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Substrate-induced Formation of a Recognition Structure in a Polypeptides - Lipid Membrane System

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We prepared poly(γ-methyl L-glutamate) bearing Ser, His, Asp, and Glu at the amino terminals to act as the serine protease binding site. The number average degree of polymerization of the polypeptides was 51. We incorporated these polypeptides into a dipalmitoylphosphatidylcholine bilayer membrane and formed a transmembranous assembly in the membrane. We investigated the recognition of N-acetyltyrosine ethyl ester, a typical substrate of the serine protease, on the polypeptide assembly in the membrane. This polypeptide assembly was composed of four kinds of polypeptides and found to act as a receptor for this substrate. This behavior may have arisen from a substrate-induced rearrangement of the four kinds of polypeptides in the membrane, forming a substrate binding structure similar to that in serine protease.

Key words: Polypeptide Assembly, Recognition Structure, Substrate-Induced Rearrangement

1. INTRODUCTION

The binding of a signaling molecule onto a receptor is the initial event for signal transduction in living systems. It has been recognized that the specific location of the amino acid yields the molecular recognition domain in the receptor and is closely related to the induced signal transfer on and through the biological membrane. It has been reported [1-6] that artificial systems composed of an ordered assembly of functional groups show specific binding behavior for some substrates. Stewart et. al., reported [7] on the design and synthesis of a polypeptide having chymotrypsin-like catalytic activity. Their receptor model consisted of four parallel amphiphilic helixes, covalently linked by their carboxy terminals. Their free amino terminals were composed of Ser, His, Asp and Glu units acting as the serine protease catalytic site. This receptor model selectively hydrolyzed chymotrypsin ester substrates in a manner similar to chymotrypsin. On the other hand, immunoglobulin can recognize a great number of molecules, owing to the hypervariable region in its binding site [8-9].

In this study, we attempt to prepare an artificial receptor that has an antibody-like diversity in its recognition structure. Here, we report our first example of such an artificial molecular recognition system, *i.e.,* the substrate-induced formation of the recognition structure in polypeptide-lipid membrane systems. In keeping with the receptor model of Stewart et. al., we prepared four kinds of polypeptides that had Ser, His, Asp and Glu at the amino terminals, and constructed their corresponding polypeptide bundles in the lipid membrane with no covalent bonds between them. In our system the active structure is generated by the formation of a polypeptide assembly that is induced by the interaction between the substrate and the amino terminals of the polypeptides in the membrane. This result indicates the possibility of constructing various antibody-like recognition systems by preparing unit polypeptides that have various functional groups at their terminals.

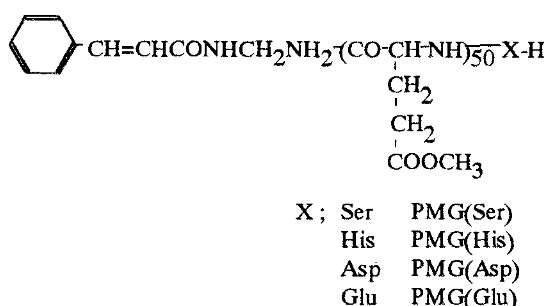
2. EXPERIMENTAL

2. 1 Materials

Poly(γ-methyl L-glutamate) (PMG) was obtained by polymerization of the N-carboxy-anhydride of L-glutamic acid γ-methyl ester in dimethylformamide (DMF), in the presence of the initiator N-cinnamoyl-N-aminoethylamine. A number average degree of polymerization of 50 was estimated from the molar ratio of the cinnamoyl moiety to the γ-methyl L-glutamate residues of PMG. This ratio was determined from the absorbance of the PMG in DMF, at 332 nm, based on the molar extinction coefficient of N-cinnamoyl-N-aminoethylamine.

