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Interaction of Poly[*N*-(3-aminopropyl)glycine] and Its Derivatives with Lipid Membrane

Yoshiko YAGI, Shunsaku KIMURA, and Yukio IMANISHI*
Department of Polymer Chemistry, Kyoto University,
Yoshida Honmachi, Sakyo-ku, Kyoto 606
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Poly[*N*-(3-aminopropyl)glycine] (PolyNAPGly, **5**) and its derivatives were synthesized as model peptides of the membrane-bound enzymes, and investigated on their interactions with lipid membrane. PolyNAPGly (**5**) was shown to be distributed to dipalmitoylphosphatidyl choline bilayer membrane without destruction of the vesicle structure. Dodecyl groups were introduced to the side chains of PolyNAPGly (**5**), and found to facilitate binding of the polymer to lipid membrane. However, the introduction of the excess amount of hydrophobic groups to PolyNAPGly (**5**) caused the formation of polymer micelles, and the addition of such polymers to lipid vesicles destroyed the membrane structure. The derivative of PolyNAPGly (**5**) (n=35) bearing one or two dodecyl groups and some N^{ε}-benzyloxycarbonylhistidyl groups was found to be bound tightly to lipid membrane without destruction of the membrane structure. The addition of these polypeptides to vesicles decreased the membrane fluidity.

The significance of the membrane-bound enzymes in cell physiology is well recognized. For example, in the case of signal transduction through cell membrane, binding of a hormone to its receptor on the cell membrane induces the activation of phospholipase C to break down phosphatidylinositol to yield diacylglyceride, which is known to trigger the activation of protein kinase C in the presence of Ca^{2+} ion. The well-known protein kinase C is a cytosolic enzyme, but associated with lipid membrane in cooperation with Ca^{2+} and diacylglycerol.[1] Under these conditions, it is activated for phosphorylation of proteins, which is the key step for the regulation of the protein function.[2] Another instance of membrane-bound enzyme in relation to the signal transduction is the hormone-sensitive adenylate cyclase, activated by binding of a hormone to the receptor through GTP-binding protein.[3] These functions of membrane-bound enzymes must be deeply dependent on the nature of membrane environments.[4]

Peptide hormones have also been shown to interact specifically with lipid membrane, and their physiological activities have been discussed in terms of their conformation and orientation in lipid membrane. For example, Kaiser and Kezdy have succeeded in synthesizing biologically active peptides which possess a sequence designed to take a specific secondary structure.[5] Furthermore, Schwyzer has shown that the mode of interaction of opioid peptides with lipid membrane can be correlated with the receptor subtype selection.[6] These reports emphasize the significance of amphiphilic structures of peptides in their interactions with lipid membrane. In the present investigation, we aimed at the elucidation of fundamental aspects of interactions between enzymes and lipid membrane using enzyme-model polypeptides.

Various kinds of polymer models of enzymes have been investigated in a homogeneous solution. Kiefer et al. synthesized branched poly(ethyleneimine)s containing nucleophilic catalytic and hydrophobic groups, and observed that they efficiently hydrolyzed hydrophobic esters.[7] The flexibility of polymer structures was suggested as one of the key factors of catalytic efficacy. Overberger and his coworkers have reported on efficient solvolytic activity of poly(*N*-vinylimidazole) and copolymers.[8] However, application of such polymers to a membrane system has not been reported, probably because they were not distributed well to lipid membrane or because they collapsed membrane structures.

The interactions of cationic polymers with lipid membrane has been studied on poly(lysine).[9,10] Poly(lysine) did not interact with a neutral lipid membrane, but was distributed into a negatively charged membrane due to electrostatic and hydrophobic interactions. The solution conformation and the chirality of the polypeptide affect the mechanism of the interactions.

We chose poly[*N*-(3-aminopropyl)glycine] (PolyNAPGly) as a starting polypeptide and its derivatives were prepared by the reaction of amino groups in side chains. These polymers are characterized by flexible chains for the *N*-substituted amide linkages: lack of amide protons to form a hydrogen bond and cis/trans isomerization around the amide bond.[11] This flexibility would be favorable for the polymers to be partitioned into lipid membrane and to function as a catalyst by a conformational adjustment at membrane surface.

