To release this stress, several helices together with several surrounding lipids form a membrane-spanning pore comprising a dynamic, peptide−lipid supramolecular complex, which allows not only ion transport but also rapid flip-flop of the membrane lipids (*10*). Upon disintegration of the pore, a fraction of the peptide molecules stochastically translocates into the inner leaflet (*11*, *12*).

Table 1: Amino Acid Sequences of the Peptides

peptide	sequence[a]
magainin 2	GIGKFLHSAKKFGKAFVGEIMNS
I	Ac-CGGGIGKWLHSAKKFGKAFVGEIMNS-NH_2
II	Ac-GIGKWLHSAKKFGKAFVGEIMNSGGC-NH_2
PGLa	GMASKAGAIAGKIAKVALKAL-NH_2
III	Ac-CGGGMASKAGAIAGKIAKVAWKAL-NH_2
IV	Ac-GMASKAGAIAGKIAKVAWKALGGC-NH_2

[a] Amino acid substitutions are underlined.

Interestingly, although the individual peptides are potent, mixtures of magainin 2 and PGLa show marked functional synergism in bacteria, tumor cells, and artificial lipid membranes (*13*−*17*). Furthermore, the 1:1 mixture of these peptides becomes hemolytic, losing the selectivity of magainin 2 and PGLa alone. These phenomena are of biological and clinical importance, but little is known about the mechanism. We suggested previously that these peptides form a 1:1 stoichiometric complex of high potency in membranes with a Gibbs free energy of −15 kJ/mol (*17*). The putative complex will be a magainin 2−PGLa heterodimer composed of either parallel or antiparallel helices.

In this study, to detect the heterodimer by chemical cross-linking, analogues of magainin 2 and PGLa with a Cys residue at either terminus were synthesized (Table 1). Our cross-linking study indicated that the **I**−**III** and **II**−**IV** pairs with Cys at the same sides effectively formed heterodimers only in membranes, suggesting the formation of a parallel heterodimer between the two antimicrobial peptides. The isolated heterodimers exhibited an order of magnitude higher membrane permeabilization activity compared with the monomeric species.

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MATERIALS AND METHODS

Materials. The peptides were synthesized by the standard fluoren-9-ylmethoxycarbonyl- (Fmoc-)[1] based solid-phase method, as previously described (*8*). The purity of the synthesized peptides was determined by analytical reversed-phase-high performance liquid chromatography (RP-HPLC) and ion spray mass spectroscopy. Egg yolk L-α-phosphatidylcholine (PC) and egg yolk L-α-phosphatidylglycerol (PG) were obtained from Sigma (St. Louis, MO). NMR grade 2,2,2-trifluoroethanol was purchased from Aldrich (Milwaukee, WI). All other chemicals were from Wako (Tokyo, Japan) and were of special grade. Buffers were prepared from NANOpure water (Barnstead, Dubuque, IA).

Lipid Vesicles. Large unilamellar vesicles (LUVs) were prepared and characterized as described elsewhere (*10*). Briefly, a lipid film, after being dried under vacuum overnight, was hydrated with the desired aqueous phase and vortex-mixed to produce multilamellar vesicles (MLVs). The suspension was subjected to five freeze−thaw cycles and then extruded through polycarbonate filters (100 nm pore size filter, 21 times). Small unilamellar vesicles (SUVs) for circular dichroism (CD) measurements were produced by sonication of freeze−thawed MLVs in ice−water under a nitrogen atmosphere. The lipid concentration was determined in triplicate by phosphorus analysis.