PMG that had Fmoc-Ser(tBu) at the amino terminal was obtained from the coupling reaction between PMG and Fmoc-Ser(tBu) in DMF, conducted over a period of 24 hours, in the presence

of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide and N-hydroxybenzotriazole. The amino terminal Fmoc protecting group was finally removed by treating with a DMF solution containing 20 vol% piperidine, for 30 minutes. PMG that had Ser at the amino terminal (PMG(Ser)) was then obtained by removing the tBu protectiong group from the Ser residue by treatment in a trifluoroacetic acid solution, containing 5 vol% water, at room temperature for 1.5 hours. PMG that had His, Asp and Glu at the amino terminals (PMG(His), PMG(Asp) and PMG(Glu)) were obtained in a similar manner, using Fmoc-His(Tri), Fmoc-Asp(OtBu) and Fmoc-Glu(OtBu) , respectively (Scheme 1).



Scheme 1. Chemical structures of PMG(Ser), PMG(His), PMG(Asp) and PMG(Glu).

The dipalmitoylphosphatidylcholine (DPPC) vesicles were prepared by sonicating an aqueous suspension of DPPC (0.1 mg/mL) with a Branson Sonifier model 250 in a buffer solution (50 mM N-2-hydroxyethylpipereazine-N'-2-ethanesulfonic acid (HEPES) - NaOH, pH 7.2), in a nitrogen atmosphere at 0 °C

2.2 Method

We determined the partition of the polypeptides between the vesicle and external aqueous phase by adding the DMF solution of the polypeptides (1 mg/mL) to the vesicle solution (1 mL) and incubating this for 10 minutes at 25°C. The molar ratio of the polypeptides to DPPC was 1.0×10^{-2}. The vesicle solution thus obtained was passed through a Bio-gel P-6DG gel filtration column (15$\phi \times$50 mm, Bio-Rad Lab.) by using a 50 mM HEPES-NaOH buffer (pH 7.2) as an eluting buffer to collect the vesicle and external polypeptides fraction. A fluorescence technique employing 4-fluoro-7-nitrobenzofurazan (NBD) as a probe was used to quantitate the polypeptide molecules in the vesicle and external aqueous phase. It is well-known that NBD reacts rapidly with primary amino groups yielding a useful fluorescent probe [10]. Therefore an ethanol solution of NBD (0.1 M) was added to the vesicle and external polypeptides fraction and sonicated for one minute at 0°C. The concentration of NBD was 5.0×10^{-6} M. These solutions were incubated for one minute at 60°C and then rapidly quenched to 0°C. The excitation wavelength of NBD at the amino terminal of each polypeptide was found to be 470 nm. We observed the fluorescence at 525 nm with a spectrofluoro-photometer (JASCO FP-FFF).

The orientation of the polypeptides in the vesicle membrane was estimated from the proportions of these amino terminals located at the outer leaf membrane of the vesicle. The vesicle fraction containing the polypeptides obtained in the above manner was reacted with NBD. In this case, the fluorescence intensity, Ia, is proportional to the number of the polypeptides whose amino terminals are oriented to the external phase. However, the sonication of the vesicle fraction induced a random orientation of the polypeptides in the membrane, and NBD could only react with half the number of the polypeptides in the membrane. Therefore, the fluorescence intensity, Ib, is taken to be proportional to half the number of polypeptides in the membrane. The orientation of the polypeptides in the vesicle membrane could be calculated from Ia / 2 Ib.

The recognition of the substrate, N-acetyltyrosine ethyl ester (Ac-TyrOEt), on the vesicle containing the polypeptides was investigated in the following manner. We added the buffer solution containing Ac-TyrOEt to the vesicle fraction (1 mL) containing the polypeptides obtained as described above, and incubated this at 25 °C for 10 minutes. The concentration of Ac-TyrOEt was 5.0×10^{-6} M. The solution was finally passed through a P-6DG gel filtration column. The amount of Ac-TyrOEt binding to the vesicle containing the polypeptides was estimated by the fluorescence intensity of Ac-TyrOEt in the vesicle fraction. The excitation wavelength used 275 nm, gave a fluorescence at 304 nm.

