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Location of melittin fragment carrying spiropyran in phospholipid bilayer membrane determined by thermal isomerization

Eiichi Kato, Takehiko Ueda, Shunsaku Kimura, Yukio Imanishi *

Department of Polymer Chemistry, Kyoto University, Yoshida Honmachi, Sakyo-ku, Kyoto 606-01, Japan

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Abstract

Melittin fragments carrying spiropyran were synthesized, and their distribution in phospholipid bilayer membrane was studied by using spiropyran as a probe. Spiropyran was connected to the side chain of a Glu residue (Glu(OSp)), and the residue was replaced for the fourth position of melittin (1–7) fragment (M7Sp). M7Sp showed a high affinity for phospholipid membrane. The spiropyran group of M7Sp was converted to a merocyanin group by UV irradiation, which reduced the amount of the peptide bound to the membrane to the half of the initial amount. The location of the merocyanin group of M7Sp in the membrane was evaluated by the rate of thermal isomerization from merocyanin to spiropyran, which is sensitive to the microenvironment of merocyanin. A large fraction of the merocyanin group isomerized rapidly back to a spiropyran form, indicating that M7Sp is located in a relatively hydrophobic region of the membrane. Although the interaction of the peptide with phospholipid membrane is affected by photoisomerization of the spiropyran substituent, spiropyran was shown to be a useful tool to evaluate the location of the peptide in the lipid membrane.

Key words: Peptide/phospholipid membrane interaction; Thermal isomerization; Spiropyran; Melittin fragments; Membrane probe; Photocontrol

1. Introduction

Conformation, orientation, and location of membrane-bound peptides are key factors for understanding the biological activity of such peptides on the molecular basis [1]. For example, opioid peptide hormones are distributed to a specific region of the membrane taking a definite conformation. This process is considered to enhance encountering and binding to a certain type of opioid receptor embedded in the membrane [2]. The location of peptides in the membrane has been investigated by various methods such as fluorescence [3], fluorescence quenching [4], ATR-IR [5], NMR [6], differential scanning calorimetry [7], and hydrophobic photolabeling [8], etc. Although the fluorescence method is very sensitive to the microenvironment, it provides average information on the location in the membrane. This point imposes several limitations on

* Corresponding author.

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the application of the fluorescence method, when the location of peptide molecules in the membrane is variable according to conformation and they are distributed according to the Boltzmann rule among several sites of similar potential energies. Parallax method based on the fluorescence quenching determines the location of the fluorophore in an Å unit [4]. However, the location of the quenching group fluctuates in some occasions strongly in the membrane [9]. Distance geometry using NOE measurement is a powerful technique to determine the location exactly. However, this method is applicable only to the case of micelles [6]. Since each method is not almighty, a novel method should be explored.

In the present study, we have used spiropyran as an environmental probe. Because the rate of thermal isomerization from merocyanin to spiropyran is very sensitive to the polarity of microenvironment [10], the location of merocyanin group in the membrane can be determined by the kinetics of thermal isomerization.

Melittin, the main component of bee venom, undergoes membrane-lysis. The interaction of melittin with phospholipid membranes has been extensively studied [11–24], and different types of distribution of melittin in the membrane have been proposed, wedge-like model [25] and transbilayer model [26]. The primary sequence of melittin possesses an amphiphilic property, in which the melittin 1–20 fragment is composed predominantly of hydrophobic residues and the rest 21–26 fragment hydrophilic. Since melittin 8–26 fragment did not show membrane-lysis activity, the hydrophobic melittin 1–7 fragment is essential for strong interactions with phospholipid membrane [27–29]. We have replaced Ala^4 residue of the melittin 1–7 fragment with Glu residue having a spiropyran group in the side chain (Glu(OSp)), and studied on interaction of the peptide with phospholipid membrane using a spiropyran group as a probe. A melittin 4–7 fragment having Glu(OSp) for the N-terminal residue and a protected Lys^7 for the C-terminal residue was synthesized as a reference compound. The location of the merocyanin group of these peptides in the membrane was estimated from the kinetics of thermal isomerization.

