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Interaction of Glucagon with Dimyristoylphosphatidylcholine in Vesicular and Discoidal Complexes

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Glucagon fragments dimyristoylphosphatidylcholine liposomes into discoidal complex under appropriate conditions. The concentration of glucagon required to fragment the vesicles increases with increasing pH, which appears to be related to the glucagon binding. It was also observed that the fragmentation is facilitated by NaCl, which is also due to increased glucagon binding. From the quenching of Trp fluorescence by doxyl group located at various positions of the acyl chain of the lipid, Trp of glucagon was found to be located close to the bilayer surface in the vesicular complex. However, the Trp fluorescence was quenched by the doxyl group in the discoidal complex to an equal extent regardless of the position of this spin label in the acyl chain. This and the results of second derivative UV spectroscopy of Tyr suggested that segments including Tyr-13 and Trp-25 are involved in the discoidal complex formation and that the orientation of glucagon is not normal to the bilayer surface.

Key words: discoidal complex, fragmentation, glucagon, liposome, vesicular complex.

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Although the mechanism of signal transduction by peptide hormones with less than 50 amino acids is not generally understood, the first step is their binding to specific membrane receptors. Interaction of peptide hormones with the phospholipid bilayer, however, is still important because it may facilitate their binding to protein receptors on the membrane surface (*1-3*). It has been thought that peptide hormones in dilute aqueous solution do not have an appreciable structure, although evidence is accumulating that some secondary structures exist (*4, 5*). When they bind to the amphiphilic surface of a bilayer or micelles, however, there is a tremendous increase in the secondary structure (*6, 7*).

Glucagon is a 29-amino acid polypeptide hormone containing regularly spaced hydrophobic amino acid residues at every third or fourth position along the chain (*1*). Such sequences can fold into amphiphilic helical structures which are assumed to be important for interacting with either their receptor or the lipid bilayer (*2, 3*). The conformational transition of glucagons upon interaction with dimyristoylphosphatidylcholine (DMPC) was observed by CD measurement (*7*). The CD spectra of glucagon in aqueous solution showed a predominantly disordered structure but in the presence of DMPC, they revealed an appreciable increase in α-helix. Epand and his coworkers found that glucagon binds to DMPC vesicles below the phase-transition temperature (T_m) of 23°C but not above T_m (*8, 9*). These investigators were the first to report a discoidal complex between DMPC and glucagon which was obtained by adding the hormone suspension to a test tube lined with a thin film of DMPC on the inside wall, followed by vortexing at 45°C (*9, 10*). The nature of the peptide/lipid interaction has not been elucidated, however.

Many proteins are known to break down phospholipid vesicles into the discoidal form under appropriate conditions (*11-14*). A general pattern emerging from these studies is that the protein must have readily accessible amphiphilic α-helical segments which presumably line the peripheral surfaces of exposed acyl chains of the lipid bilayer patches. It is expected that the proteins, which form discoidal complex with lipid bilayer, are also capable of interacting with vesicles without fragmenting them when the lipid/protein ratio is high. There have been no extensive comparative studies on the mode of protein interactions with intact vesicles and with discoidal bilayer patches. In the present investigation, we addressed this problem by studying the interaction between lipid vesicles and glucagon, which has an advantage over other proteins in being a short peptide.

MATERIALS AND METHODS

Materials—Glucagon mixture from bovine and porcine pancreases, DMPC, and 5-, 7-, 12-, and 16-doxyl stearic acid methyl esters were obtained from Sigma. DMPC showed a single spot when visualized with iodine after thin-layer chromatography. All other chemicals were obtained in the highest grade available.

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; CD, circular dichroism; L/P ratio, lipid-to-protein molar ratio.

