ELSEVIER

Biochimica et Biophysica Acta 1195 (1994) 157–163

BBA Biochimica et Biophysica Acta

Self-assembly of mastoparan X derivative having fluorescence probe in lipid bilayer membrane

Katsuhiko Fujita, Shunsaku Kimura *, Yukio Imanishi

Department of Material Chemistry, Faculty of Engineering, Kyoto University, Yoshida Honmachi, Sakyo-ku, Kyoto, 606-01 Japan

Received 19 April 1994

Abstract

A mastoparan X (MPX) derivative having an anthryl group at the C-terminal residue was synthesized (MPX-A), and its conformation, orientation and aggregation in phospholipid bilayer membrane were studied. The efficiency of intramolecular energy transfer from the Trp residue to the anthryl group at high peptide dilution suggested α-helical conformation in the lipid membrane, which is consistent with the previous report by NMR of MPX concentrated in the membrane. Either emission from the Trp residue or the anthryl group of MPX-A in the lipid membrane was quenched by 5-doxylstearic acid, suggesting that MPX-A is located at the membrane surface with the helix axis oriented parallel to the surface. The dependence of the excited energy transfer and the fluorescence depolarization of MPX-A on the peptide concentration revealed that MPX-A aggregated in the lipid membrane to form a defined structure.

Keywords: Mastoparan X; Lipid membrane; Aggregation; Alpha-helical peptide; Amphiphilic helix; Orientation

1. Introduction

Conformation, orientation and aggregation of biologically active peptides in the phospholipid bilayer membrane are important information for understanding the structure-function relationship of the peptide [1]. For example, helical peptides having a primary amphiphilic structure form ion channel in lipid membrane by aggregation of the peptides spanning across the membrane [2–4]. It is well known that peptide hormones take a regular conformation in lipid membrane, which is essential for interaction with the receptor in the membrane [5].

Mastoparans, which are tetradecapeptides isolated from wasp venom, show various biological activities such as degranulation of mast cell [6], stimulation of G proteins [7], and facilitation of phospholipase A_2 [8]. Ion channel formation by taking a helix-bundle structure in lipid membrane has also been suggested [9]. CD [10] and NMR [11,12] spectroscopy have shown that mastoparans take partially helical conformation of an amphiphilic structure in lipid membrane. These spectroscopic methods, however, require much higher concentrations of peptide than in biological conditions. For example, mastoparans degranulate mast cell at micromolar concentrations [13], while approximately 100-fold concentration is required for NMR measurement. The interaction of peptides with phospholipid bilayer membrane is sensitively influenced by the peptide concentration. For example, the orientation of magainin in a lipid bilayer membrane was changed with varying the concentration [14]. It is, therefore, important to study the interaction of mastoparans with a lipid bilayer membrane by other sensitive spectroscopic method. In the present investigation, fluorescence spectroscopy is used to investigate the conformation, orientation and aggregation of the peptide at the biological concentrations.

Abbreviations: Ant, anthrylmethyl group; BOP reagent, benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; HOBt, *N*-hydroxybenzotriazole; MPX, mastoparan X; MPX-A, MPX derivative having an anthryl group at the C terminal residue; F-MPX-A, MPX-A with fomylated Trp; SUV, small unilamellar vesicle; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; TMP, trimethyl phosphate.

* Corresponding author.

SSDI 0005-2736(94)00143-D

The anthryl group was connected to the C-terminal residue of MPX, which has a Trp residue at the third position [6]. MPX-A, therefore, possesses two different fluorescent probes at both ends of a chain. The conformation of MPX-A was estimated by measuring intramolecular excitation energy transfer from the Trp residue to the anthryl group. The orientation of MPX-A in lipid bilayer membrane was assessed by fluorescence quenching of the probes by a water-soluble quencher. Furthermore, the aggregation of MPX in lipid membrane was investigated by measuring intermolecular energy transfer from MPX to F-MPX-A, in which the Trp residue was formylated to be non-fluorescent [15].

2. Materials and methods

2.1. Materials

Boc-protected amino acids, 25% HBr/acetic acid, DCC and HOBt were purchased from Kokusan Kagaku, Japan, and BOP reagent, TFA, TFMSA, and 9-hydroxymethylanthracene were purchased from WAKO Pure Chemicals, Japan. MPX was obtained from Peninsula Lab., USA. DMPC and 5-, 12- and 16-doxyl stearic acids were purchased from Sigma, USA.