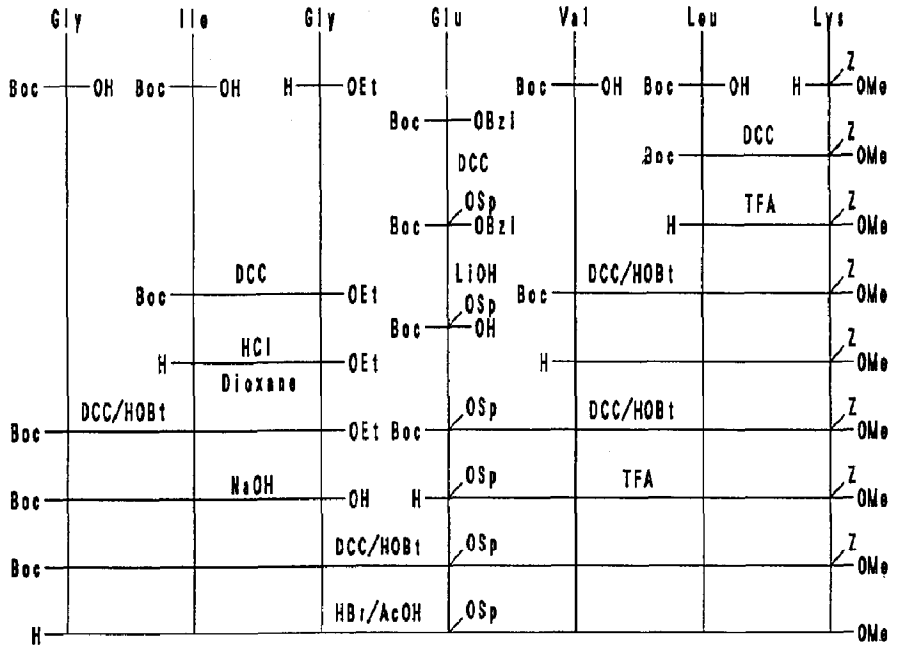


Fig. 1. Synthesis of three melittin fragments carrying a spiropyran group.

2. Experimental

2.1. Peptide synthesis

The melittin fragments were synthesized by the conventional liquid-phase method (Fig. 1). All intermediates and final compounds were identified by ^{1}H NMR, and the purity was checked by thin layer chromatography (TLC). Analytical TLC was performed on Merck silica gel 60 F_{254} aluminium plates, with detection by UV light and/or the ninhydrin test. Light was shut off during the evaporation procedure.

Boc-Glu(OSp)-OBzl A photochromic compound, 1-(β-hydroxyethyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2H-benzopyran] (HO-Sp), was prepared by the reaction of 1-(β-hydroxyethyl)-3,3-dimethylindolenium iodide and 5-nitrosalicylaldehyde in the presence of piperidine [30,31]. HO-Sp (1 eq. mol) was reacted with Boc-Glu-OBzl (γ free, 1 eq. mol) in the presence of 4-pyrrolidinopyridine (0.1 eq. mol) using dicyclohexylcarbodiimide (DCC) as a coupling reagent. The product was purified by eluting through a silica gel column (Lobar Si60, Sigma) using a mixture of ethyl acetate and toluene (1:1, v/v) as eluant. $R_f = 0.78$ (ethyl acetate/toluene, 1/1, v/v).

TFA · H-Glu(OSp)-OH (M1Sp) Boc-Glu(OSp)-OBzl (100 mM) was dissolved in acetone and 1.5-fold molar excess of LiOH solution (0.4 N) was added dropwise under stirring over 1 h. After further stirring for 1.5 h at 24°C, the solution was acidified with citric acid solution (10%) and extracted with ethyl acetate. The combined extract was concentrated and eluted through a silica gel column (Lobar Si60, Sigma) using ethyl acetate as an eluant to obtain Boc-Glu(OSp)-OH (Boc-M1Sp). R_f = 0.43 (ethyl acetate/methanol, 7/3, v/v). Boc group was removed by the treatment of trifluoroacetic acid (TFA) containing 10% anisole at 0°C for 30 min. White precipitate was obtained by the addition of excess amount of diethyl ether.