N^{ε}-Benzyloxycarbonylhistidyl (ZHis) and dodecyl groups were introduced into the polymers as a nucleophilic catalytic moiety and as an anchor to lipid membrane, respectively, and the interactions of these polymers with lipid membrane were investigated. Perturbation of lipid membrane is represented by the change in membrane fluidity and by the leakage of carboxyfluorescein (CF) entrapped in the vesicle. Perturbation is discriminated from destruction of vesicle structure.

Experimental

Synthesis. Synthetic scheme of the polymer is shown in Fig. 1.

***N*-(3-Aminopropyl)glycine (1):** Bromoacetic acid (3.5 g) in ethanol was added dropwise to 1,3-propanediamine (18.5 g) in ethanol. After refluxing for 72 h, ethanol was evaporated, and 5 M NaOH (25 ml; 1 M=1 mol dm^{-3}) was added and evaporated. The water addition/evaporation was repeated, and the residue was dissolved in ether/water, and evaporated. The obtained oil was used for the next step without further purifications.

***N*-Benzyloxycarbonyl-*N*-[3-(benzyloxycarbonylamino)-propyl]glycine (2):** Benzyloxycarbonyl chloride (0.1 M) was added to 2.5 M NaOH aqueous solution (40 ml) of *N*-(3-aminopropyl)glycine at 0 °C under a vigorous stirring. After stirring for 24 h at room temperature, the aqueous phase was washed with ether and acidified with HCl, and extracted with ethyl acetate. The ethyl acetate solution was washed with water and dried over Na_2SO_4. After eluting on silica gel with chloroform/methanol/acetic acid (95/5/3 v/v) as an eluant, the oil was crystallized from ether/ethanol, yield 4.2 g (42%); mp 96—98 °C. Found: C, 62.88 ; H, 6.07; N, 7.09%. Calcd for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 6.96%.

Poly[*N*-[3-(benzyloxycarbonylamino)propyl]glycine] (4): Compound **2** (8.5 g) was reacted with thionyl chloride (10 ml) at 60 °C for 20 min. The solution was added into hexane (200 ml). The obtained white precipitate was recrystallized from benzene, yield 3.4 g (56%); mp 86—87 °C. The purified *N*-carboxy anhydride (NCA) (**3**) was polymerized in dichloromethane (40 ml) using hexylamine as an initiator (25.9 mg). After 70 h, the solution was added into ether (200 ml) to precipitate the product. The obtained polymer was reprecipitated from acetone. The degree of polymerization of the polymer was estimated to be 35 by vapor-pressure osmometry.

Poly[*N*-(3-aminopropyl)glycine] (PolyNAPGly) (5): Compound **4** (0.5 g) in acetic acid (20 ml) was treated with 3 M HBr/acetic acid (2 ml) at room temperature for 1 h. The solution was added into ether to precipitate the product. The white solid obtained was purified by dialysis (Spectrapore 1000, Ieda Inc.), yield 106 mg (30%).

Poly[*N*-[3-(dodecylamino)propyl]glycine] (Poly(C_{12})$_n$-NAPGly) (6,7): PolyNAPGly(**5**) (0.1 g) in dimethyl sulfoxide (2 ml) was reacted with dodecyl iodide (15.2 mg, in the case of n=1) in the presence of lutidine (1 ml) under a nitrogen atmosphere at 60 °C for 60 h. The solution was added to acetic acid/ether (1/4 v/v), and the obtained solid was purified by dialysis, yield 24 mg (25%). It was shown by NMR measurement that dodecyl groups were introduced on 2.8% of amino groups in the polymer.