2.2. Synthesis

MPX-A was synthesized by condensation of 1st and 2nd fragments (Fig. 1). Boc-Ser(Ant)-NH_2 was obtained by the reaction of Boc-Ser-NH_2 with 9-bromomethylanthracene [16], which was prepared from 9-hydroxymethylanthracene by treating with PBr_3. The first fragment composed of six residues of the N-terminal part of MPX-A and F-MPX-A was synthesized by the conventional liquid phase method using DCC and HOBt as coupling reagents. The second fragment composed of seven residues of the C-terminal part was synthesized by the solid phase method using oxime resin developed by Kaiser et al. [17]. The protected second fragment was cleaved from the resin by incubation with H-Ser(Ant)-NH_2 for 25 h. Both fragments were purified with a Sephadex LH-20 column using DMF as eluant and recrystallization. The two fragments were coupled by using DCC and HOBt to obtain a fully protected peptide, Z-MPX-A. The product was purified with a Sephadex LH-20 column using DMF as eluant, and was treated with hydrazine monohydrate to remove the formyl group [18]. Z groups were removed by the treatment with TFMSA to obtain crude MPX-A [19]. On the other hand, Z-MPX-A was treated by HBr/acetic acid to obtain crude F-MPX-A [20]. Each crude product was purified by the partition chromatography using a Sephadex G-50 column, which was equilibrated with the lower phase of 1-butanol/acetic acid/water (4:1:5) and eluted with the upper phase. Fractions of the main peak were collected and concentrated by evaporation [21]. The residues were purified further by a reverse-phase HPLC column (Cosmosil 5C18, Nakalai Tesque, Japan) using acetonitrile/water (3:2, v/v) mixture containing 0.1% TFA as eluant [22]. MPX-A and F-MPX-A were identified by NMR spectroscopy and amino acid analysis.

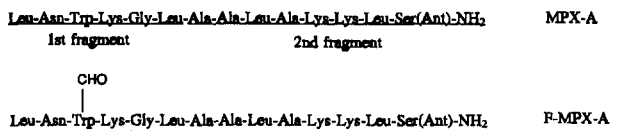

Fig. 1. Molecular structures of MPX derivatives.

2.3. Liposome preparation

Small unilamellar vesicles (SUV) composed of DMPC were prepared by sonication of the lipid dispersion in a phosphate buffer (50 mM, pH 7.0) followed by ultra-centrifugation at $100\,000 \times g$ as reported previously [23]. The lipid concentration was determined by the phospholipid assay kit, Dia Color PL (Ono Yakuhin, Japan) [24]. All measurements using the SUV were carried out at 30°C, which is above the phase-transition temperature of DMPC liposome [25].

2.4. Measurements

CD, UV and fluorescence spectra were measured on a JASCO J-600 spectropolarimeter using a cell with an optical path length of 2 mm, a JASCO UVIDEC-1 UV/VIS spectrophotometer and a Hitachi MPF-4 fluorophotometer, respectively. Fluorescence depolarization was measured by an equipment installed on the MPF-4 fluorophotometer according to the method as reported previously [26]. The stock solution of peptide (approx. 3 mM) in twice distilled TMP was prepared, and the accurate concentration was determined with the absorbance of Trp or the anthryl group.

The dissociation constant of the peptide-containing DMPC vesicles was determined from the increase of the fluorescence intensity of the peptide by the addition of DMPC vesicles according to the method reported previously [27].

Fluorescence quenching experiments were carried out as follows. A dry film of DMPC containing various doxylstearic acids was dispersed in a phosphate buffer (50 mM, pH 7.0). The dispersion was sonicated for 5 min at 35°C. The peptide (1.1 μM) was incubated with the liposome ([DMPC] = 0.37 mM) for 15 min, and the fluorescence intensity was measured at 30°C. The excitation and monitor wavelengths were 280 and 350 nm

for the Trp residue and 360 and 415 nm for the anthryl group, respectively.

Fluorescence quantum yield of Trp residue in MPX was determined using quinine sulfate in 1 N sulfuric acid as standard [28]. Excitation energy transfer efficiency was determined from the excitation spectra of the energy acceptor, the anthryl group [29]. The distance between the Trp residue and the anthryl group was estimated on the basis of the Forster's theory [30].

