

# Photoinduced Structural and Functional Changes of an Azobenzene-Containing Amphiphilic Sequential Polypeptide

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**ABSTRACT:** We prepared a photoresponsive amphiphilic sequential polypeptide,  $\bar{M}_n = 10\,630$ , composed of two amphiphilic  $\alpha$ -helical copolypeptides, poly( $\gamma$ -methyl L-glutamate)-co-(L-glutamic acid), jointed by an azobenzene as a model of photoreceptors. We investigated the photoinduced structural and functional changes of the photoresponsive polypeptide in an aqueous solution and a dipalmitoylphosphatidylcholine bilayer membrane system, respectively. In the dark, the photoresponsive amphiphilic sequential polypeptides formed a micelle in aqueous solution and a transmembrane bundle in the bilayer membrane, respectively. The transmembrane bundle in the membrane acted as an ion permeable path. UV light irradiation induced a bending of the polypeptide by a trans–cis photoisomerization of the azobenzene moiety. The photoinduced structural changes of the polypeptide resulted in a disaggregation of the micelle in aqueous solution and a destabilization of the transmembrane bundle, respectively. The behavior arose from a photoinduced denaturation of the amphiphilic character of the polypeptides. After removal of the light, the initial micellar aggregate in aqueous solution did not recover. However, the polypeptides in the membrane returned to the original transmembrane bundle structure. As a result, photoinduced reversible structural changes of the polypeptide in the bilayer membrane could regulate a transmembrane ion transport.

## Introduction

It has been recognized that the high-order structural changes of membrane proteins induced by external stimulation is an essential process in life phenomena such as signal transduction. For example, the first step of the signal transduction in photoreceptors is caused by the structural changes of G-protein induced by a photoisomerization of retinal in rhodopsin.<sup>1</sup> Recently, amphiphilic polypeptides with  $\alpha$ -helical bundles were synthesized as a model of functional proteins.<sup>2,3</sup> The investigations on stimulus–response coupling in these artificial model systems should provide major information in understanding the signal transduction mechanism.

In a previous study, we reported a preparation of an amphiphilic sequential polypeptide by a monolayer reaction method<sup>4</sup> and a bundle arrangement of the polypeptides in bilayer membranes.<sup>5–7</sup> The polypeptides formed an amphiphilic  $\alpha$ -helix that is hydrophilic on one face (which consists of L-glutamic acid side chains) and hydrophobic on the opposite face (which comprises  $\gamma$ -methyl L-glutamate side chains). The amphiphilic  $\alpha$ -helices were incorporated into the bilayer membrane to form the transmembrane bundle with a hydrophilic inner pore surrounded by hydrophilic L-glutamic acid side chains and a hydrophobic exterior, composed of hydrophobic  $\gamma$ -methyl L-glutamate, in contact with the hydrocarbon region of the membrane. The transmembrane bundle consisting of the amphiphilic sequential polypeptides provided an ion permeable path through the membrane.

Here we report photoinduced high-order structural changes of a photoresponsive amphiphilic sequential polypeptide, consisting of two amphiphilic  $\alpha$ -helical segments jointed by an azobenzene residue. The polypep-

ptide formed a micellar aggregate in aqueous solution and a transmembrane bundle which provided an ion permeable path in a lipid bilayer membrane by self-association in the dark. Photoirradiation of the aggregate induced a disaggregation of the micelle in aqueous solution and a destabilization of the transmembrane bundle in the bilayer membrane, blocking the ion permeable path. This could be explained by the bending of the polypeptide due to the trans–cis photoisomerization of the azobenzene. Preliminary results of the photocontrol of the structure of the photoresponsive amphiphilic sequential polypeptide were reported previously.<sup>8</sup>

## Experimental Section

**Materials.** A poly( $\gamma$ -methyl L-glutamate) with an azobenzene moiety in the main chain (MAzoM) was obtained by the polymerization of the *N*-carboxyanhydride of L-glutamic acid  $\gamma$ -methyl ester in dimethylformamide (DMF) solution with *p,p'*-diaminoazobenzene as an initiator. The molar ratio of the anhydride to the initiator was 60. The polymerization occurred at room temperature over 24 h.<sup>9</sup> The DMF solution was poured into water, and precipitated MAzoM was purified by reprecipitation from DMF into water until no unreacted diaminoazobenzene was detected spectroscopically. A number average molecular weight of 11 000 was estimated from the molar ratio of the azobenzene moiety to the  $\gamma$ -methyl L-glutamate residues of MAzoM. The ratio was determined by the absorbance at 375 nm on the basis of the molar extinction coefficient of the azobenzene in MAzoM dimethylformamide solution.