Cross-Linking in Solution. A magainin analogue (**I** or **II**) and a PGLa analogue (**III** or **IV**) were dissolved in either 25 mM carbonate buffer (pH 9.3) containing 150 mM NaCl and 1 mM EDTA or TFE/carbonate buffer (3/2 v/v) and mixed in 1.5 mL Eppendorf tubes (total volume 40−60 μL). Concentrated peptide stock solutions were routinely prepared in 1 M HCl to avoid oxidation and diluted with the carbonate buffer just prior to the experiment. The pH was adjusted to 9.3 with 0.1 M NaOH. The final concentration of each peptide was 0.5 mM. The mixtures were reacted (oxidized) for 6 h at 30 °C, and then the reaction was stopped by addition of 30 μL of 0.1 M HCl per 20 μL of sample. RP-HPLC measurements were performed on a Shimadzu gradient HPLC system consisting of two LC-9A pumps, a manual injector, an SPD-6A UV spectrophotometric detector operating at 280 nm, and a C-R3A recorder. Analyses were carried out on a Cosmosil $5C_4$-AR-300 column (250 × 4.6 mm i.d., nacalai tesque, Kyoto, Japan) at a flow rate of 1 mL/min at 30 °C. Mobile phase A was 0.05% trifluoroacetic acid in acetonitrile, and the B phase was 0.05% trifluoroacetic acid in water. The linear gradient conditions (B%) were as follows. **I**−**III**: 0−5 min, 73%; 5−15 min, 73−67%; 15−70 min, 67−60%. **I**−**IV**: 0−5 min, 73%; 5−15 min, 73−65%; 15−45 min, 65−60%; 45−60 min, 60−46%. **II**−**III**: 0−5 min, 73%; 5−15 min, 73−67%; 15−70 min, 67−61%. **II**−**IV**: 0−5 min, 73%; 5−15 min, 73−63%; 15−45 min, 63−60%; 45−75 min, 60−32%.

Cross-Linking in Membranes. A magainin analogue (**I** or **II**) and a PGLa analogue (**III** or **IV**) were dissolved in the carbonate buffer, and aliquots of 50 μL of each solution (0.3 mM) were mixed together. LUVs composed of PG/PC (1/1 mol/mol) prepared in the carbonate buffer were added to the peptide mixture in 1.5 mL Eppendorf tubes. The concentrations of each peptide and lipid were 90 μM and 2.25 mM, respectively. The mixtures were incubated for 2 h at 30 °C. The reaction was stopped by addition of 10 μL of 0.1 M HCl and 10 μL of 8 M guanidine hydrochloride per 100 μL sample. The latter reagent was added to facilitate desorption of the peptides from the membranes. The samples were delipidated by the Bligh−Dyer method (*18*) and analyzed by RP-HPLC. The recovery of the peptide was always greater than 70%.

Calcein Leakage. LUVs were prepared by use of 70 mM calcein/1 mM EDTA (pH 7.4) as a hydrating solution. Dye-containing vesicles were separated from free dye on a Bio-Gel A-1.5m column with 10 mM Tris/150 mM NaCl/1 mM EDTA (pH 7.4) buffer as the eluent. Calcein-free LUVs prepared by use of the Tris buffer as a hydrating solution were mixed with dye-loaded liposomes to adjust the lipid concentration to the desired value. The release of calcein from the LUVs was fluorometrically monitored on a Shimadzu RF-5000 spectrofluorometer at an excitation wavelength of 490 nm and emission wavelength of 520 nm at 30 °C. The maximum fluorescence intensity corresponding to 100% leakage was determined by the addition of 10% w/v Triton X-100 (20 μL) to 2 mL of the sample. The apparent percent leakage value was calculated according to the equation % apparent leakage $= 100(F - F_0)/(F_t - F_0)$. F and F_t denote the fluorescence intensity before and after addition of the detergent, respectively. F_0 represents the fluorescence of intact vesicles.

Circular Dichroism (CD) Spectra. CD spectra were measured on a Jasco J-720 apparatus interfaced to an NEC PC-9801 microcomputer, using a 1 mm path-length quartz cell to minimize the absorbance due to buffer components. The instrumental outputs were calibrated with nonhygroscopic ammonium *d*-camphor-10-sulfonate (*19*). Eight scans were averaged for each sample, and the averaged blank spectra (the vesicle suspension) were subtracted. The peptide concentration was 25 μM. The absence of any optical artifacts was confirmed as described elsewhere (*20*).

RESULTS

Peptide Design. A Cys residue was introduced at either the N or C terminus of each peptide with a Gly-Gly linker for flexibility. Both terminal charges, which will affect the reactivity of the SH group (*21*), were blocked by acetylation and amidation. Trp residues, which were useful for the determination of peptide concentration, were introduced at the fifth position of magainin 2 and the 18th position of PGLa. It has been confirmed that these substitutions did not affect the synergism between these peptides (*17*). As shown below, these Cys-containing derivatives exhibited properties (conformation and membrane permeabilization) very similar to those of the parent peptides.