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Preparation of Phospholipid Vesicles—Multilamellar vesicles (MLV) were prepared by evaporating the solvent from DMPC/chloroform solution under a nitrogen gas flow and evacuating for at least 30 min to remove any remaining trace of solvent. The dried thin film of DMPC on the vial inner wall was suspended in a buffer solution and then vortexed. Subsequently, this MLV suspension was warmed at 37°C for 5 min and then vortexed again. Small unilamellar vesicles (SUV) were produced by sonicating this MLV suspension with a Branson Sonifier 450 under nitrogen. After the sonication, the vesicle dispersion was centrifuged for 1 min at 6,000 rpm to remove any probe particles, as well as MLV. Buffers used were 20 mM glycine (pH 3.0), a mixture of 15 mM citrate and 15 mM glycine (pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5), a mixture of 15 mM citrate and 15 mM Tris (pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0), a mixture of 15 mM MOPS and 15 mM sodium borate (pH 7.0, 7.5, 8.0, 8.5, and 9.0), 20 mM sodium phosphate (pH 2.0, 3.0, 6.0, and 7.0), MES (pH 6.0), MOPS (pH 7.0), 20 mM Tris (pH 8.0), and 20 mM borate (pH 9).

Phospholipid concentration was determined according to the method of Vaskovsky *et al.* (*15*). Glucagon stock solution was dissolved in distilled water. In order to facilitate the dissolution, a small volume of HCl solution was added. The glucagon stock solution was divided into small volumes and stored at −70°C. The concentration of glucagon stock solution was determined spectrophotometrically at the wavelength of 278 nm using the absorption coefficient of 2.38 $cm^2 \cdot mg^{-1}$ in 0.01 N HCl solution (*16*).

Binding of Glucagon to Phospholipid—The equilibrium interaction of glucagon with DMPC was studied with a Shimadzu RF-5301PC spectrofluorometer at 20°C. The intrinsic fluorescence intensity maximum wavelength was determined with excitation of 295 nm. Binding kinetics was followed spectrofluorometrically with emission at 350 nm.

Discoidal Complex Formation—The discoidal complex formation was followed by light-scattering with the above spectrofluorometer at the wavelength of 600 nm. The cuvette was thermostatically controlled and the temperature of the glucagon solution before mixing was adjusted to be the same as that of the vesicle suspension already in the cuvette. The sample was continuously stirred with a small magnetic bar placed in the cuvette below the light path.

Isolation of Discoidal Complex—Aliquots of glucagon stock solution were added to DMPC MLV suspended in different buffer solutions containing 500 mM NaCl at 20°C. Stable discoidal complex was formed by warming the solutions at 40°C for 10 min, and kept on ice for 10 min prior to the chromatography. A transparent 300 μl sample was loaded on a Sepharose CL-4B gel column (55×0.9 cm) with bed volume of 32 ml and the column was eluted either with 50 mM ammonium acetate buffer (pH 7.0) containing 500 mM NaCl or with 20 mM glycine buffer (pH 3.0) containing 500 mM NaCl. The eluate was assayed for lipid and glucagon according to the method of Vaskovsky *et al.* (*15*) and modified Lowry method (*17*), respectively. In the subsequent routine separation for CD and second derivative UV spectroscopy experiments, the discoidal complex was isolated from free glucagon using a desalting column (1.1×4.5 cm) of Sephadex G-100.

Fluorescence Quenching by Spin-Labels—SUVs were prepared from a chloroform solution of DMPC and one of the following fatty acid spin labels: 5-, 7-, 12-, and 16-doxyl stearic acid methyl esters. The fluorescence measurements were performed as in the binding experiments, with a Jasco FP-770 spectrofluorometer.

Second-Derivative UV Spectroscopy—All absorption spectra were recorded with a Perkin Elmer Lambda 16 UV/VIS spectrophotometer and the second derivative spectra were obtained with a PC. Because the glucagon-DMPC complex was stable only below the phase transition temperature of DMPC (*8, 9*), all experiments were performed at 20°C. When glucagon-DMPC complex was incubated at 37°C, the intensity of the fluorescence emission spectrum of glucagon decreased and large lipid aggregates started to form.

CD Measurements—These measurements were performed with a Jasco model J-710 spectropolarimeter. The temperature of the sample was maintained at 20°C with a thermostated cell holder. The effect of light-scattering was minimized by using SUV (*18*). Glucagon was added to a premixed solution containing SUV and incubated for 30 min. The CD of each sample was measured within 2 h. With a longer incubation time, an appreciable portion of DMPC is hydrolyzed into lysophosphatidylcholine and stearic acid at low pH (*19*).