A photoresponsive amphiphilic sequential polypeptide, am.-MAzoM, was prepared by a selective saponification of MAzoM side chains according to a monolayer reaction method, which was described in detail elsewhere.<sup>4</sup> A known amount of MAzoM dissolved in 1,2-dichloroethane was placed at an air–water interface in a L-B trough (Nippon Laser & Electronics Lab., NL-LB-240-MWC) with a syringe. The solution spread over the interface, and the solvent evaporated, leaving the MAzoM monolayer. The area occupied by the monolayer was decreased by moving a hydrophobic barrier to form a solid condensed state of the monolayer. When the area per mono-

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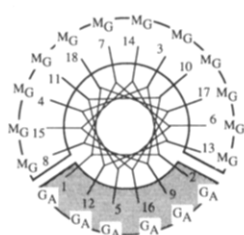
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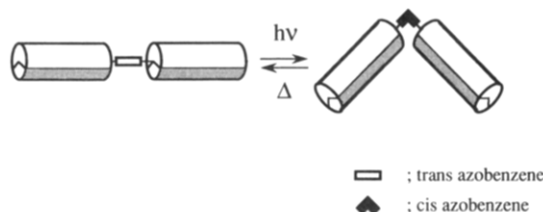
Chart 1. Schematic Picture of am.-MAzoM

Top view

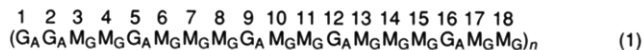


shaded surface represents location of L-glutamic acid side chains,  $G_A$ , and unshaded surface is  $\gamma$ -methyl L-glutamate,  $M_G$ .

Side view



mer residue reached  $15 \text{ \AA}^2$ , aqueous potassium hydroxide was injected into the aqueous phase beneath the solid condensed monolayer to selectively saponify MAzoM side chains oriented into the aqueous phase. The final concentration of KOH in the aqueous phase was 0.1 M. After 10 min, excess hydrochloric acid was added to convert the copolypeptide to its acidic form. The L-glutamic acid content of the resulting am.-MAzoM was confirmed by  $^1\text{H-NMR}$  spectra of am.-MAzoM in trifluoroacetic acid. The spectra showed the decrease in the peak at 3.9 ppm associated with the side chain  $-\text{OCH}_3$  group. From the degree of the decrease in the  $-\text{OCH}_3$  peak area, the L-glutamic acid content of am.-MAzoM was estimated to be 34 mol %. The value of L-glutamic acid content, 34 mol %, suggests the possibility that the  $\gamma$ -methyl L-glutamate side chains with an angle of  $120^\circ$  ( $120^\circ/360^\circ = 1/3$ ) on the cross-section of the  $\alpha$ -helix could be saponified along the helix axis (Chart 1). On the basis of Chart 1, a hypothetical sequence of the polypeptide components could be written as



where  $M_G$  and  $G_A$  denote  $\gamma$ -methyl L-glutamate and L-glutamic acid residues, respectively. The sequence of the polypeptide components was determined by enzymic hydrolysis of am.-MAzoM with *Staphylococcus aureus*, Strain V8 protease. This enzyme specifically cleaves peptide bonds<sup>10</sup> on the carboxyl-terminal side of glutamic acid. On the basis of the hypothetical sequence in eq 1, the hydrolysis of am.-MAzoM by the protease should yield unit sequences  $M_G M_G M_G G_A$  and  $M_G M_G G_A$  and  $G_A$  monomer. From a gel filtration chromatographic analysis<sup>4</sup> (Wakopak WB-G-30 column, Wako-junyaky-kougyo Co., Ltd.) of am.-MAzoM hydrolyzed with the protease, three kinds of oligopeptide were generated in the enzymic hydrolysis. The molecular weights of the oligopeptides could be estimated to be 623, 417, and 132 from the calibration curve of poly(ethylene oxide). These molecular weights were rather close to those calculated for  $M_G M_G M_G G_A$  (576),  $M_G M_G G_A$  (433), and  $G_A$  (147). Therefore, the polypeptide component of am.-MAzoM obtained has the sequence shown in eq 1.