Cross-Linking Conditions. Generally, two oxidation reactions simultaneously proceed during incubation; concentration-dependent dimerization due to S−S formation and concentration-independent side chain oxidation. The latter should be avoided. To facilitate the former reaction, a relatively high peptide concentration of 0.5 mM was used

[1] Abbreviations: CD, circular dichroism; Fmoc, fluoren-9-ylmethoxycarbonyl; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PC, egg yolk L-α-phosphatidylcholine; PG, egg yolk L-α-phosphatidylglycerol; P/L, peptide-to-lipid molar ratio; RP-HPLC, reversed-phase high-performance liquid chromatography; SUVs, small unilamellar vesicles; TFE, 2,2,2,-trifluoroethanol.

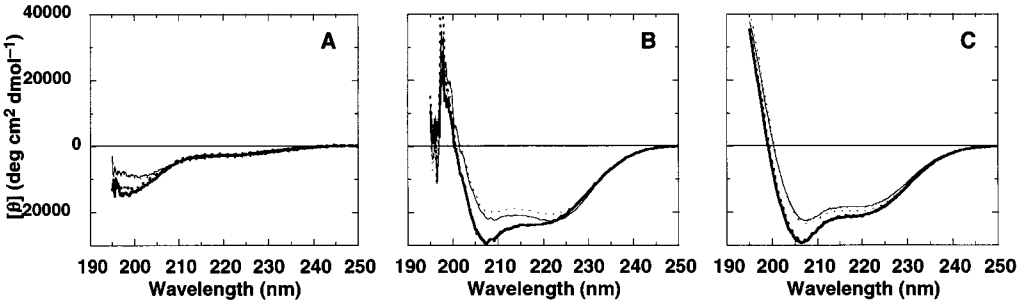

FIGURE 1: CD spectra at 30 °C. CD spectra of the peptides (25 μM) were measured in (A) 25 mM carbonate/150 mM NaCl/1 mM EDTA buffer (pH 9.3), (B) 2 mM PG/PC LUV suspension (pH 9.3), and (C) TFE/carbonate buffer (3/2 v/v). Peptides: thin solid line, **I**; thin dotted line, **II**; thick solid line, **III**; thick dotted line, **IV**.

in solution experiments. In membrane experiments, a lower peptide concentration was sufficient because the peptides were locally concentrated in the membrane phase. Furthermore, an alkaline pH of 9.3 was chosen because the SH group with a pK_a value of 9−9.5 should be deprotonated for S−S formation (*21*). This pH would not be expected to significantly affect the ionization states of the Lys residues, the pK_a values of which incorporated into the membrane active melittin peptide are ca. 10 in both aqueous and lipidic environments (*22*). Especially in negatively charged lipid bilayers used in this study, this effect would be negligible because the local pH value of the membrane surface was lowered compared to the bulk phase (*23*).

The reaction time was limited by the appearance of side chain-oxidized byproducts. For example, the peptide mixtures were incubated in buffer until 6 h, where minor peaks due to byproducts started to appear in HPLC charts (see Figure 1A). Longer incubation made the accurate estimation of the composition of the main products difficult. Redox buffer has often been used to facilitate reshuffling of disulfides (*24*). We did not use this buffer because a number of byproducts (mixed disulfides) were formed.

Cross-Linking in Buffer. CD spectra (Figure 1A) showed that the four Cys-introduced peptides assumed unordered structures in buffer similarly to the parent peptides (*17*, *23*). Figure 2A shows the HPLC chart of an equimolar mixture of **I** and **III** that had been reacted in alkaline buffer for 6 h at 30 °C. Five major peaks were assigned by ion spray mass spectroscopy to **I** monomer, **III** monomer, **I** homodimer, **I**−**III** heterodimer, and **III** homodimer in the order of increasing retention time. More than 80% of the starting materials were converted to the dimeric species. The composition of the reaction mixture is summarized in Figure 3A. The ratio of **I** homodimer:**I**−**III** heterodimer:**III** homodimer was 1:2.16:1.06, which was almost identical to the stochastic value expected for random reaction, i.e., 1:2:1. Similar results were obtained for the **I**−**IV** (Figure 3B) and **II**−**IV** (Figure 3D) combinations. In the case of the **II**−**III** pair (Figure 3C), the ratio was close to, but somewhat deviated from, the random reaction value. The slower oxidation might be related to this deviation.