3. Results and discussion

3.1. Distribution to lipid membrane

Fluorescence spectra of MPX-A were measured with increasing amount of DMPC vesicles (Fig. 2). Emission of MPX-A in a buffer solution appeared at 350 (from the Trp residue) and 415 nm (from the anthryl group) upon excitation at 280 nm. Under the condition, the 82% of photoenergy at 280 nm is absorbed by the indolyl group, and the rest by the anthryl group. The emission of the anthryl group is mainly due to the energy transfer from the Trp residue. The emission of the Trp residue in a buffer solution shifted to a shorter wavelength and strengthened the intensity by the addition of DMPC vesicles, indicating distribution of MPX-A to the phospholipid bilayer membrane. The dissociation constant, K_d, of MPX-A with DMPC membrane was calculated from the intensity change of the anthryl group which was directly excited at 360 nm as well as the Trp residue with varying lipid concentrations. K_d (apparent) [27] was 0.18 mM, which is comparable to 0.11 mM for MPX with the DMPC membrane and 0.12 mM for MPX with the egg-yolk lecithin membrane [31]. Therefore, the introduction of the anthryl group to MPX and the replacement of Ile residue of MPX with Leu residue do not strongly influence the affinity of peptide with lipid membrane.

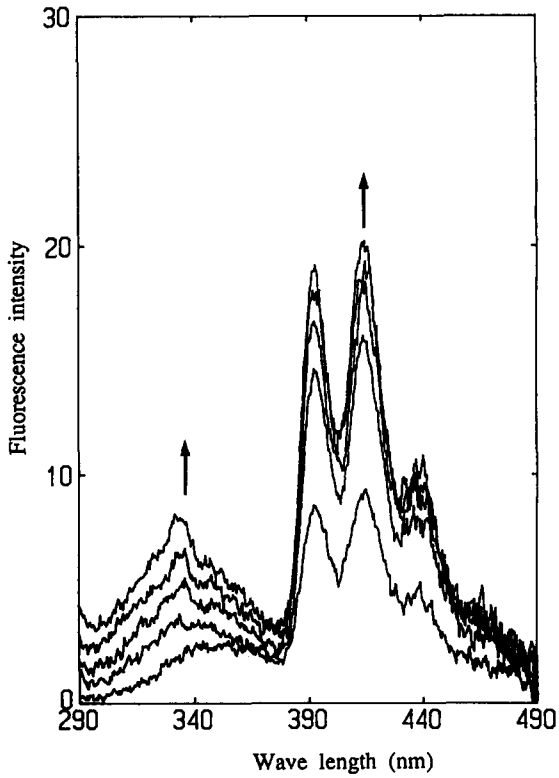

Fig. 2. Fluorescence spectra of MPX-A with varying concentrations of DMPC vesicle: 0, 77, 150, 230 and 380 μM from the bottom to the top. Excitation wavelength: 280 nm. [MPX-A] = 1.1 μM.

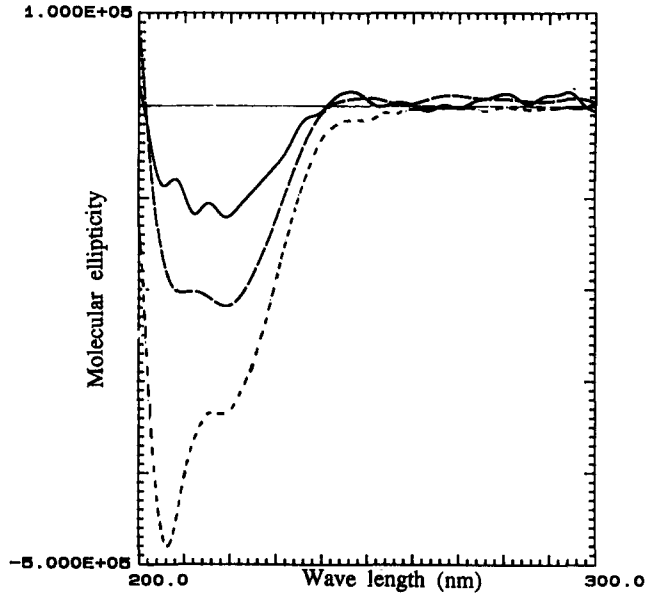

Fig. 3. CD spectra of MPX-A in 50 mM phosphate buffer, pH 7.0 (———), 10 mM SDS micelle (— — —), and TMP (- - - - -). [MPX-A] = 1.8 μM.