TFA · H-Glu(OSp)-Val-Leu-Lys(Z)-OMe (M4Sp) HCl · Val-Leu-Lys(Z)-OMe was synthesized according to the scheme shown in Fig. 1. BocM1Sp was coupled with H-Val-Leu-Lys(Z)-OMe using DCC in dimethylformamide (DMF) for 1 day. The product was purified by elution through a Sephadex LH-20 column using methanol as an eluant to obtain Boc-Glu(OSp)-Val-Leu-Lys(Z)-OMe. $R_f = 0.75$ (ethyl acetate). Boc group was removed by the treatment with TFA containing 10% anisole at 0°C for 30 min. The solution was evaporated, and the residue was recrystallized from diethyl ether/petroleum ether mixture. $R_f = 0.63$ (chloroform/methanol/acetic acid, 95/5/3, v/v/v).

2HBr Gly-Ile-Gly-Glu(OSp)-Val-Leu-Lys-OMe (M7Sp) Boc-Gly-Ile-Gly-OH ($R_f = 0.67$ with butanol/acetic acid/water, 10/1/3, v/v/v) was prepared according to the scheme shown in Fig. 1. To a DMF solution of Boc-Gly-Ile-Gly-OH (22 mg) and TFA Glu(OSp)-Val-Leu-Lys(Z)-OMe (71 mg), DCC (16 mg), N-hydroxybenzotriazole (13 mg) and triethylamine (9.1 μl) were added at 0°C. After stirring overnight at room temperature, the solution was concentrated, and ethyl acetate was added. The precipitate was filtered off, and the filtrate was evaporated. The residue was purified by elution through a Sephadex LH-20 column using DMF as an eluant. The main fraction was subjected to elution through a silica gel column using a mixture of ethyl acetate and methanol (7:3, v/v) as eluant, and the fraction of $R_f = 0.85$ was collected.

The protected heptapeptide (20 mg) was dissolved in HBr/acetic acid (25 wt%, 200 μl) containing 1% anisole. After standing for 1 h, diethyl ether was added. The precipitate was purified by elution through a Sephadex LH-20 column using methanol as an eluant. The main fraction was further purified by elution through a reverse-phase HPLC column (COSMOSIL 5C18 column) using a mixture of variable contents of acetonitrile (20%–100%) and water containing 0.1% TFA. The final product was identified by amino acid analysis.

2.2. Measurements

Absorption and fluorescence spectra were recorded on a Jasco Ubest-50 UV/VIS spectrophotometer and a Hitachi MPF-4 fluorescence spectrophotometer, respectively. A high-pressure mercury lamp (100 W) was used for light source,

and filters of UV-31, UVD-36C, and solution filter of nickel sulfate were used for UV irradiation, and Y50 for visible-light irradiation.

2.3. Binding constant

M1Sp, M4Sp or M7Sp, was incubated with DPPC multilamellar vesicles (MLV, potassium borate buffer, pH 9.0, 2 mM) at a molar ratio of [DPPC]/[peptide] = 100. The borate buffer was used because of the following fluorescamine assay. For the other experiments in the buffer or DPPC dispersion, a Hepes buffer solution containing 0.1 M NaCl was used as standard. Under these conditions, melittin is shown to exist predominantly as monomer [32]. The dispersion was subjected to visible-light irradiation to convert merocyanin group completely to spiropyran group, and was divided into two portions. One portion was irradiated by UV light, and both portions were centrifuged (15000 rpm, 6 min) to sediment the peptide bound to MLV. Concentrations of the peptides in the supernatant were determined by the fluorescence method using fluorescamine for labeling free amino groups. Binding constants of melittin fragments to DPPC MLV were calculated according to

$$K = \frac{[\text{peptide}]_0 - [\text{peptide}]_{sup}}{[\text{peptide}]_{sup}[\text{DPPC}]}, \quad (1)$$

where $[\text{peptide}]_0$ and $[\text{peptide}]_{sup}$ represent the initial concentration of peptide and the concentration of peptide in the supernatant, respectively.