Cross-Linking in Membranes. Figure 1B shows the CD spectra of the peptides bound to PG/PC (1/1) SUVs. The double minima around 208 and 222 nm indicated that the pepties conformed to α-helices in the membranes. These spectra were very similar to those of the native peptides (*17*). Figure 2B shows the HPLC pattern of an equimolar mixture of **I** and **III** that had been reacted in the presence of PG/PC

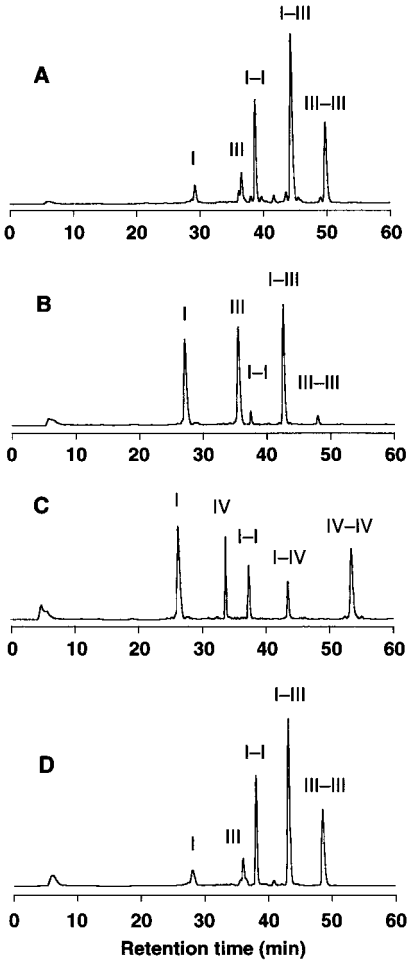

FIGURE 2: Representative RP-HPLC charts. Magainin 2 analogue−PGLa analogue 1:1 mixtures were incubated for oxidation under various conditions and analyzed by RP-HPLC (detection, absorbance at 280 nm). Each peak was identified by ion spray mass spectroscopy. For example, **I**, **I**−**I**, and **I**−**III** represent monomer of **I**, homodimer of **I**, and heterodimer composed of **I** and **III**, respectively. **I** and **III** (0.5 mM each) were reacted in (A) carbonate buffer (pH 9.3) and (D) TFE/carbonate buffer (3/2 v/v) for 6 h at 30 °C. (B) **I** and **III** (90 μM each) and (C) **I** and **IV** (90 μM each) were reacted in 2.25 mM PG/PC LUVs (pH 9.3) for 2 h at 30 °C.

LUVs for 2 h at 30 °C. The distribution of the dimeric species, which is summarized in Figure 4A (closed bars), was completely different from that in buffer (Figures 2A and 3A). The population of the **I**−**III** heterodimer overwhelmed those of the homodimers. This was not due to slow oxidation because a longer incubation (12 h) yielded similar results (Figure 4A, hatched bars). Figure 4D shows that the **II**−**IV**

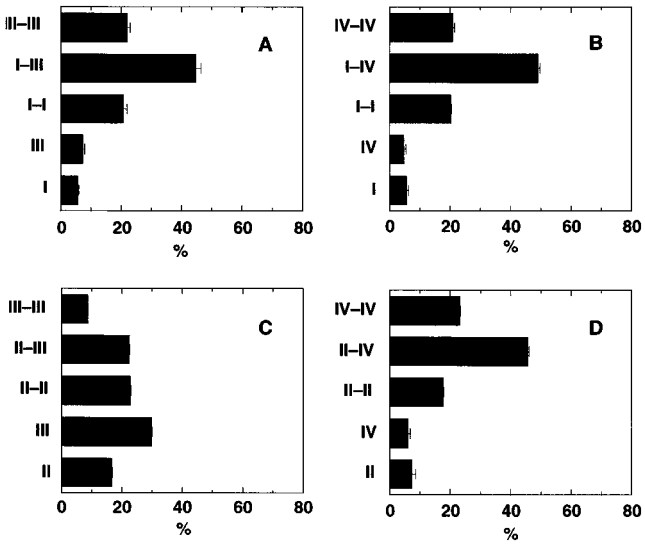

FIGURE 3: Cross-linking in buffer. Magainin 2 analogue−PGLa analogue 1:1 mixtures (0.5 mM each) were oxidized in carbonate buffer (pH 9.3) for 6 h at 30 °C. The products were analyzed by RP-HPLC. The percentage compositions of peak areas are shown by bars. Peptide combinations: (A) **I** and **III**; (B) **I** and **IV**; (C) **II** and **III**; (D) **II** and **IV**.