Poly[*N*-[3-[[N^{α}-(benzyloxycarbonyl)histidyl]amino]propyl]-glycine] (PolyZHis$_n$NAPGly) (8,9): A solution of PolyNAPGly (50 mg) in dimethyl sulfoxide (2 ml) was reacted overnight with $N^{\alpha,im}$-bis(benzyloxycarbonyl)histidine *p*-nitrophenyl ester (15.8 mg, in the case of n=4) in the presence of triethylamine (100 ml) under a nitrogen atmosphere at 30 °C. Then the solution was added to ether (100 ml). The obtained oil was dissolved in *N*,*N*-dimethylformamide (2 ml), and pH was adjusted to 9 with 2 M NaOH. After standing for 30 min for deblocking the protected imidazolyl group, the solution was acidified by acetic acid, and added to ether. The obtained polymer was purified by dialysis, yield 12.5 mg (20%). Histidyl groups were introduced on 12.5% of total amino groups in the polymer.

Preparation of Small Unilamellar Vesicles (SUV). The lipid dispersion in a buffer (2-[4-(2-hydroxyethyl-1-piperazyl]ethanesulfonic acid (HEPES) (pH 7.2) or tris(hydroxymethyl)aminomethane (Tris) (pH 9.0) (10 mM), NaCl (0.1 M), and EDTA (0.1 mM)) was sonicated under a nitrogen atmosphere above the phase transition temperature for 20 min using a probe type of ultrasonic disruptor (TOMY SEIKO Co., Ltd., Japan) at the power of range 3, and ultracentrifuged at 100000 g to remove aggregates other than small unilamellar vesicles[12] and any titanium from the probe.[13] Vesicles, in which CF was trapped, were prepared according to the method reported by Barbet et al.[12]

Lipid concentration was determined by the method reported by Raheja et al.[14]

Measurements. Fluorescence measurements were carried out on a Hitachi MPF-4 fluorescence spectrophotometer. Fluorescence depolarization was measured by a modified MPF-4 apparatus installed with a fixed polarizer at the monitor side and a rotating polarizer at the excitation side which is driven by a stepping motor to 0° or 90° with respect to another polarizer. A depolarizer was set in front of the polarizer at the excitation side. At each position of the rotating polarizer, four data points were measured, processed by microcomputer, and averaged out. One polarization value was obtained at every 30 s. Excitation wavelengths of 8-anilino-1-naphthalenesulfonoic acid (ANS), 1,6-diphenyl-1,3,5-hexatriene (DPH), and CF were 380, 360, and 490 nm, respectively.

The concentration of polymer is represented in terms of the peptide residue unit.

Results

Derivatives of PolyNAPGly (5). PolyNAPGly (**5**) derivatives carrying one and two dodecyl groups in a polymer chain are represented as PolyC_{12}-NAPGly(**6**) and Poly(C_{12})$_2$-NAPGly (**7**), respectively. Other derivatives are PolyZHis$_4$-NAPGly (**8**), PolyZHis$_5$-NAPGly (**9**), and PolyC_{12}ZHis$_3$-NAPGly (**10**), which contain in a polymer chain four ZHis groups, five ZHis groups, and one dodecyl and three ZHis groups, respectively (Fig. 1). These derivatives were purified by dialysis to eliminate contamination by low molecular weight compounds.

CF Leakage from Vesicles by Polymer Addition. CF leakage from vesicles induced by the addition of the polymers represents perturbation to the membrane structure.[12] Figure 2 shows the time course of the CF leakage from dipalmitoylphosphatidyl choline (DPPC) vesicles induced by the addition of the polymers above the phase transition temperature of DPPC membranes. The addition of PolyNAPGly (**5**) and PolyZHis$_5$-NAPGly (**9**) did not increase the leakage so much, but the addition of Poly(C_{12})$_2$-NAPGly (**7**) and PolyC_{12}-ZHis$_3$-NAPGly (**10**) increased the CF leakage. Therefore, the polymers are tightly bound to the lipid