RESULTS AND DISCUSSION

Interaction of Glucagon with DMPC Vesicles—In addition to the usual L/P dependency, the fragmentation of DMPC vesicles by glucagon was found to be dependent on pH as well as NaCl concentration. Figure 1 shows the time courses of pH-dependent fragmentation of DMPC MLV at L/P ratio of 10 as monitored by light-scattering. It was

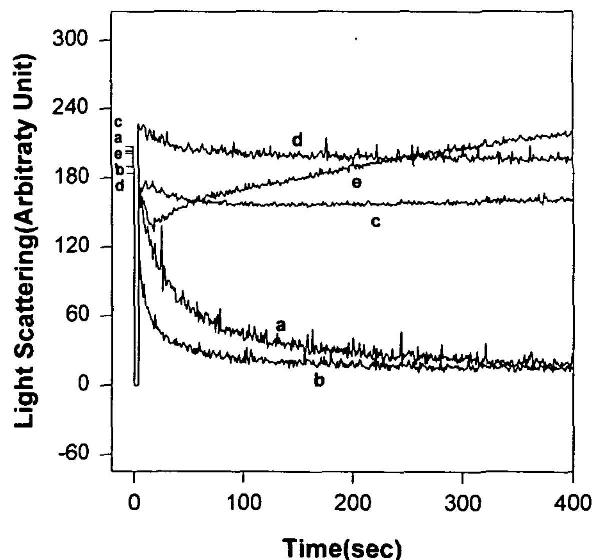

Fig. 1. **Time-courses of fragmentation of vesicles by glucagon in the presence of 0.5 M NaCl at several pH values.** Changes in light-scattering after DMPC MLV was mixed with glucagon at 20°C were monitored with a spectrofluorometer. The concentration of glucagon was 10 μM and the L/P ratio was 10. (a) pH 2.0 (20 mM NaP_i buffer); (b) pH 3.0 (20 mM NaP_i buffer); (c) pH 4.0 (20 mM sodium citrate); (d) pH 6.0 (20 mM NaP_i); (e) pH 7.0 (20 mM NaP_i buffer). The intensities of scattered light from vesicles alone were almost same, regardless of pH (≈200±10).

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already shown that some proteins and peptides, including glucagon, fragment DMPC vesicles into discoidal complexes and this process causes a decrease in the light-scattering of MLVs (*20, 21*). The protein-bound intact vesicle is defined as "vesicular" complex and the fragmented vesicle complex is called a "discoidal" complex because it has a plate-like shape (*10, 22*). At below pH 4, the vesicles were fragmented very rapidly, while above this pH value, no fragmentation can be seen at this L/P ratio. The scattering intensity at pH 2 and 3 approached but did not quite reach the value of the detergent-solubilized vesicles.

Even at high pH, however, it was found that the vesicles were fragmented when the L/P ratio was low enough. Figure 2 shows the extent of fragmentation as a function of P/L ratio at a number of pH values. In these experiments, the concentration of phospholipid was fixed and the glucagon concentration was increased. As the pH was increased, the concentration of glucagon required to completely fragment the vesicles increased. Even at pH values as high as 9, the fragmentation was still appreciable. It is of interest to note that the concentration of glucagon required to fragment the vesicles sharply increased between pH 3.5 and 5. That this pH-dependency qualitatively correlates with glucagon binding to vesicles can be seen in Fig. 3, which shows the effect of pH on glucagon binding to vesicles as monitored by measuring the changes in maximum emission wavelength of Trp of glucagon in the presence of DMPC vesicles. Glucagon contains a single Trp residue at position 25. There is a blue shift of the maximum emission wavelength from 350 to 340 nm and the quantum yield increases when glucagon binds to DMPC vesicles. These observations suggest that the polarity of the environment of Trp-25 decreases as a result of the binding of the C-terminal region of glucagon to the phospholipid, indicating possible penetration of this region into the hydrophobic interior of the vesicle.