Anilidonaphthalene-modified am.-MAzoM (AN-am.-MAzoM) was obtained by condensation of the L-glutamic acid residue of am.-MAzoM and 1-amino-8-anilidonaphthalene in dimethylformamide, containing dicyclohexylcarbodiimide and *N*-hydroxybenzotriazole.<sup>11</sup> The anilidonaphthalene content, 3 mol %, in the polypeptide was determined from the fluorescence intensity at 470 nm of the DMF solution of AN-am.-MAzoM on the basis of the fluorescence intensity of a model compound, 1-propionamido-8-anilidonaphthalene (pro-AN). The excitation wavelength was 350 nm.

Dipalmitoylphosphatidylcholine (DPPC) vesicles containing am.-MAzoM were prepared as follows. DPPC and am.-MAzoM were each dissolved in dimethylformamide. These solutions

were mixed and poured into a glass flask, forming a thin film on the inner surface of the flask after evaporating the solvent. Buffer solution (50 mM [*N*-(2-hydroxyethyl)piperazin-*N'*-yl]-2-ethanesulfonic acid (HEPES)-2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), pH 6.8) was added to the flask, and was sonicated by a Branson Sonifier Model 250 under a nitrogen atmosphere at  $0^\circ\text{C}$  in the dark to prepare the vesicles. Dynamic light-scattering measurements (Otsuka Electronics Co., Ltd. DLS-700) showed that the diameter of the vesicle was 90 nm.

## Methods

**Spectroscopic Measurements.** Absorption spectra of am.-MAzoM in an aqueous solution containing 0.1 M KCl at pH 6.9 and in a DPPC vesicle solution were measured with a spectrophotometer (Hitachi, 330) to monitor the photoisomerization of the azobenzene moiety of am.-MAzoM. The concentration of am.-MAzoM in the aqueous solution was  $1.92 \times 10^{-6}$  M. The concentration of DPPC in the aqueous solution was 0.5 mg/mL. The molar ratio of the am.-MAzoM molecules to DPPC was fixed at  $7.4 \times 10^{-3}$ .

Fluorescence spectra of AN-am.-MAzoM in the vesicle and pro-AN in various solvents were measured with a spectrofluorometer (Jasco FP-777). The concentration of DPPC was 0.2 mg/mL. The molar ratio of the AN-am.-MAzoM molecule to DPPC was fixed at  $7.4 \times 10^{-4}$ . The concentration of pro-AN was fixed at  $3.0 \times 10^{-5}$  M. The excitation wavelength was 350 nm. Fluorescence spectra of anilidonaphthalene in am.-MAzoM aqueous solution containing 0.1 M KCl at pH 6.9 and in various solvents were also measured. The concentrations of anilidonaphthalene and am.-MAzoM were fixed at  $4.2 \times 10^{-6}$  and  $5.0 \times 10^{-6}$  M, respectively. The excitation wavelength was 348 nm.