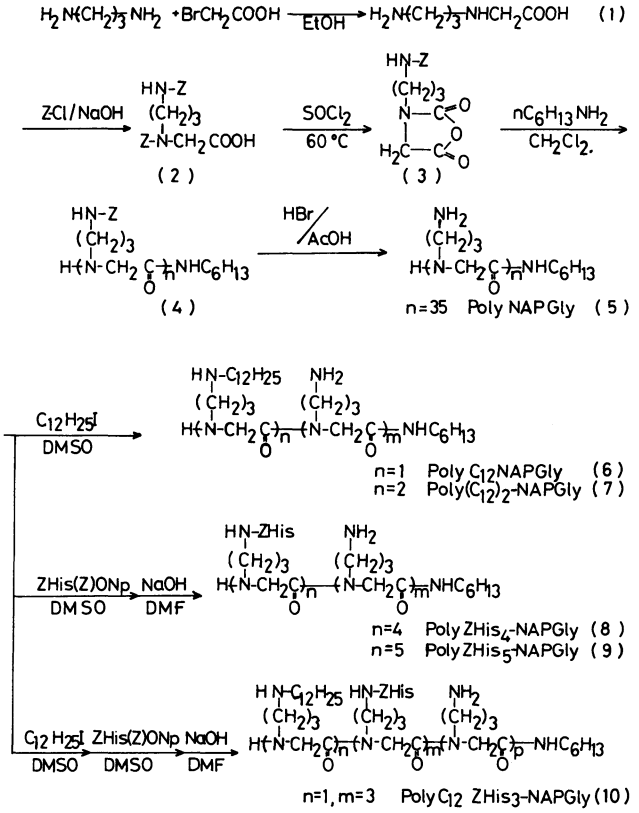

Fig. 1. Synthetic scheme of PolyNAPGly and its derivatives.

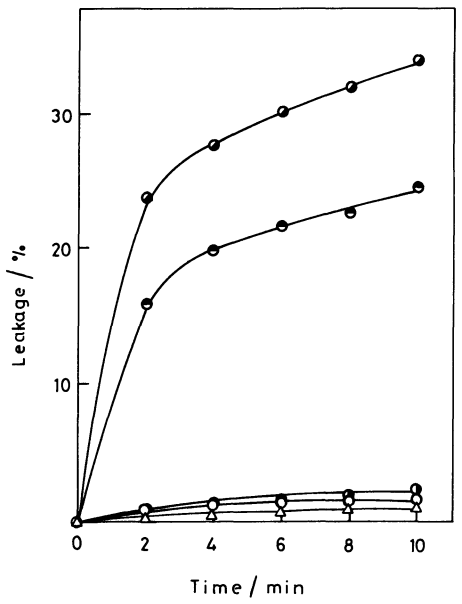

Fig. 2. Time course of CF leakage from DPPC vesicles by the addition of polymers at 46°C and at pH 9.0. DPPC lipid, 17 μM; polymer, 3.2 μM. △, Buffer; ○, PolyNAPGly (**5**); ◓, Poly(C_{12})$_2$-NAPGly (**7**); ◑, PolyZHis$_5$-NAPGly (**9**); ◕, Poly-C_{12}ZHis$_3$-NAPGly (**10**).