Figure 4 shows that the extent of vesicle fragmentation by glucagon increases with increasing NaCl concentration. That this is due to increased glucagon binding to the vesicles is shown by Fig. 5. At pH 3 as well as at pH 7, the extent of binding, as indirectly monitored by the decrease in the wavelength of the Trp fluorescence intensity maximum,

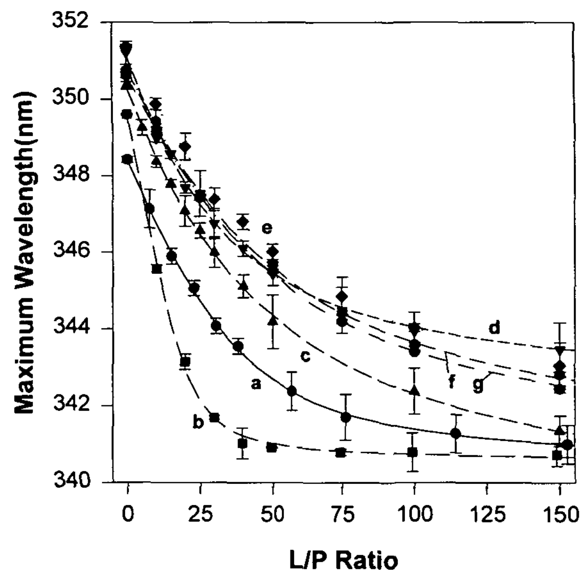

Fig. 3. **pH-dependent binding of glucagon to vesicles.** Changes in the maximum fluorescence wavelengths with increasing L/P ratio were observed at several pHs (2.0 (a), 3.0 (b), 4.0 (c), 5.0 (d), 7.0 (e), 8.0 (f), and 9.0 (g)). The concentrations of glucagon and NaCl were 1 μM and 0.5 M, respectively, in all samples.

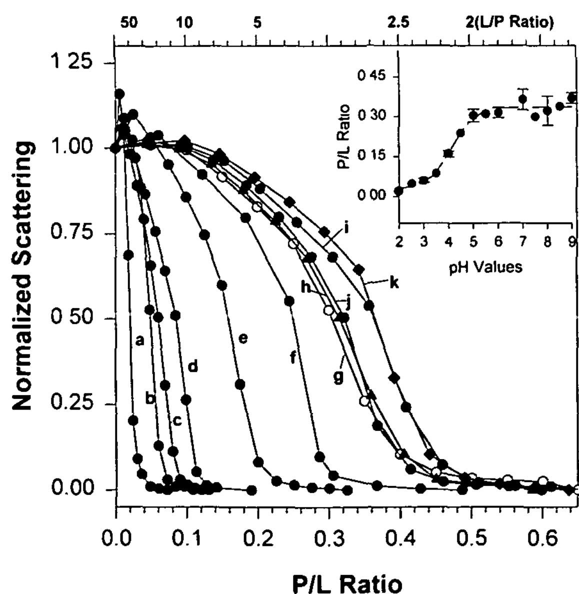

Fig. 2. **Normalized light scattering values of MLV/glucagon mixture as a function of P/L ratio.** DMPC and NaCl concentrations were fixed at 0.1 mM and 0.5 M, respectively. The pHs used were 2.0 (a), 2.5 (b), 3.0 (c), 3.5 (d), 4.0 (e), 4.5 (f), 5.0 (g), 6.0 (h), 7.0 (i), 8.0 (j), and 9.0 (k). The inset represents the P/L ratio required for 50% fragmentation plotted against pH.

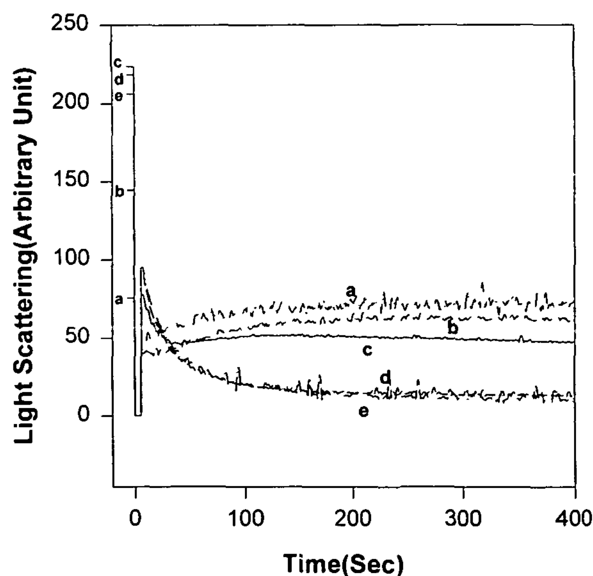

Fig. 4. **Effect of NaCl on the fragmentation of vesicles.** Changes in light-scattering, after mixing of DMPC MLV with glucagon at pH 3.0 and various NaCl concentrations, were followed. The concentration of glucagon was 10 μM and the L/P ratio was 10. The NaCl concentrations were 0 M (a), 0.05 M (b), 0.1 M (c), 0.2 M (d), and 0.5 M (e). The intensities of scattered light from vesicles alone were 82.3 (a), 146 (b), 215 (c), 212 (d), and 198 (e), respectively.