3.2. Conformation of MPX-A

Fig. 3 shows CD spectra of MPX-A in various media. A negative Cotton effect appearing at 220–230 nm region indicates the occurrence of α-helical conformation. The peptide concentration was chosen at 1.8 μM to reduce the aggregation effect on the spectra as low as possible. Although the CD patterns are not of the double-minimum type which is typical for α-helix, the apparent helix content was estimated from the intensity at 222 nm [32]. It was 22% in buffer, 41% in SDS micelle, and 70% in TMP. CD spectra of MPX-A in SDS micelle were measured with changing concentrations from 0.7 to 4.0 μM, but no significant variation in CD spectra was detected. MPX-A, therefore, is expected to take α-helical conformation in lipid membrane. However, DMPC vesicles aggregated in the presence of MPX-A of 10 μM, which made the CD measurement difficult due to the light-scattering effect.

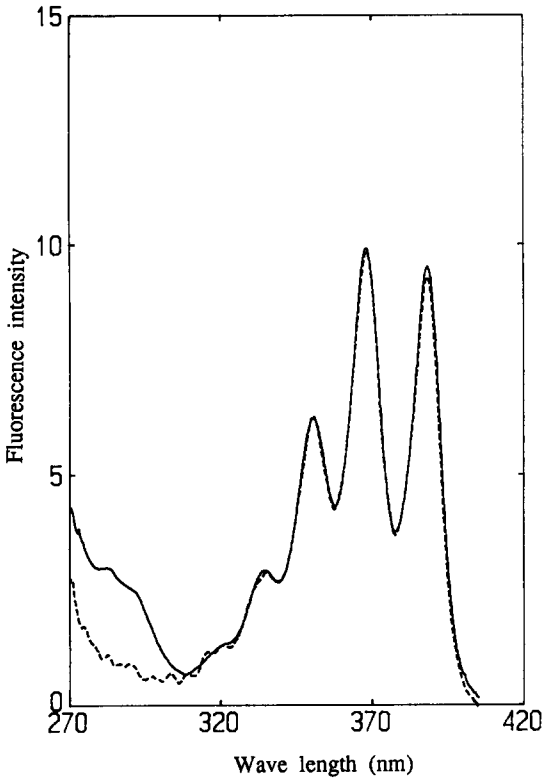

Fig. 4. Excitation spectra of MPX-A (———) and F-MPX-A (- - - -) in the presence of DMPC vesicles. Monitor wavelength; 415 nm. Peptide concentration; 0.43 μM. [DMPC] = 4.2 mM.

The conformation of MPX-A in lipid membrane was investigated by fluorescence spectroscopy. The efficiency of excited energy transfer from the Trp residue to the anthryl group of MPX-A was calculated from the excitation spectrum of MPX-A (Fig. 4). The molar ratio of MPX-A and DMPC was 1:9800. Under these conditions, intramolecular energy transfer is absolutely predominant over intermolecular process, because at most one peptide molecule is included in one vesicle by calculation. F-MPX-A, which has a non-fluorescent Trp, was used as reference. The energy-transfer efficiency was calculated from the intensity at 283 nm to be 34%. This value corresponds to a distance of 2.49 nm between the Trp residue and the anthryl group of MPX-A according to the Forster's theory [29]. The distance between the indole nitrogen of Trp residue and the center of anthryl group along the molecular axis of MPX-A was calculated to be 2.05 nm on the basis of a fully α-helical conformation and a *trans* configuration of side chains [33]. On the other hand, a distance of 4.0 nm can be obtained for an extended conformation. Therefore, MPX-A is considered to take a highly helical structure in lipid membrane. The result at high peptide dilution appears consistent with the previous NMR assignment of the conformation of the vesicle-bound peptide at the concentrated condition [11,12].

It should be noted, however, that the helix content might not be so high in lipid membrane, if peptide molecules are allowed to undergo an accordion vibration. In this case, the two fluorescent probes should approach within the critical distance for the energy transfer during the fluorescence life time of the Trp residue, and the energy-transfer efficiency increases. This happens to MPX-A in a TMP solution. The energy-transfer efficiency of MPX-A in a TMP solution was calculated to be 65%, which corresponds to a distance of 2.13 nm between the Trp residue and the anthryl group, suggesting a fully helical conformation. However, the helix content was 70% from the CD measurement.