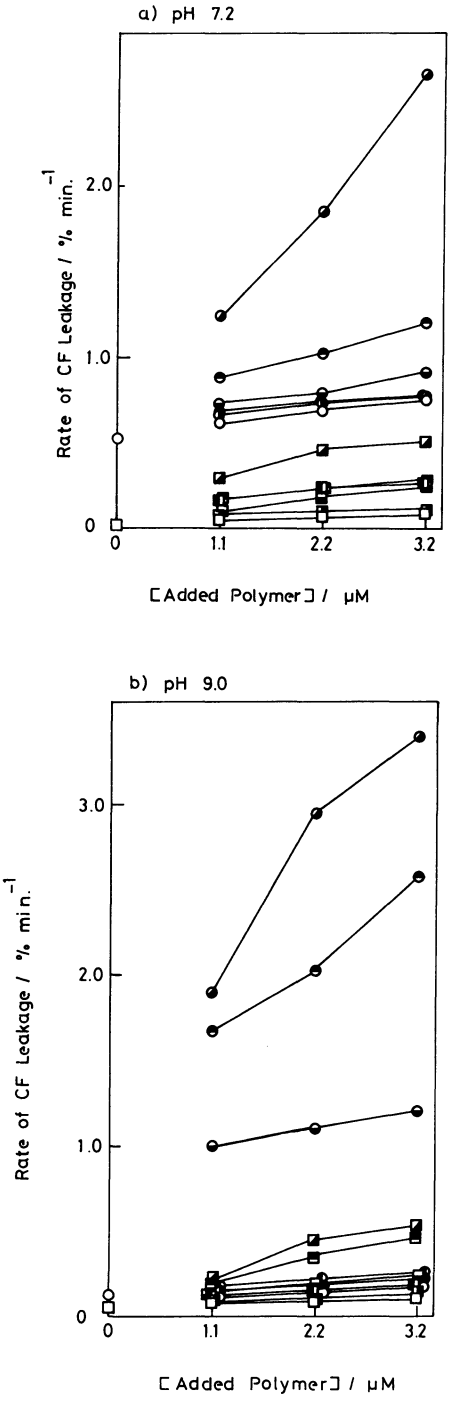

Fig. 3. The dependence of CF leakage from DPPC vesicles on the concentration of added polymers. The rate of CF leakage was determined at 10 min after the polymer addition. DPPC lipid, 17 μM. a) Experiment at pH 7.2, b) experiment at pH 9.0. □ and ○, PolyNAPGly (**5**); ◘ and ◒, PolyC_{12}-NAPGly (**6**); ◘ and ◒, Poly(C_{12})$_2$-NAPGly (**7**); ◧ and ◐, PolyZHis$_4$-NAPGly (**8**); ◧ and ◑, PolyZHis$_5$-NAPGly (**9**); ◪ and ◕, PolyC_{12}ZHis$_3$-NAPGly (**10**). Square and circle symbols represent the experiments carried out at 33°C and 46°C, respectively.

membrane by the action of the dodecyl groups as anchoring groups.

Dependence of CF Leakage on pH, Temperature, and Amount of Added Polymer. The rate of CF leakage from DPPC vesicles was determined at 10 min after the polymer addition. Figure 3 shows the rates of CF leakage at different pH values, temperatures, and concentrations of added polymers. The rate of CF leakage at pH 9.0 was larger than that at pH 7.2, while it is considered that distribution to and permeation across lipid membrane of CF are more difficult at pH 9.0 than at pH 7.2 because of its pK_a values of 4.5 and 6.3.[12] The rate of CF leakage was small at 33 °C, which is lower than the phase transition temperature. However, it was enhanced at 46 °C, which is higher than the phase transition temperature. The higher the hydrophobicity of the polymer is, the more the CF leakage was enhanced. In the case of less hydrophobic polymer, the CF leakage was not increased so much with increasing concentration of added polymers. This indicates weak interactions of these polymers with bilayer membranes and a small number of binding sites in the lipid membranes for these polymers. On the other hand, the CF leakage induced by the addition of Poly(C_{12})$_2$-NAPGly (**7**) and PolyC_{12}ZHis$_3$-NAPGly (**10**) increased with an increase in the concentration of added polymers, indicating a strong binding of these polymers to the membrane.

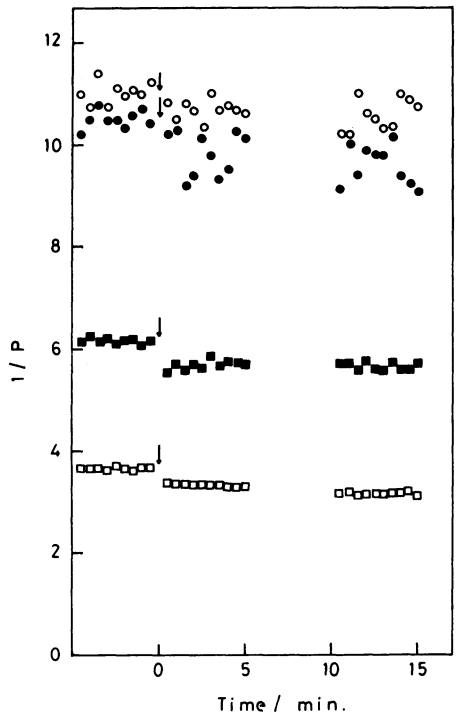

Fig. 4. Change in membrane fluidity of DPPC vesicles on the addition of Poly(C_{12})$_2$-NAPGly(7) at pH 9.0. DPPC lipid, 84 μM; Polymer, 20 μM.
□, DPH at 33 °C; ■, ANS at 33 °C; ○, DPH at 46 °C; ●, ANS at 46 °C.