2.4. Thermal isomerization

A peptide solution (1.0×10^{-5} M) was subjected to UV irradiation for 30 min. The changing absorption intensity at the maximum wavelength of merocyanin group was followed at 25°C. Thermal isomerization was also examined in the presence of DPPC small unilamellar vesicles (SUV), which were prepared as follows. Dried DPPC was dispersed in a Hepes-buffered solution (10 mM Hepes, 0.1 M NaCl, pH 7.4). The dispersion was sonicated at 50°C under N_2 atmosphere. After centrifugation at 100000 g, SUV were obtained.

2.5. Leakage of 5/6-carboxyfluorescein (CF) encapsulated in DPPC vesicles

CF-encapsulated DPPC vesicles were prepared by the method reported by Barbet et al. [33]. The excitation and monitor wavelengths of CF were 470 and 515 nm, respectively. Complete release of CF was determined by addition of Triton X-100 (0.3 wt%). Peptides having a spiropyran form in ethanol were kept in dark, and added to the vesicle dispersion keeping the ethanol content less than 1% of the total volume. The temperature was regulated by circulating water thermostated at 25°C.

3. Results and discussion

3.1. Binding of melittin fragment to DPPC membrane

Melittin fragment taking either spiropyran or merocyanin form was incubated with DPPC vesicles, and the concentration of the peptide in aqueous phase was determined. The binding constant of melittin fragment to DPPC membrane was calculated according to the method described in section 2, and is summarized in Table 1 together with the relative amount of melittin fragment bound to the membrane. M1Sp having either spiropyran or merocyanin was practically insoluble in lipid membrane. In contrast, M4Sp was not detected in the aqueous phase, indicating a high affinity for DPPC membrane due to the

Table 1
Binding of melittin fragments to DPPC membrane [a]

Fragment	Without UV irradiation		After UV irradiation	
	K (M^{-1})	% [b]	K (M^{-1})	% [b]
M1Sp	126	0.75	120	0.71
M4Sp	large	> 99	large	> 99
M7Sp	116000	87.4	11600	41.1

[a] [DPPC] = 60 μM, [peptide] = 0.6 μM, at 25°C.
[b] The percentage of peptide distributed to DPPC membrane.

Table 2
Thermal isomerization of merocyanin-containing peptides in various media

Fragment [a]	Solvent [b]	F	k_{fast}	$1-F$	k_{slow}	λ_{max} (nm)
HO-Sp	EtOH	1.0	0.110	–	–	540
BocM1Sp	97% DOx	1.0	4.33			572
BocM1Sp	90% DOx	1.0	0.642			562
BocM1Sp	80% DOx	1.0	0.186			551
BocM1Sp	60% DOx	1.0	0.025			548
BocM1Sp	50% DOx	–	< 0.001			536
M1Sp	EtOH	0.261	0.638	0.739	0.063	543
M1Sp	DPPC	–	< 0.001	–	–	–
M4Sp	EtOAc	0.497	2.59	0.503	0.676	–
M4Sp	EtOH	0.258	1.38	0.742	0.089	550
M4Sp	DPPC	0.163	0.244	0.837	0.018	565–575
M4Sp	DGOH	0.364	0.072	0.636	0.003	–
M7Sp	EtOH	0.097	0.551	0.903	0.006	520
M7Sp	DPPC	0.836	0.135	0.164	0.024	545

[a] Concentration of melittin fragments was 0.1 μM.
[b] EtOH, DOx, EtOAc, and DGOH represent ethanol, dioxane, ethyl acetate, and diglycerol, respectively.
See the text about F, k_{fast}, and k_{slow}.

hydrophobic property. On the other hand, M7Sp showed different membrane affinities depending on the nature of the chromophoric group (spiropyran or merocyanin). M7Sp having a spiropyran group possessed a high-binding constant. However, upon UV-light irradiation about half of the bound peptides was dissociated from the lipid membrane. The merocyanin form is zwitterionic and makes the peptide more hydrophilic, resulting in a reduced affinity for phospholipid membrane.