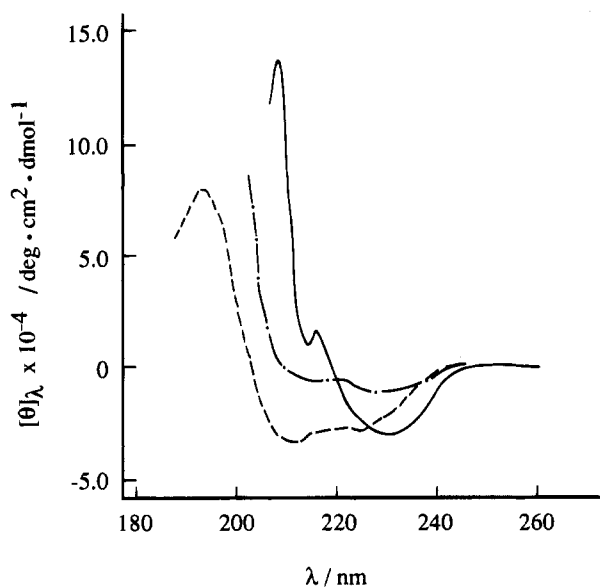
Circular dichroism spectra of am.-MAzoM in trimethyl phosphate (TMP), an aqueous solution containing 0.1 M KCl at pH 6.9, and a DPPC vesicle solution, respectively, were measured with a Jasco J-600 spectropolarimeter. The secondary structure was estimated from the molar ellipticity. The concentration of am.-MAzoM was fixed at  $1.92 \times 10^{-6}$  M. The concentration of DPPC was 0.2 mg/mL.

**Permeation Measurements.** DPPC vesicles containing am.-MAzoM in the 50 mM HEPES-Tris buffer solution, pH 6.8, containing 0.1 M potassium gluconate were prepared by sonication in a manner similar to that above. The concentration of DPPC was 10 mg/mL. The molar ratio of am.-MAzoM molecules to DPPC was  $7.4 \times 10^{-4}$ . A 200  $\mu\text{L}$  aliquot of the aqueous suspension of DPPC vesicles containing am.-MAzoM in the membrane and potassium gluconate in the interior thus obtained was then passed through an Econo-Pac 10DG column (Bio-Rad Laboratories, bed volume 10 mL) by using 0.1 M sodium gluconate in 50 mM HEPES-Tris buffer, pH 6.8, as an eluting buffer to remove the external potassium gluconate. The fractions containing the vesicles were collected and added to the buffer solution containing 0.1 M sodium gluconate. The total volume was 50 mL. The resulting vesicles had an ionic concentration gradient between the interior (0.1 M potassium ion) and exterior (0.1 M sodium ion) under isotonic conditions. The amount of ion exchange across the vesicular bilayer before and after UV light irradiation was determined with an ion meter (Orion Research, microprocessor ionalyzer/901) at  $20^\circ\text{C}$ .

**Irradiation.** The light source used for UV light (250 nm  $< \lambda < 380$  nm) irradiation was a 500 W super-high-pressure mercury lamp (Ushio, USH-500D) equipped with a Toshiba UV-D33S glass filter.

## Results and Discussion

**Conformation of a Photoresponsive Amphiphilic Sequential Polypeptide.** We have already reported that several amphiphilic sequential polypeptides associate to form high-order micellar aggregates in aqueous solution<sup>4</sup> and transmembrane bundles in bilayer membranes.<sup>5,6</sup> Here we demonstrate a photoinduced structural control of aggregates composed of photoreponsive amphiphilic sequential polypeptides, am.-MA-

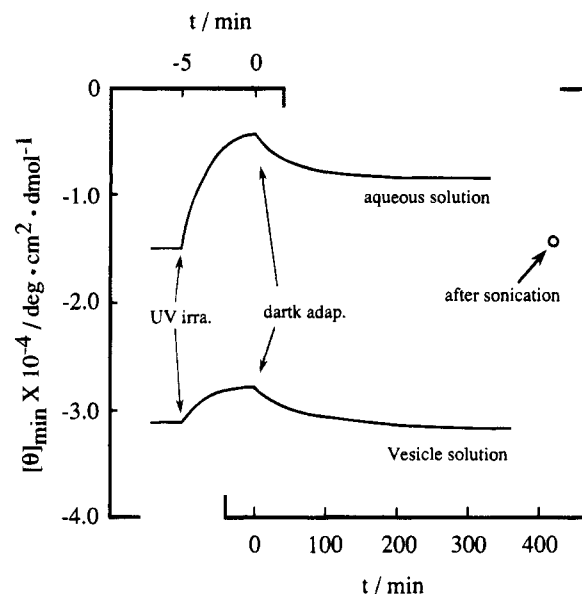


**Figure 1.** CD spectra of am-MAzoM in TMP (---), an aqueous solution containing 0.1 M KCl at pH 6.9 (- · -), and a DPPC vesicle (—).

zoM, which have an azobenzene moiety in the main chain. An azobenzene is known to undergo a trans-cis photoisomerization, involving changes in the geometry and polarity of the photochrome. The overall geometry of the azobenzene changes from planar to nonplanar,<sup>12,13</sup> and the dipole moment increases from 0.5 to 3.1 D.<sup>14</sup> In our case, the isomerization of the azobenzene moiety of the am-MAzoM main chain induced a bending of the am-MAzoM polypeptide.