3.3. Orientation of MPX-A in lipid membrane

The location and orientation of the helical MPX-A in lipid membrane were investigated by fluorescence quenching of Trp residue and the anthryl group with various doxylstearic acids [34]. The results are shown in Fig. 5 according to the Stern-Volmer plot. The emission from both probes was most strongly quenched by 5-doxylstearic acid in the presence of DMPC vesicles, indicating that the location of both chromophores are close to the membrane surface. However, the accurate distance between the fluorophores and the bilayer center could not be determined by using the parallax method [35]. Possible reasons are (i) the electrostatic interaction between the peptide and the quenchers, which deviate the two-dimensional concentration of the quenchers around the peptide from the average concentration under the homogeneous distribution in the membrane, and (ii) disturbance of the membrane structure at the binding site of the peptide, which changes the depth of the quencher in the membrane. Although the quenching rate of the Trp residue can not be directly compared with that of the anthryl group due to different fluorescence-life time, the Trp residue is considered near to the aqueous phase and the anthryl group is slightly deep from the water/lipid interface. Therefore, it is considered that MPX-A is located at the surface of a phospholipid bilayer membrane, and the helix axis of MPX-A oriented parallel to the membrane surface.

3.4. Aggregation of MPX-A in lipid membrane

Aggregation of MPX in lipid membrane was studied by measuring the intermolecular energy transfer from the Trp residue of MPX to the anthryl group of F-MPX-A in the presence of DMPC vesicles. The equimolar mixture of MPX and F-MPX-A was added to DMPC vesicles. The efficiency of intermolecular energy-transfer was calculated from the excitation spectra, and the results are summarized in Fig. 6. The

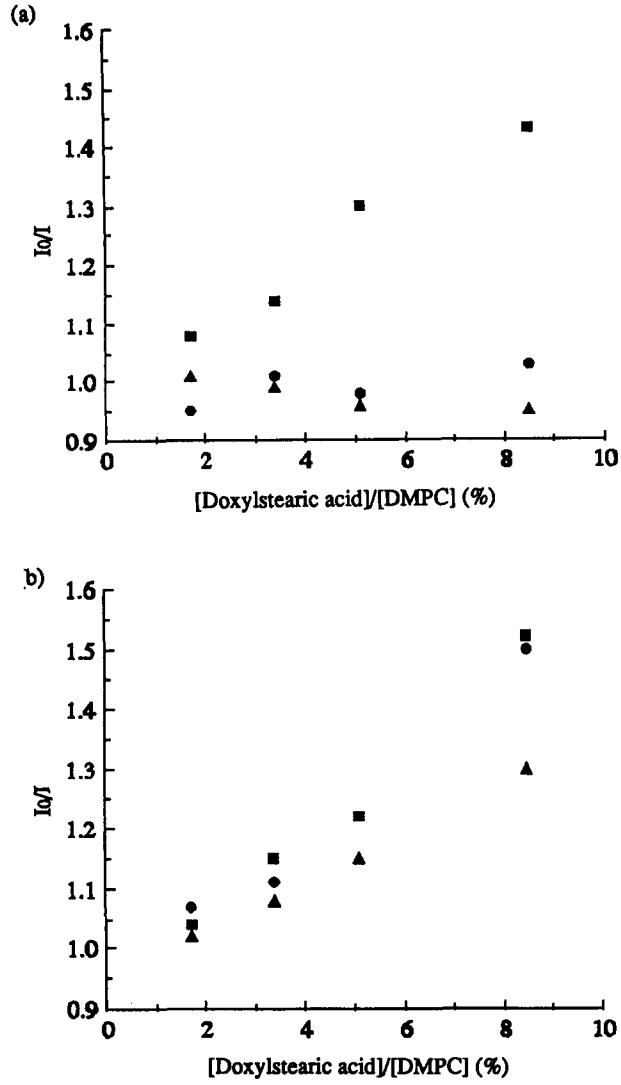

Fig. 5. Fluorescence quenching of MPX-A by 5- (■), 12-(●), and 16-doxylstearic acids (▲) in the presence of DMPC vesicles. Fluorescence quenching of (a) Trp and (b) the anthryl group. [DMPC]/[MPX-A] = 337. Other conditions are indicated in the Materials and methods.