3.2. Location of merocyanin-carrying peptide in lipid membrane as judged by thermal isomerization

The merocyanin form is sensitive to the polarity of microenvironment, and the maximum-absorption wavelength (λ_{max}) shifts towards shorter wavelength as the environmental polarity increases [10]. λ_{max} of BocM1Sp was measured in different compositions of dioxane/water mixtures (Table 2). A blue-shift of λ_{max} is evidently observed with increasing water content in the solution.

More drastic change was observed in the rate of thermal isomerization from merocyanin to spiropyran of BocM1Sp. The isomerization rate in 60% dioxane was 1/173-fold of that in 97% dioxane, and the isomerization in 50% dioxane was very low. The faster isomerization in less polar media can be explained in terms that the zwitterionic form of merocyanin is not stable in apolar media.

The kinetic studies of thermal isomerization of M1Sp, M4Sp, and M7Sp, however, revealed that the decay of merocyanin content could not be expressed by a single-component first-order reaction (Fig. 2). In these cases, two, fast and slow, processes should be taken into consideration in

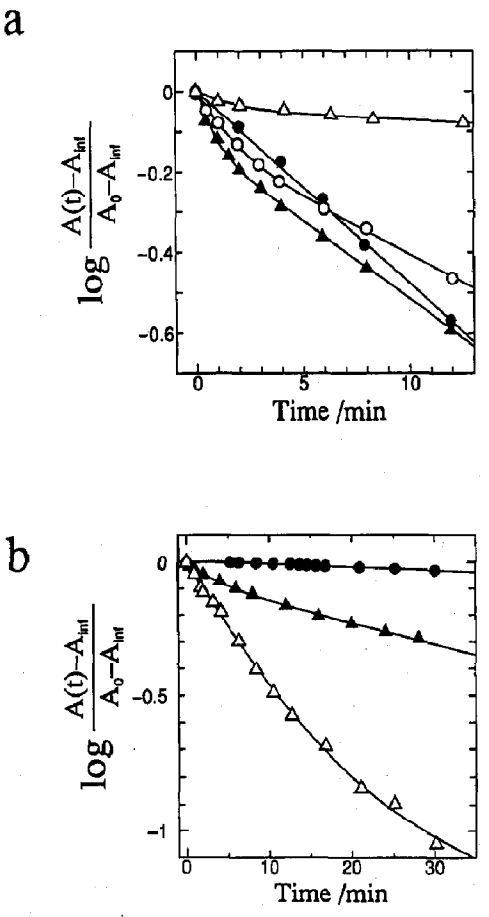
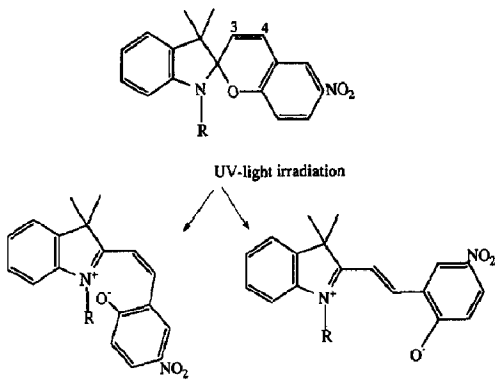


Fig. 2. First-order plots for the thermal isomerization of the merocyanin group of HO-Sp (●), M1Sp (○), M4Sp (▲), and M7Sp (△), in ethanol (a) and in the presence of DPPC vesicles (b). [peptide] = 10 μM. [DPPC] = 1.6 mM.



Scheme 1. Two forms of merocyanin on the basis of cis–trans isomerization.

the analysis of the experimental data shown in Fig. 2, which were well fitted by

$$\frac{A_t - A_{\inf}}{A_0 - A_{\inf}} = F \exp(-k_{\text{fast}} t) + (1 - F) \exp(-k_{\text{slow}} t), \quad (2)$$

where A_0, A_t, and $A_{\inf}$ are the absorbance at $\lambda_{\max}$ at time zero, t, and infinite, respectively. F is the fraction of peptides having the decay rate constant of k_{fast}. k_{fast}, k_{slow}, and F are summarized in Table 2.