The Change in Membrane Fluidity upon the Polymer Addition. The change in membrane fluidity induced by the addition of a polymer was investigated by measuring fluorescence depolarization of ANS and DPH in the membrane (Fig. 4). The fluorescent probes, ANS and DPH, reflect the fluidity of the surface region and the hydrophobic domain of lipid membrane, respectively. Figure 5 shows the relative change in fluorescent polarization induced by the polymer addition. It was found that the addition of every polymer increases the polarization, that is, it decreases the membrane fluidity. Poly(C_{12})$_2$-NAPGly (**7**) and PolyC_{12}ZHis$_3$-NAPGly (**10**) were shown to effectively decrease the membrane fluidity below the phase transition temperature.

Discussion

Dissolution of the polymers into lipid bilayer membrane without a serious destruction of the membrane structure is indispensable for polymers to be a model compound of membrane-bound enzymes. PolyNAPGly (**5**) was expected to satisfy the conditions as follows. i) A propyl group should act as an anchor group for the polypeptide to be bound to membrane. ii) The backbone of the polypeptide is flexible due to *N*-substituted amide bonds, hence the polypeptide easily fits to the membrane surface. iii) PolyNAPGly (**5**) is difficult to go deeply into the membrane, because it is positively charged at the neutral pH and the lack of amide protons makes carbonyl groups unshielded from solvents. Taking these points into consideration, it is suggested that the polypeptides tend to be incorporated into the membrane surface compartment.

The synthetic polymers are soluble in water. The molecular structures of these polymers are composed of hydrophilic and hydrophobic regions. However, they were designed not to form a polymer micelle by avoiding the introduction of hydrophobic substituents excessively to the polymer. The absence of micelle formation was confirmed by a hydrophobic fluorescent probe, ANS.[15,16] The derivatives neither enhanced nor shifted the emission from ANS in aqueous solution. This indicates the absence of hydrophobic environment imposed on ANS by micelle formation. An extensive introduction of hydrophobic groups to PolyNAPGly (**5**) makes it a polymer micelle, which is an unfavorable structure to collapse the vesicles. For instance, the polymer bearing nine ZHis groups in a chain enhanced the emission intensity of ANS in solution, indicating the formation of polymer micelle. The addition of this polymer to CF-trapped vesicles induced a complete leakage of CF immediately after polymer addition, indicating the destruction of vesicle structures. Such polymers were not used in the present investigation. After all, the amount of dodecyl and ZHis substituents in a polymer chain should be less than two and five, respectively, in order for the