3. RESULTS AND DISCUSSION

3.1 Structure of the Polypeptides in the Vesicle Membrane

Polypeptides PMG(Ser), PMG(His), PMG-(Asp) and PMG(Glu) were found to adopt α-helical conformations in DPPC vesicles. We reported previously [11,12] that the hydrophobic α-helical polypeptides were incorporated into the vesicle membrane to form membrane-spanning helical bundles. Figure 1 shows the partition of the four kinds of polypeptides between the DPPC vesicle membrane and the external aqueous phase. The partition of the polypeptide to the membrane phase increased with an increase in the hydrophobicity [13] of the amino terminal residue of the polypeptide. Figure 2 shows the relationship between the orientation of the amino terminal of the polypeptide

and the hydrophobicity of its amino terminal

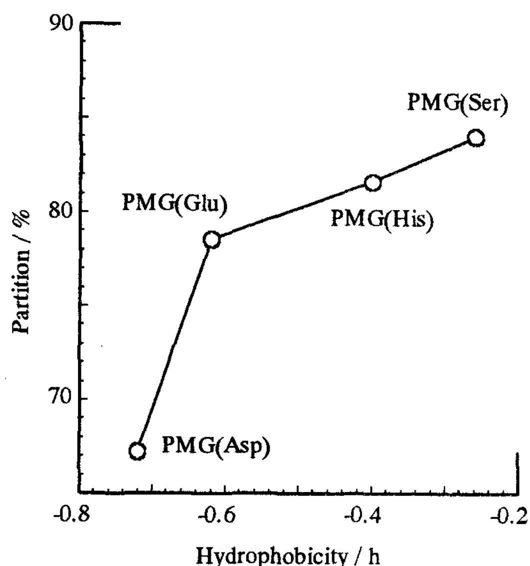


Figure 1. The partition of the polypeptides between the vesicle membrane and the external aqueous phase.

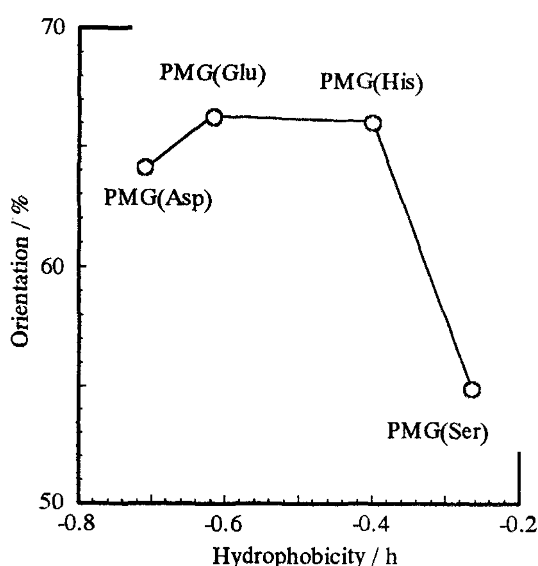


Figure 2. The orientation of the polypeptides in the vesicle membrane.

residue. The amino terminal residues of PMG(Asp), PMG(Glu) and PMG(His) were oriented mainly towards the external aqueous phase. On the other hand, the proportion of the relative hydrophobic Ser residue of the PMG(Ser) amino terminal located at the outer leaf membrane of the vesicle was found to be lower than for those of PMG(Ser), PMG(Glu) and PMG(His). These results suggest that the partition of the polypeptides between the vesicle membrane and the external aqueous phase is proportional to the hydrophobicity of the amino terminal residue, and that the polypeptide is incorporated in the membrane from the hydrophobic terminal of the polypeptide.

When we observed the shape of the polypeptides in the DPPC vesicle with an atomic force microscope, intramembraneous particles with diameters of approximately 20 nm were clearly visible. The size of these particles suggest that they are a membrane-spanning α-helical bundle consisting of an assembly of polypeptide molecules, since a single α-helical polypeptide molecule would not be large enough to form particles of the size we observed

3.2 Recognition of the Substrate on the Vesicle Containing the Polypeptides

We investigated the recognition of the substrate, Ac-TyrOEt, on the DPPC vesicle containing the four kinds of polypeptides (PMG(Ser), PMG(His), PMG(Asp) and PMG(Glu)), by the ratio of the amount of Ac-TyrOEt binding on the vesicle to the total added Ac-TyrOEt. Figure 3 shows the recognition of Ac-TyrOEt on the pure DPPC vesicle and the DPPC vesicles containing PMG(Ser), PMG(His), PMG(Asp) and PMG(Glu), respectively. The recognition of Ac-TyrOEt on the pure vesicle and on the vesicles containing only one kind of polypeptide was found to be between 20 and 26%. The recognition of Ac-TyrOEt on the vesicle containing the four kinds of polypeptides was 56%, considerably larger than those values found for both the pure vesicle and the vesicles containing only one