The conformation of am-MAzoM was characterized by circular dichroism (CD). Figure 1 shows the CD spectra of am-MAzoM in trimethyl phosphate (TMP), in an aqueous solution containing 0.1 M KCl at pH 6.9, and in a DPPC vesicle, respectively. The CD spectra of the polypeptide in TMP exhibited two negative bands at 208 and 222 nm typical of the right-handed  $\alpha$ -helix. The molar ellipticity at 222 nm,  $[\theta]_{222}$ , was  $-28\,181\text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ . On the other hand, the CD spectra of am-MAzoM in aqueous solution and in the vesicle were red-shifted to 227 and 228 nm, respectively, and the 208 nm band was reduced. The same distortions of the spectra have been shown with aggregated  $\alpha$ -helical polypeptides.<sup>15–17</sup> Thus, am-MAzoM has an  $\alpha$ -helical conformation and several amphiphilic  $\alpha$ -helical rods self-associated to form an aggregate in both aqueous solution and the vesicle. This probably means that in the bilayer membrane the am-MAzoMs are arranged so that all side chains facing the outside are hydrophobic and the inside are hydrophilic (reversed micelle), while in water the opposite assembly occurs (micelle). Furthermore, the  $[\theta]_{222}$  values of am-MAzoM in an aqueous solution and in the DPPC vesicle were  $-10\,025$  and  $-20\,793\text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ , respectively. This implied that the  $\alpha$ -helical conformation of am-MAzoM was stabilized in the DPPC vesicle membrane.

UV light irradiation at 20 °C induced a trans to cis photoisomerization in the azobenzene moiety of am-MAzoM. The main absorption band at 375 nm assigned to the trans  $\pi$ - $\pi^*$  transition decreased, indicating that the azobenzene moiety in the main chain of am-MAzoM can be isomerized from trans to cis form. From the changes in the absorbance at 375 nm, it was estimated that 60% of the trans form was converted to the cis isomer. To elucidate the effect of the UV light irradiation



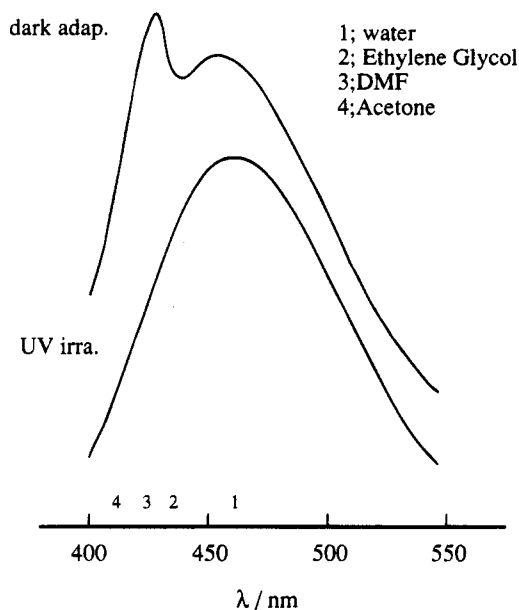
**Figure 2.** Photoinduced changes in the minimum ellipticity,  $[\theta]_{\min}$ , at 227 and 228 nm of am-MAzoM in an aqueous solution containing 0.1 M KCl at pH 6.9 and the DPPC vesicle, respectively.