Occurrence of two components in the kinetics of thermal isomerization indicates two different states of the merocyanin group in these peptides. Two possible forms of merocyanine are a contact ion pair and a solvent separated ion pair or free ions of merocyanin, which might be brought about by cis–trans isomerization of C^3–C^4 (numbering in a spiropyran form) double bond (Scheme 1). In the merocyanin form, other ion species in the peptide molecule such as N-terminal ammonium group might interact with the anionic site of merocyanin. The thermal isomerization recombination in the form of the contact ion pair must be faster than in the form of the free ions, because in the former two sites for recombination are in a close proximity. For example, the fraction of k_{fast} species of M4Sp is 49.7% in ethyl acetate, which should be ascribed to the contact ion-pair species. The fraction decreases to 25.8% in ethanol. The increasing solvent polarity may produce the free ions more than the contact ion pair by UV irradiation, since the former species should be stabilized in the polar environment. It should be noted that the conversion between the free ions and the contact ion pair is hindered, because it requires the cis–trans isomerization. It is, therefore, conceivable that the microenvironment of a merocyanin group is probed from the fraction of k_{fast} species. The higher fraction of k_{fast} species is, the more hydrophobic the microenvironment of the merocyanin group should be.

When an aqueous solution of M1Sp was irradiated with UV lamp, the absorption intensity of the merocyanin group increased slightly. The rate of thermal isomerization of the merocyanin group was extremely slow. In the presence of DPPC vesicles, too, the absorption intensity of the merocyanin group of M1Sp produced by UV-light irradiation did not decay significantly, indicating that M1Sp stayed in the aqueous phase. The result is consistent with the binding assay, which showed that the fraction of M1Sp bound to DPPC vesicles was less than 1%. M4Sp showed a high proportion of k_{slow} species (83.7%), indicating that the merocyanin group is located in the polar region of phospholipid membrane. On the other hand, in the case of M7Sp, the k_{fast} species was predominant (83.6%), indicating that the merocyanin group is located in the hydrophobic region of lipid membrane. Although the affinity of M7Sp for phospholipid membrane was lower than that of M4Sp, the merocyanin group of M7Sp was found to reside in the hydrophobic core region of lipid bilayer membrane. It is therefore concluded that the location of the merocyanin group in lipid membrane is determined by the interaction between the peptide moiety and the phospholipid membrane.

3.3. Maximum absorption wavelength of merocyanin group in DPPC vesicles

Spectral changes with time of M4Sp and M7Sp under UV-light irradiation are shown in Fig. 3. The absorption due to merocyanin group of M4Sp increased the intensity and underwent a blue-shift. The blue-shift of M4Sp from 575 to 565 nm suggests that the location of the merocyanin group

in the lipid membrane changed from the hydrophobic core region of lipid bilayer membrane toward the hydrophilic membrane surface due to increased hydrophilicity upon UV-light irradiation. On the other hand, M7Sp increased the intensity of maximum absorption with very little shift upon UV-light irradiation. Since about 60% of M7Sp taking a merocyanin form stayed in aqueous phase (Table 1), the contribution of this fraction dominates over the absorption spectra, resulting in very little shift of maximum absorption.

3.4. Leakage of CF entrapped in DPPC vesicles

The effect of photoisomerization of spiropyran group on the interaction of the spiropyran-carrying peptide with phospholipid membrane was examined by CF leakage from DPPC vesicles. The

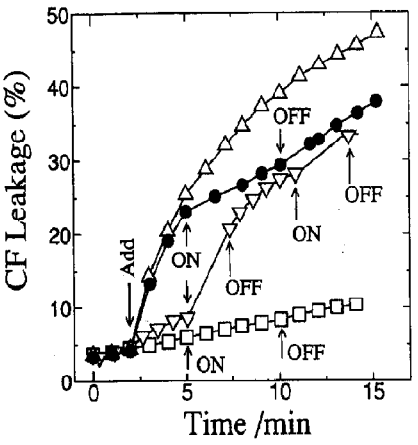


Fig. 4. CF leakage from DPPC vesicles induced by the addition of M1Sp (□), M4Sp (▽), and M7Sp (●). Arrows of Add, ON, and OFF represent the addition of the peptide, UV-light irradiation, and visible-light irradiation, respectively. (△) represents the effect of M7Sp addition without UV-light irradiation. [DPPC] = 60 μM. [peptide] = 0.2 μM. Temperature = 25°C.