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increases with increasing NaCl concentration.

It was reported that increase of pH from 2 to 7 did not have any effect on the CD spectrum of glucagon in solution in the absence of added electrolyte (*23*), indicating that there is little variation in the conformation of glucagon within this pH range. But, it was also observed here that the α-helical content of glucagon increases with the addition of NaCl at pH 3, but not at pH 7. Figure 6 shows that glucagon has more α-helical structure when bound to DMPC SUV at

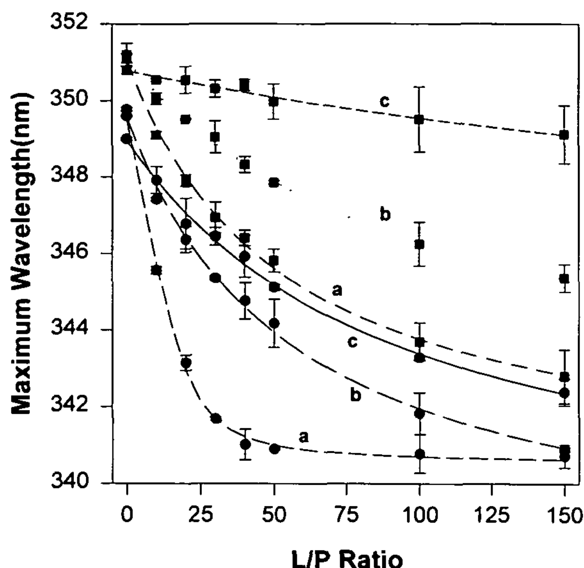

Fig. 5. **Dependency of glucagon binding to DMPC vesicles on NaCl concentration.** The binding efficiencies of glucagon to DMPC SUVs, as monitored by measuring the shift in the maximum emission wavelength, at pH 3.0 (●) and pH 7.0 (■) were obtained at NaCl concentrations of 0.5 M (a), 0.1 M (b), and 0 M (c). The concentration of glucagon was 1 μM in all samples.

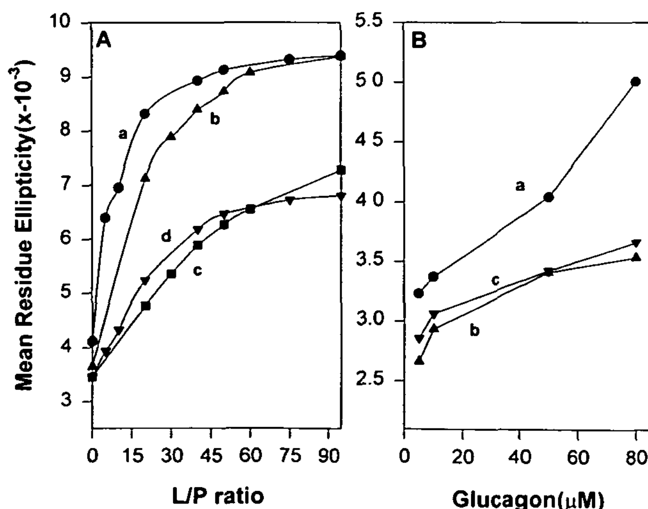

Fig. 6. **Mean residue ellipticities at 222 nm of SUV-bound glucagon (A) and free glucagon (B).** (A) The glucagon concentration was fixed at 40 μM and lipid concentration was increased. a: pH 3.0, 0.5 M NaCl; b: pH 3.0, 0.1 M NaCl; c: pH 3.0, 0 M NaCl; d: pH 7.0, 0.5 M NaCl. (B) a: pH 3.0, 0.5 M NaCl; b: pH 3.0, 0.1 M NaCl; c: pH 7.0, 0.5 M NaCl.

pH 3 than at pH 7. Thus, it appears that the extent of binding (Fig. 5) qualitatively parallels the α-helix content of glucagon in solution. The binding experiments were done with SUV, while the fragmentation was carried out with MLV. Still, it is clear that the extent of fragmentation is dictated by the extent of binding.