on the conformation of am-MAzoM, we measured the changes in  $[\theta]_{227}$  (in the aqueous solution) and  $[\theta]_{228}$  (in the vesicle) of am-MAzoM, upon UV light irradiation and dark adaptation (Figure 2). In the aqueous solution,  $[\theta]_{227}$  decreased on UV light irradiation and, after removal of the light, the value increased and attained an equilibrium level, but the original value was not recovered, unless the solution was sonicated. On the other hand, in the bilayer membrane system, the  $[\theta]_{228}$  value decreased on UV light irradiation and completely recovered its initial value upon dark adaptation. These results suggested that the UV light irradiation induced the denaturation of amphiphilic character of am-MAzoM owing to the trans-cis photoisomerization of the azobenzene moiety resulting in the destabilization of the aggregate. The difference between the recovery behavior in the aqueous solution and the bilayer membrane system could be explained as follows. In the aqueous solution, UV light irradiation induced a precipitation of am-MAzoM due to the disaggregation of the aggregate. On the other hand, in the bilayer membrane system, the am-MAzoM whose azobenzene moiety was cis could be incorporated in the membrane, so that re-association of am-MAzoM could occur upon dark adaptation.

#### Structure of Aggregates Composed of Photoreponsive Amphiphilic Sequential Polypeptides.

The structure of am-MAzoM aggregates in an aqueous solution and in DPPC vesicles was also studied by fluorescence. It is well-known that fluorescence characteristics, such as emission maxima and fluorescence intensity, of anilino-naphthalene derivatives are very sensitive to the environmental polarity.<sup>18</sup> The emission maxima of anilino-naphthalene derivatives shift to higher wavelengths with an increase solvent polarity.

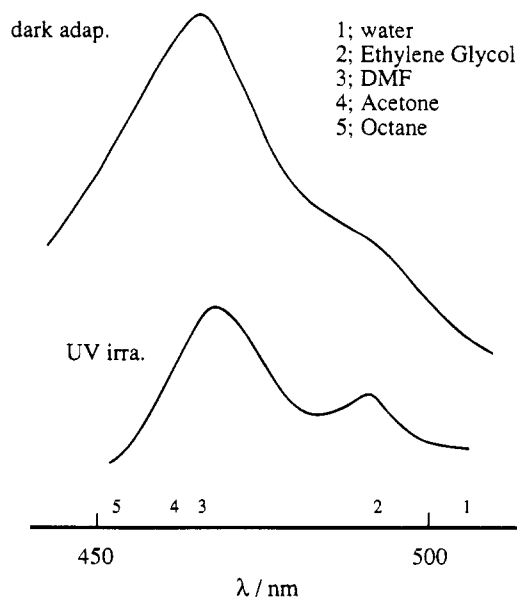
In an aqueous solution, the formation of an am-MAzoM aggregate was followed by the incorporation of a hydrophobic fluorescence probe, anilino-naphthalene, into the aggregate. Figure 3 shows the fluorescence spectra of anilino-naphthalene in an am-MAzoM aqueous solution containing 0.1 M KCl at pH 6.9 before and after UV light irradiation. The excitation wavelength of anilino-naphthalene was 348 nm. Also indicated are



**Figure 3.** Changes in fluorescence spectra of anilinonaphthalene in an am-MAzoM aqueous solution containing 0.1 M KCl at pH 6.8.

the emission maxima of anilinonaphthalene in various solvents. Upon dark adaptation, two emission maxima were observed at 429 and 455 nm. The emission of anilinonaphthalene at 429 nm corresponded to that of a medium with a polarity between that of acetone and DMF, whereas that of 455 nm is characteristic of the emission in water. The emission at 429 nm implied that anilinonaphthalene was incorporated into the hydrophobic region in an am-MAzoM aqueous solution, suggesting that anilinonaphthalene was incorporated into the interior of an am-MAzoM micelle. The emission at 455 nm was assigned to the emission of anilinonaphthalene in the external aqueous solution. Upon UV light irradiation, the emission band at 429 nm vanished. This suggested that the photoisomerization of the azobenzene moiety of am-MAzoM induced the disaggregation of the micelle, with a release of anilinonaphthalene from the micelle to the external aqueous phase. The fluorescence spectra of anilinonaphthalene in am-MAzoM aqueous solution did not change upon dark adaptation, thus the photoinduced structural changes of am-MAzoM micelle were irreversible.