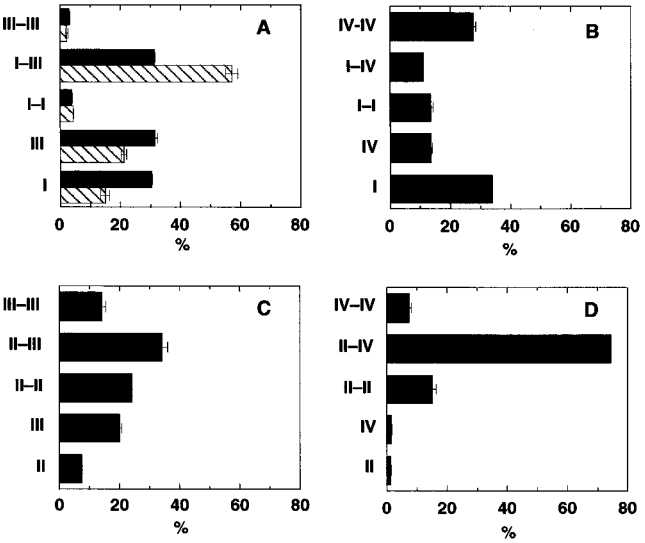

FIGURE 4: Cross-linking in membranes. Magainin 2 analogue−PGLa analogue 1:1 mixtures (90 μM each) were oxidized in 2.25 mM PG/PC LUVs (pH 9.3) for 2 h (solid bars) or 12 h (hatched bars) at 30 °C. The percentage compositions of peak area are shown by bars. Peptide combinations are the same as in Figure 4.

combination with Cys at the C termini also exhibited selective formation of the heterodimer. In contrast, the combinations of peptides with Cys at the opposite termini did not preferentially produce heterodimers (Figures 2C and 4B for the **I**−**IV** pair and Figure 4C for the **II**−**III** pair).

Cross-Linking in TFE/Buffer. To determine whether the different cross-linking results in buffer and membranes were due to the differences in peptide conformations, cross-linking experiments were carried out in a TFE/buffer mixture, where the peptides also assumed helical structures as confirmed by CD spectra (Figure 1C). The results of the experiments (Figures 2D and 5) were similar to those in buffer (Figures 2A and 3).

Membrane Permeabilization Activity. The membrane permeabilizing activities of the four analogue peptides and the two isolated parallel heterodimers (**I**−**III** and **II**−**IV**) were

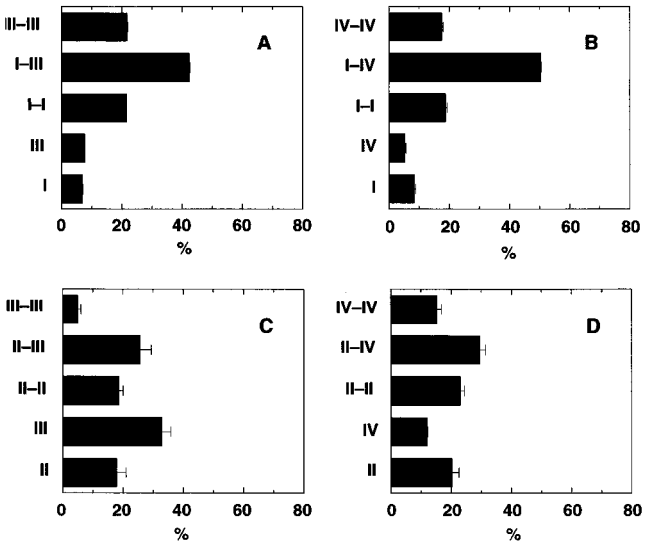

FIGURE 5: Cross-linking in TFE/buffer. Experiments similar to those shown in Figure 3 were carried out in TFE/carbonate buffer (3/2 v/v).

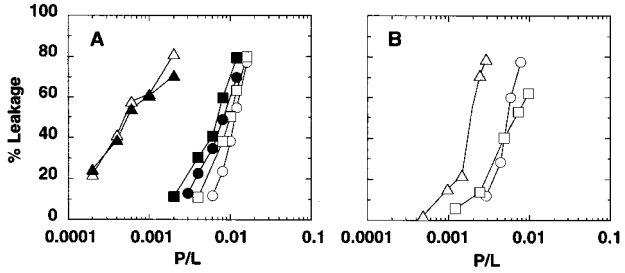

FIGURE 6: Membrane permeabilizing activities. Each peptide was incubated with PG/PC LUVs entrapping 70 mM calcein dye in 10 mM Tris/150 mM NaCl/1 mM EDTA (pH 7.4) at 30 °C. The percent dye leakage during a 5 min incubation is plotted as a function of P/L. The peptide concentrations of the dimeric species are expressed as concentrations reduced to monomers. (A) Peptide: ○, **I**; ●, **II**; □, **III**; ■, **IV**; △, **I**−**III**; ▲, **II**−**IV**. (B) Peptide: ○, F5W-magainin 2; □, PGLa; △, a physical 1:1 mixture of F5W-magainin 2 and PGLa. Data taken from ref *17*.