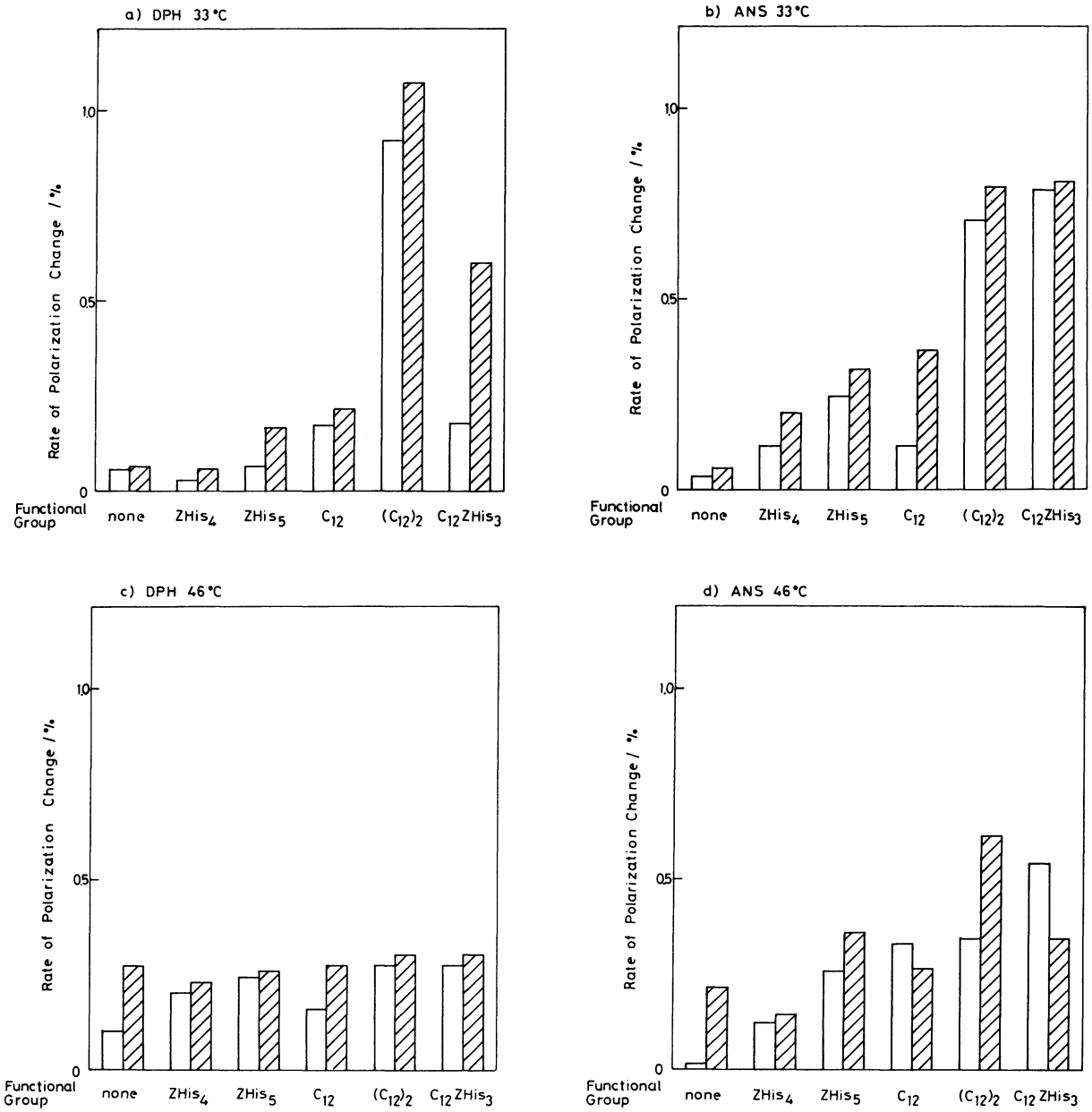

Fig. 5. The relative change in fluorescence polarization of DPH and ANS in DPPC vesicles induced by the addition of polymers. The relative change in fluoresence polarization is represented by $(P-P_0)/P_0$, where P designates the average polarization value at 5 min after the addition of polymer (20 μM), and P_0 means the polarization value in the absence of polymer. The temperature and used fluorescent probe are indicated at the top of each figure. The observed P_0 values are a) 0.285 (pH 7.2) and 0.275 (pH 9.0), b) 0.163 (pH 7.2 and 9.0), c) 0.096 (pH 7.2) and 0.091 (pH 9.0), and d) 0.098 (pH 7.2) and 0.095 (pH 9.0).
□, pH 7.2; ▨, pH 9.0.

derivatives of PolyNAPGly(**5**) to interact with lipid membrane without a serious damage of the membrane structure.

In the presence of DPPC vesicles, the addition of PolyNAPGly (**5**) changed the polarization of ANS in the lipid membrane. The varied polarizations of ANS should be attributed to the fluidity change of the membrane on interaction of PolyNAPGly (**5**) with the lipid membrane. While, CF leakage from DPPC vesicles was not influenced so much by the addition of PolyNAPGly (**5**), indicating the absence of the serious damage of the lipid membrane. These observations strongly support that PolyNAPGly (**5**) is a suitable polymer for the investigation of membrane-bound enzymes.