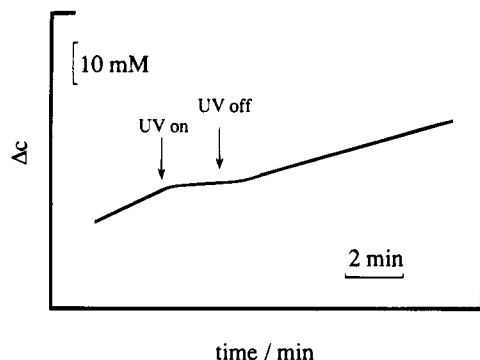
We investigated the aggregated structure of am-MAzoM in a DPPC bilayer membrane using anilinonaphthalene-modified am-MAzoM, AN-am-MAzoM. It should be noted that the fluorophore was necessarily introduced into the hydrophilic face of the amphiphilic  $\alpha$ -helix. We estimated the environmental polarity around the hydrophilic face of the am-MAzoM aggregate from the wavelength of emission maxima of AN-am-MAzoM in the bilayer membrane. Figure 4 shows fluorescence spectra of AN-am-MAzoM in a DPPC vesicle before and after UV light irradiation. The emission maxima of a model compound, pro-AN, in various solvents are also shown in this figure. The excitation wavelength of AN-am-MAzoM was 350 nm. Before UV light irradiation, an emission maximum was observed at 466 nm. The environmental polarity of the fluorescent-labeled hydrophilic face of am-MAzoM in the vesicle corresponded to that of dimethylformamide. This implies that the hydrophilic face of the amphiphilic  $\alpha$ -helix is accessible to neither the hydrocarbon chains of DPPC nor the aqueous phase. A simple understand-



**Figure 4.** Changes in fluorescence spectra of AN-am-MAzoM in a DPPC vesicle.

ing of the intermediate polarity around the hydrophilic face of am-MAzoM is that the face is in contact with the hydrophilic faces of other am-MAzoMs in the bilayer membrane. Thus, am-MAzoMs were incorporated to the DPPC bilayer membrane and associated to form the transmembrane bundle whose internal surface consists of hydrophilic glutamic acid side chains and external surface, hydrophobic methyl L-glutamate side chains, in contact with the hydrocarbon region of the DPPC membrane. In addition, we have reported that the amphiphilic sequential polypeptides, which are the polypeptide components of am-MAzoM, formed the transmembrane bundle in the bilayer membrane with freeze-fracture electron microscopy.<sup>5</sup> The diameter of the bundle was ca. 40 Å. This may support the bundle formation of am-MAzoM, since am-MAzoM consisted of the same amphiphilic sequential polypeptides. On the other hand, UV irradiation induced a change in the fluorescence spectra of AN-am-MAzoM in the DPPC vesicle; i.e., the emission maximum at 466 nm shifted toward 469 nm, and a maximum could clearly be observed at 490 nm. This distortion of the fluorescence spectra could be explained as follows. The photoisomerization of the azobenzene moiety in am-MAzoM destabilized in transmembrane bundle so that the hydrophilic face of am-MAzoM was partly exposed to the aqueous solution. This change was reversible.

**Photoinduced Ion Permeation Changes through the Bilayer Membrane.** Figure 5 shows the rate of potassium ion permeation through the bilayer membrane containing am-MAzoM from the vesicular interior to the aqueous phase before and after UV light irradiation. The ion permeability through the bilayer membrane was relatively high before UV light irradiation. On UV light irradiation, the permeability was drastically decreased by a factor of 5.8. The permeability returned almost to its original value by keeping the vesicle in the dark after UV irradiation. These responses of the permeability to photostimulation seem to be closely related to the structure of am-MAzoM in the bilayer membrane. The transmembranous bundle composed of am-MAzoMs may act as a permeable path for the ion. The photoinduced decrease in the permeability can be explained in terms of the closure of that



**Figure 5.** Photoinduced ion permeability changes across the DPPC bilayer membrane containing am.-MAzoM at 20 °C.

path owing to the partial transfer of am.-MAzoM from the membrane interior to the aqueous phase.

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