The time-courses of binding and fragmentation are compared in Fig. 7. Binding was monitored by following the intensity changes of the emission peak at 350 nm after glucagon solution was added to make the L/P ratio 10. The fragmentation was followed in terms of light-scattering changes of DMPC MLV. The intensity of the Trp emission peak changed almost simultaneously with that of light-scattering values. This means that the fragmentation of liposomes occurs concomitantly with the binding of glucagon.

Characterization of DMPC-Glucagon Complex—That the C-terminal region of glucagon containing the Trp-25 is in contact with the acyl chain of phospholipid in the glucagon-lipid complex was shown already by fluorescence spectroscopy. The N-terminal half, which contains both of the Tyr residues (Tyr-10 and Tyr-13) in glucagon, was also found to be bound to phospholipids. The absorption of UV light by Tyr residues is largely masked by the stronger

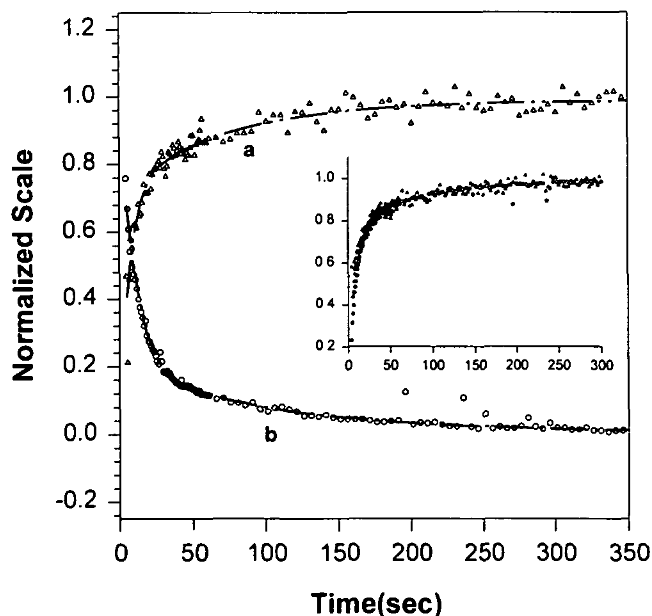

Fig. 7. **Comparison of the time-courses of glucagon binding (a) and vesicle fragmentation (b).** The binding process was monitored in terms of the Trp-emission at 350 nm with excitation at 295 nm. The fragmentation was followed by measuring light-scattering at 600 nm. A 0.1 mM DMPC MLV solution which also contained 0.5 M NaCl and 20 mM glycine (pH 3.0) was pre-incubated in a spectrofluorometer cell at 20°C for 30 min and then a small volume of glucagon solution was added (the final concentration of glucagon was 10 μM). The L/P ratio of glucagon MLV mixture was 10. Three experimental results were averaged. These time-courses were curve-fitted into an exponential decay curve. The binding (△) and fragmentation (○) curves were best fitted to the equations $Y=Y_0-(A_1e[-(X-X_0)/t_1] + A_2e[-(X-X_0)])$ and $Y=Y_0+A_1e[-(X-X_0)/t_1]+A_2e[-(X-X_0)]$. The experimental parameters obtained are $t_1=6.61$, $A_1=0.665$, $t_2=72.7$, $A_2=0.2638$ for the binding and $t_1=9.93$, $A_1=0.762$, $t_2=100$, $A_2=0.199$ for the fragmentation. The inset shows that binding and fragmentation kinetics overlap.