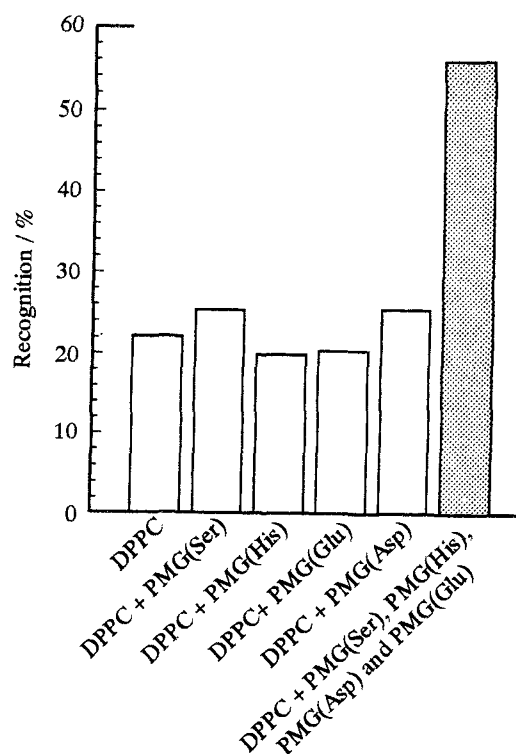


Figure 3. Recognition of Ac-TyrOEt on the vesicle containing the polypeptides.

kind of polypeptide. This result suggests that the membrane-spanning bundles consisting of PMG(Ser), PMG(His), PMG(Asp) and PMG(Glu) in the vesicle membrane act as recognition sites for Ac-TyrOEt.

We have attempted to demonstrate the substrate-induced rearrangement of the four kinds of polypeptides assemblies in the vesicle membrane, forming the substrate-binding structure. It is well-known [14] that cinnamoyl groups produce a dimer when irradiated by UV light. In our case, the photo-dimerization of the carboxyl terminal cinnamoyl moieties of the polypeptides would partially fix the arrangement of the polypeptide assembly and hinder the substrate-induced rearrangement of the polypeptides due to the interaction between the substrate and amino terminal residues in the vesicle membrane. We irradiated the DPPC vesicle containing the four kinds of polypeptides with UV light (250 nm < λ < 380 nm) from a 500-watt high-pressure mercury lump, equipped with a Toshiba UV-D33S glass filter, for 30 minutes at 25°C. The exact value of the photo-dimerization of the cinnamoyl moiety of the polypeptide is not clear at present. The recognition of Ac-TyrOEt on the vesicle containing the four kinds of the polypeptides decreased after UV light irradiation (Table 1).

Table 1. Recognition of Ac-TyrOEt on the vesicle containing the four kinds of polypeptide before and after UV light irradiation.

	Recognition / %
before UV light irradiation	56
after UV light irradiation	48

This result implies that the photo-dimerization of the polypeptides in the vesicle membrane hinders the formation of the Ac-TyrOEt recognition structure. In other words, the added substrate, Ac-TyrOEt, induces a rearrangement of the polypeptide assembly in the vesicle membrane to form a substrate-binding domain due to the interaction between the substrate and amino terminal residues.

4. CONCLUSION

The α-helical polypeptides having Ser, His, Asp, and Glu, at the amino terminal were incorporated into the vesicle membrane to form a membrane-spanning bundle. The amino terminal Ser, His, Asp and Glu residues could form substrate-recognition structures similar to the substrate binding domain of serine protease, by rearranging the four kinds of polypeptides in the bundle due to the interaction between the substrate and the amino terminal residues. The substrate-induced formation of the molecular recognition structure in this system is an example of the mimetic systems of the antibody-like diversity of the recognition. It may be possible to produce such an artificial antibody-like recognition system by using various functional groups as a unit at the binding site.

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