efficiency increased with increasing peptide concentration in lipid membrane. It is notable that the experimental conditions were limited such that the peptide/lipid molar ratios were lower than $7.5 \cdot 10^{-4}$ where the energy-transfer efficiency was calculated to be 4% in the case of a homogeneous dispersion of peptide molecules in the outer leaflet of a lipid bilayer membrane [35,36]. Since much higher efficiency was observed at the peptide/lipid molar ratios lower than $7.5 \cdot 10^{-4}$, the peptide molecules appear to aggregate at these low concentrations.

The fluorescence depolarization of the anthryl group of F-MPX-A was also measured under the same conditions, and the results are shown in the same figure (Fig. 6). The degree of depolarization value increased with increasing the peptide concentration in the membrane similarly to the energy-transfer efficiency. The increase of the degree of depolarization can be explained by an energy migration between the anthryl groups due to aggregation of peptide molecules [2]. It is, therefore, considered that MPX and F-MPX-A aggregate in lipid membrane at very low concentrations.

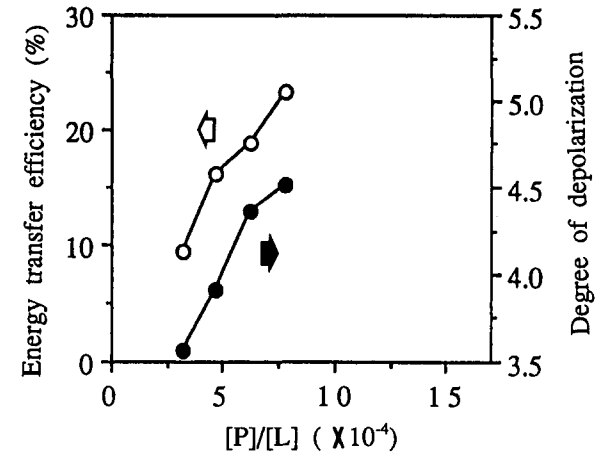

Fig. 6. Energy-transfer efficiency (○) and fluorescence depolarization (●) of an equimolar mixture of MPX and F-MPX-A with varying peptide/lipid molar ratios. Excitation wavelength; 280 nm, monitor wavelength: 415 nm. [DMPC] = 2.1 mM. Peptide concentrations varied.

The peptide aggregation in lipid membrane was also indicated by measuring excitation spectra of MPX-A with varying peptide/lipid molar ratios. The energy-transfer efficiency was plotted against the peptide/lipid molar ratio in Fig. 7. The efficiency increased with increasing peptide concentrations and reached to a plateau above a peptide/lipid molar ratio of $7 \cdot 10^{-4}$. The increase of the energy-transfer efficiency at these low peptide concentrations can be explained by consid-

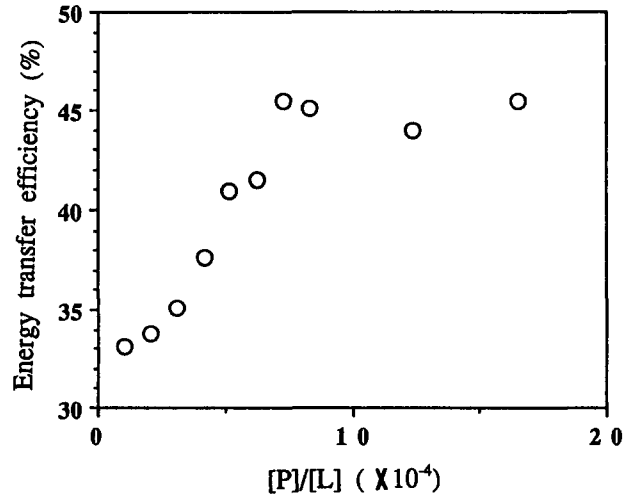

Fig. 7. Energy-transfer efficiency of MPX-A with varying peptide/lipid molar ratios. [DMPC] = 4.0 mM, [MPX-A] = 0.43 – 7.0 μM