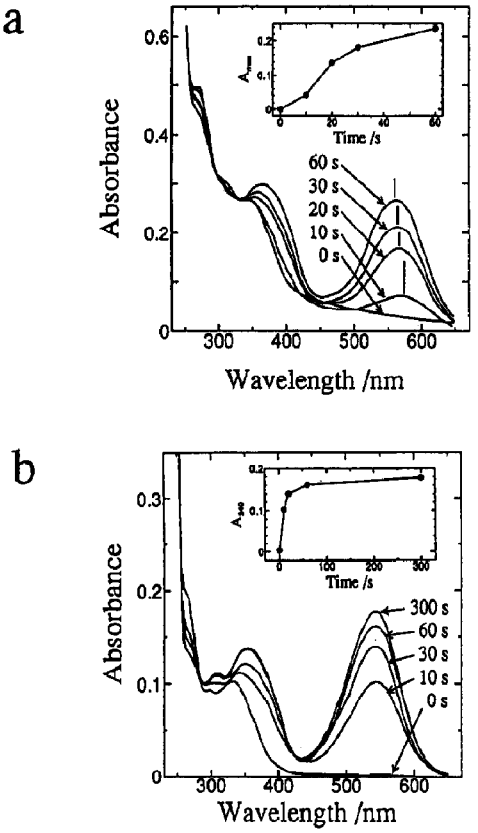


Fig. 3. Absorption spectra under UV-light irradiation of M4Sp (a) and M7Sp (b) in the presence of DPPC vesicles. [peptide] = 8.5 μM. [DPPC] = 0.64 mM.

experimental results are shown in Fig. 4. UV-light irradiation did not influence CF leakage by the M1Sp addition, which did not bind to the membrane. M4Sp taking a spiropyran form did not influence the leakage so much, although most of the peptide added was distributed in the membrane. However, a drastic increase of CF leakage was observed upon UV-light irradiation, and it slowed down by visible-light irradiation. On the other hand, the addition of M7Sp taking a spiropyran form induced a significant CF leakage, which was slowed down by UV-light irradiation. These results can be explained as follows. The spiropyran group of M4Sp is converted to a zwitterionic merocyanin group upon UV-light irradiation, and an amphiphilic property of M4Sp is strengthened. Amphiphilic peptides disturb the membrane structure resulting in enhancement of CF leakage [34]. M7Sp possesses an amphiphilicity more or less in either spiropyran or merocyanin form, and the distribution of M7Sp disturbs the membrane structure significantly. However, the fraction of M7Sp bound to the membrane was reduced to the half upon UV-light irradiation as described before, resulting in the suppression of the CF leakage. These results demonstrate the photocontrol of peptide activity

on phospholipid membrane using a nonnatural amino acid, Glu(OSp).

Merocyanin is a zwitterionic molecule produced by the ring-opening isomerization of spiropyran, which leads to different interaction of the peptide with phospholipid membrane. Although the charge effect on the interaction cannot be neglected, merocyanin-carrying peptides retain the property of unmodified peptides for lipid membrane as shown here. Therefore, merocyanin-carrying peptides are very useful for investigation of interactions between peptides and phospholipid membrane.

4. Conclusion

We have synthesized melittin 1–7 fragment with Glu(OSp) at the fourth position and a fully protected melittin 4–7 fragment with Glu(OSp) at the N-terminal position. It was shown that thermal isomerization from merocyanin to spiropyran form sensitively probes the nature of the microenvironment of the merocyanin group in the lipid membrane. The membrane affinity of M4Sp was higher than M7Sp due to hydrophobic property of the former, but the location of the merocyanin group of M7Sp was close to the core of membrane than that of M4Sp. This observation indicates that melittin 1–7 sequence plays an important role in incorporation of melittin into phospholipid membrane.

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