examined by calcein leakage assay. Figure 6A plots the percent leakage value during a 5 min incubation as a function of the peptide-to-lipid molar ratio (P/L) on a logarithmic scale. Note that the peptide concentrations of the dimeric species are expressed as concentrations reduced to monomers. The monomeric species induced leakage in the P/L range 0.001−0.01, similarly to the parent peptides (Figure 6B). In contrast, the heterodimers permeabilized the membrane with an order of magnitude smaller P/L value.

DISCUSSION

Peptide−peptide association in membranes has been investigated mainly by spectroscopic methods, such as fluorescence resonance energy transfer (*25*) and electron spin resonance (*26*) by use of fluorescent-labeled and spin-labeled peptides, respectively. Chemical cross-linking based on disulfide bond formation, which is a useful method for determining the mode of helix−helix association in the aqueous phase (*24*, *27*), has not been utilized for membrane-associating peptides. One of the advantages of this technique is that peptide aggregates can be chemically fixed and isolated.

If a disulfide bond is formed upon random collision of two Cys-containing peptides, e.g., **I** and **III**, the distribution

of **I** homodimer:**I**−**III** heterodimer:**III** homodimer will be a stochastic ratio of 1:2:1. In contrast, if **I** and **III** form a parallel 1:1 complex by molecular recognition, the **I**−**III** heterodimer is expected to prevail because both Cys residues attached to the N termini of the peptides are in proximity. If **I** and **III** form an antiparallel heterodimer, the disulfide bond is formed only by random collision between dimers. Therefore, the dimer distribution will not be very far from the stochastic value.

In both the buffer (Figure 3) and TFE/buffer mixture (Figure 5), the distributions of the dimeric species were close to that expected in the case of random reaction for most peptide combinations. These results suggested that magainin 2 and PGLa do not recognize each other in solution phase irrespective of secondary structure (random or helix). In marked contrast, upon membrane binding (Figure 4), magainin 2−PGLa heterodimers were preferentially formed only for **I**−**III** and **II**−**IV** combinations with Cys at the same sides. These observations strongly suggested that both peptides form a parallel heterodimer in this enviromnent.

The isolated parallel heterodimers exhibited almost identical membrane permeabilization activities, which were an order of magnitude stronger than those of the monomeric species (Figure 6A). The leakage activity of a physical equimolar mixture of magainin 2 and PGLa was shown to be enhanced only by severalfold relative to those of the individual peptides (Figure 6B), in keeping with the suggestion that the peptides are in a monomer−dimer equilibrium (*17*). The markedly enhanced activities of the parallel heterodimers were not merely due to their larger sizes because the **I**−**IV** antiparallel heterodimer (data not shown) and a magainin dimer (*28*) exhibited much weaker leakage activities compared to the parallel heterodimers.

This study clarified that magainin 2 and PGLa form a parallel heterodimer of high potency in membranes, in agreement with the previous observations that the coexistence of both peptides slightly alters their secondary structures and enhances Trp fluorescence of L18W-PGLa (*17*). The complex formation appears to rely on strict molecular recognition because a single amino acid mutation significantly affected synergism (*17*). Helix formation alone is not sufficient (Figure 5), and the membrane environment is essential for complex formation. One possibility is that peptides are highly concentrated in the membrane phase. Two-dimensional arrangement of helices also limits the degree of freedom of helix orientation, promoting molecular recognition. Alternatively, negatively charged phosphate groups may be involved in complex formation between positively charged peptides. In any case, this study demonstrated that the membrane environment has a potential to induce unique molecular recognition, which would be termed "membrane-induced molecular recognition".

Recently, the roles of peptide dimerization in membrane pemeabilization induced by peptides such as magainin 2 (*28*) and melittin (*29*, *30*) have attracted a great deal of attention. Furthermore, a heterodimeric antimicrobial peptide including an interpeptide disulfide bridge has been discovered in an arboreal frog (*31*). Elucidation of the interactions of these dimeric species with lipid bilayers is crucial for understanding their mechanisms of action and the development of potent peptidic antibiotics.

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