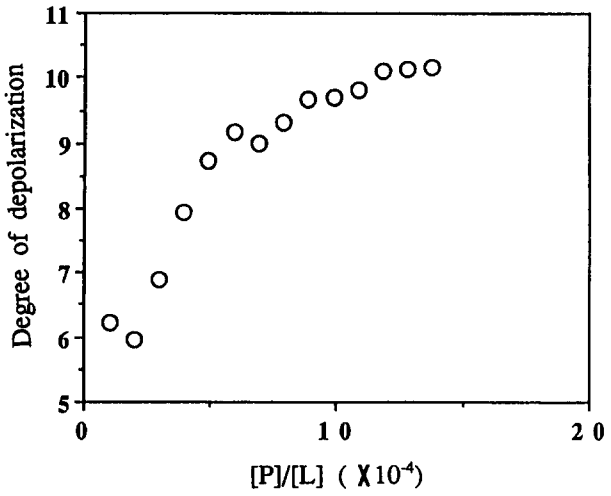

Fig. 8. Fluorescence depolarization of MPX-A with varying peptide/lipid molar ratios. Conditions are the same as those in Fig. 7.

ering aggregation of peptide molecules in lipid membrane, accompanying the intermolecular energy transfer. The fluorescence depolarization depended on the peptide/lipid molar ratios in a similar manner to the relation of the energy transfer with the peptide/lipid molar ratios (Fig. 8), supporting the aggregation of MPX-A in the lipid membrane.

Interestingly, the energy-transfer efficiency as well as the degree of fluorescence depolarization became constant above a peptide/lipid molar ratio of $7 \cdot 10^{-4}$. This observation suggests that aggregate of MPX-A having a definite size is formed above this molar ratio. Four MPX-A molecules are included in one DMPC vesicle at this peptide/lipid molar ratio under the assumption that 5000 lipids form one vesicle having a 30-nm diameter [35]. It is thus considered that the aggregate of MPX-A is an assembly of four molecules on average. However, this number remains to be studied further.

Table 1
Fluorescence properties of MPX-A

	Quantum yield of Trp in MPX	R_0 (Trp → Ant)	Energy transfer efficiency (%)	R (nm)
In SUV suspension	0.32	2.24 [a]	34	2.49
In TMP solution	0.42 [b]	2.32 [a,b]	65	2.13

R, the distance between the centers of the donor and the acceptor chromophores determined from the energy transfer efficiency. R_0, the distance at which the transfer efficiency is 50%.

[a] The orientation factor, K^2 was assumed to be 2/3, value of a random orientation.

[b] The refractive indices of 1.336 and 1.369 were adopted for the phosphate buffer and TMP, respectively.

It has been reported that several biological activities of mastoparans elicited by direct interaction with G proteins [37]. Because G proteins exist at the inner surface of a plasma membrane, mastoparans must be translocated across the membrane. It has been proposed that the inside-negative membrane potential may be the driving force for the translocation [31,38]. The assembly formation may facilitate the transmembrane translocation of mastoparans by shielding the hydrophilicity. Furthermore, the aggregation of mastoparans helps concentration of positive charges to activate G proteins as reported for G-protein activating peptides [39,40].