The effect of substituents of the polymer on binding to the membrane was investigated. The introduction of ZHis groups to PolyNAPGly (**5**) did not affect its

interaction with lipid membrane so much. However, the incorporation of dodecyl groups enhanced the binding to membrane (Fig. 2). Since PolyC$_{12}$ZHis$_3$-NAPGly (**10**) disturbed the membrane structure more extensively than PolyZHis$_4$-NAPGly (**8**), the dodecyl group of PolyC$_{12}$ZHis$_3$-NAPGly (**10**) should enhance the binding of the polymer to membrane (Fig. 3). Therefore, dodecyl group is effective in anchoring water-soluble compounds to lipid membrane. This result is consistent with that reported by Sunamoto, who succeeded in coating lipid vesicles with polysaccharide carrying a few hydrophobic alkyl chains.[17]

Cationic charges on the amino groups of the polymer also influence the affinity of the polymer with lipid membrane. Figure 3 shows that the effect of polymer addition on the CF leakage is larger at pH 9.0 than at pH 7.2. At pH 9.0 positive charges on the polymer are less than those at pH 7.2. Therefore, the polymer is bound to the neutral membrane at pH 9.0 more favorably than at pH 7.2. CF leakage did not reach 100% under the conditions in which several polymer molecules per one vesicle were added, indicating that the polymers do not completely destroy the vesicle structure. The time course of the CF leakage is biphasic (Fig. 2). One is just after the polymer addition, which is a transient state accompanied by the insertion of the polymer molecules into the membrane. The following slow leakage of CF should be attributed to a disordering of the membrane structure occurring after the complete incorporation of the polymers into the membrane.

The phase transition temperature of DPPC vesicles was raised by about 1 °C on addition of PolyNAPGly, PolyZHis$_4$-NAPGly (**8**), PolyC$_{12}$-NAPGly (**6**), or PolyC$_{12}$ZHis$_3$-NAPGly (**10**). This was detected by investigating the temperature dependence of fluorescence intensity of ANS according to the method by Mayer et al. (data not shown).[16] This observation also indicates that the polymers are absorbed on the membrane surface and slightly decreased the mobility of lipid molecules.

This effect of the polymers on the membrane fluidity was more prominent below the phase transition temperature. Based on the polarization of DPH, fluidity of the hydrophobic domain of a lipid membrane was almost independent of the nature of added polymers above the phase transition temperature, indicating that these polymers do not have serious effects on the structure of hydrophobic domain of a lipid membrane. However, below the phase transition temperature, effects of polymers on the fluidity of the hydrophobic domain of a lipid membrane were strongly dependent on the nature of polymers (Fig. 5). Especially, Poly(C$_{12}$)$_2$-NAPGly (**7**) and PolyC$_{12}$ZHis$_3$-NAPGly (**10**) perturbed markedly the structure of hydrophobic domain of lipid membrane. Dufourcq and his coworkers have reported that interactions of melittin, which is an amphiphilic polypeptide toxin, with phosphatidyl choline bilayers are stronger above the phase transition temperature than those below it.[18] However, it might be inferred that amphiphilic compounds tend to be bound more tightly to gel-state membrane than to liquid-crystalline membrane. Since the complex formed between amphiphilic compounds and lipid molecules should be highly ordered, the binding should accompany a large entropy loss. The entropy loss upon binding might be smaller for gel-state membrane than for liquid-crystalline membrane, leading to a larger loss of free energy upon binding to gel-state membrane than to liquid-crystalline membrane. For example, it has been reported that glucagon is bound tightly to gel-state membrane.[19,20] The present polymers behaved similarly to glucagon in the interaction with lipid membranes.

It should be noted that binding of polymer to lipid membrane decreased the membrane fluidity. Decrease in membrane fluidity by Ca^{2+} has been observed and attributed to the dehydration of head groups of lipid molecules by Ca^{2+} binding.[21] The decrease in membrane fluidity on the polymer addition might be explained by the same reason—that is, the ammonium and carbonyl groups of the polymer are bound to the polar groups of lipid molecules to dehydrate the membrane surface.[22]

To conclude, water-soluble polymers can be bound to lipid membrane by introducing hydrophobic groups as anchoring groups. The membrane affinity is adjustable by the number and the properties of the hydrophobic group. These points are very essential in the simulation of membrane-bound enzymes, since they affect sensitively physical properties of membrane, such as membrane fluidity and packing of lipid molecules.

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