References

[1] Sargent, D.F. and Schwyzer, R. (1986) Proc. Natl. Acad. Sci. USA 83, 5774–5778.
[2] Otoda, K., Kimura, S. and Imanishi, Y. (1993) Biochim. Biophys. Acta 1150, 1–8.
[3] Otoda, K., Kimura, S. and Imanishi, Y. (1992) Biochim. Biophys. Acta 1112, 1–6.
[4] Otoda, K., Kimura, S. and Imanishi, Y. (1993) J. Chem. Soc. Perkin Trans. 1, 3011–3016.
[5] Schwyzer, R. (1986) Biochemistry, 25, 6335–6342.
[6] Nakajima, T., Uzu, S., Wakamatsu, K., Saito, K., Miyazawa, T., Yasuhara, T., Tsukamoto, Y. and Fujino, M. (1986) Biopolymers 25, 115–121.
[7] Higashijma, T., Uzu, S., Nakajima, T. and Ross, E.M. (1988) J. Biol. Chem. 263, 6491–6494.
[8] Argiolas, A and Pisano, J.J. (1983) J. Biol. Chem. 25, 13697–13702.
[9] Mellor, I.R. and Sanson, M.S.P. (1990) Proc. R. Lond. B 239, 383–400.
[10] Higashijima, T., Wakamatsu, K., Takemitsu, M., Fujino, M., Nakajima, T. and Miyazawa, T. (1983) FEBS Lett. 152, 227–230.
[11] Wakamatsu, K., Higashijima, T., Fujino, M., Nakajima, T. and Miyazawa, T. (1983) FEBS Lett. 162, 123–126.
[12] Wakamatsu, K., Okada, A., Miyazawa, T., Ohya, M. and Higashijima, T. (1992) Biochemistry 31, 5654–5660.
[13] Hiral, Y., Yasuhara, T., Yoshida, H., Nakajima, T., Fujino, M. and Kitada, C. (1979) Chem. Pharm. Bull. 27, 1942–1944.
[14] Ludtke, S.J., He, K., Wu, Y. and Huang, H.W. (1994) Biochim. Biophys. Acta 1190, 181–184.
[15] Kimura, S. and Imanishi, Y. (1982) Helv. Chim. Acta 65, 2431–2442.
[16] Sugano, H. and Miyoshi, M. (1976) J. Org. Chem. 41, 2352–2353.
[17] Kaiser, E.T., Mihara, H., Laforet, G.A., Kelly, J.W., Walters, L., Findeis, M.A. and Sasaki, T. (1989) Science 243, 187–192.
[18] Ohno, M., Tsukamoto, S., Makisumi, S. and Izumiya, N. (1972) Bull. Chem. Soc. Jpn. 45, 2852–2855.
[19] Akaji, K., Fujii, N., Yajima, H. and Pearson, D. (1982) Chem. Pharm. Bull. 30, 349–351.
[20] Ben-Ishai, D. (1954) J. Org. Chem. 19, 62.
[21] Yajima, H., Kanai, J., Funakoshi, S., Hirai, Y. and Nakajima, T. (1980) Chem. Pharm. Bull. 28, 882–886.
[22] Wakamasu, M., Kitada, C. and Fujino, M. (1982) Chem. Pharm. Bull. 30, 7266–7269.
[23] Barenholz, Y., Gibbes, D., Litman, B.J., Goll, J., Thompson, T.E. and Carlson, F.D. (1977) Biochemistry 16, 2806–2810.
[24] Nishina, H. (1984) Med. Technol. 12, 539–545.

[25] Van Dijck, P.W.M., De Kruijff, B., Van Deenen, L.L.M., De Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576–587.
[26] Ohtoyo, T., Shimagaki, M., Otoda, K., Kimura, S. and Imanishi, Y. (1988) Biochemistry 27, 6458–6463.
[27] Surewicz, W.K. and Epand, R.M. (1984) Biochemistry 23, 6072–6077.
[28] Melhuish, W.H. (1960) J. Phys. Chem. 64, 762-
[29] Stryer, L. (1978) Annu. Rev. Biochem. 47, 819–846.
[30] Forster, T. (1965) Modern Quantum Chemistry, Academic Press, New York, p. 93.
[31] De Kroon, A.I.P.M., De Gier, J. and De Kruijff, B. (1991) Biochim. Biophys. Acta 1068, 111–124.
[32] Greenfield, N. and Fasman, G.D. (1969) Biochemistry 8, 4108–4113.
[33] Momany, F., McGuire, R.F., Burgess, A.W. and Sheraga, H.A. (1975) J. Phys. Chem. 79, 2361–2381.
[34] Chattopadhyay, A. and London, E. (1987) Biochemistry 26, 39–45.
[35] Fung, B.K.-K. and Stryer, L. (1978) Biochemistry 17, 5241–5248.
[36] Haung, C. and Mason, J.T. (1978) Proc. Natl. Acad. Sci. USA 75, 308–310.
[37] Weigarten, R., Ransnas, L., Mueller, H., Sklar, L.A. and Bokoch, G.M. (1990) J. Biol. Chem. 265, 11044–11049.
[38] De Kroon, A.I.P.M., Vogt, B., Van't Hof, R., De Kruijff, B. and De Gier, J. (1991) Biophys. J. 60, 525–537.
[39] Higashijima, T., Burnier, J. and Ross, E.M. (1990) J. Biol. Chem. 265, 14176–14186.
[40] Tomita, U., Inanobe, A., Kobayashi, I., Takahashi, K., Ui, M. and Katada, T. (1991) J. Biochem. 109, 184–189.