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absorption of Trp residues. Since, however, the second-derivative UV spectrum of Tyr is sensitive to the hydrophobicity of the environment, the degree of exposure of Tyr residues can be evaluated in terms of the ratio of the two peak-to-peak distances, even in the presence of a relatively high Trp content (*24*). The ratio of the between two peak-to-peak distances (r_a/r_b) in the second-derivative UV spectrum is proportional to the polarity of the Tyr residue. Table I gives the r_a/r_b ratios of glucagon under various conditions. This table also includes the r_a/r_b ratios of glucagon in 95% ethylene glycol as well as in various aqueous solutions for comparison. It was reported that the hydrophobicity of the protein interior is about 1.2 times that of pure ethylene glycol (*25*). The r_a/r_b values of the glucagon-DMPC complex with the L/P ratio of 10 at pH 3 and 7 were 0.72 and 1.02, respectively. Also the r_a/r_b values of the isolated discoidal complex at pH 3 and 7 were 0.52 and 0.68, respectively. These differences are thought to originate from the more extensive binding of glucagon to lipid at pH 3 than at pH 7. The second-derivative spectra suggest that the N-terminal region of glucagon, which contains Tyr-10 and Tyr-13, is also in contact with the acyl chains of phospholipids in the discoidal complex. Because of extensive light-scattering, the second-derivative UV spectrum of the vesicular complex was not obtained.

Figure 8 shows the CD spectra of glucagon under various conditions. As expected, this peptide has little secondary structure in a pH 3 buffer without NaCl and shows the largest α-helical content in TFE solution. In order to minimize the effect of free glucagon on the CD spectra, the discoidal complex was isolated by passing it through a gel filtration column. Figure 9 shows that a clear-cut separation of the discoidal complex from the free glucagon was achieved. The values of L/P ratio of the discoidal complex used in these experiments at pH 3 and 7 were found to be about 35 and 40, respectively. The discoidal complex obtained following the procedure of Jones *et al.* (*10*), where glucagon was introduced into a test tube lined with a thin film of DMPC on the side wall followed by vortexing at 45°C, had an L/P ratio of about 70. The α-helical contents of discoidal and vesicular complexes prepared at the same pH of either 3 or 7 were almost the same. The discoidal

TABLE I. **Ratios of the two peak-to-peak distances (r_a/r_b) from the second-derivative band of Tyr and Trp of glucagon under various conditions.**

Experimental conditions	r_a/r_b[a]
95% ethylene glycol	0.68
0.01 N HCl solution	1.23
pH 3 NaCl 0.5 M buffer solution	1.24
Isolated discoidal complex at pH 3 NaCl 0.5 M	0.52
Isolated discoidal complex at pH 7 NaCl 0.5 M	0.68
Glucagon-DMPC complex at pH 3 NaCl 0.5 M (L/P 20)	0.72
Glucagon-DMPC complex at pH 7 NaCl 0.5 M (L/P 20)	1.02

[a]The ratio of the values of 288-283 nm and 295-290.5 nm.

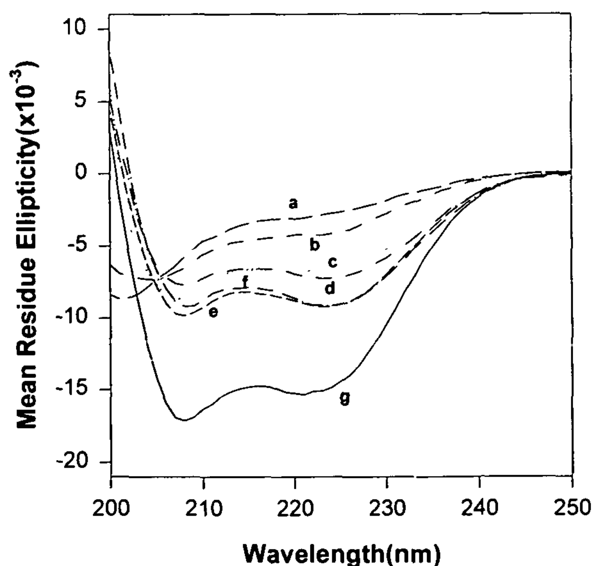

Fig. 8. **CD spectra of glucagon under various conditions.** A 50 μM glucagon solution was prepared in (a) pH 3.0 buffer containing no NaCl, (b) pH 3.0 buffer containing 0.5 M NaCl, (g) in TFE 80%. (c) and (e) are CD spectra of 40 μM glucagon in a pH 7.0 buffer containing 0.5 M NaCl and a pH 3.0 buffer containing 0.5 M NaCl, both containing 4 mM SUV. (d) and (f) are CD spectra of isolated DMPC-glucagon discoidal complex which was made under the condition of pH 7.0, 0.5 M NaCl, and pH 3.0, 0.5 M NaCl, respectively.

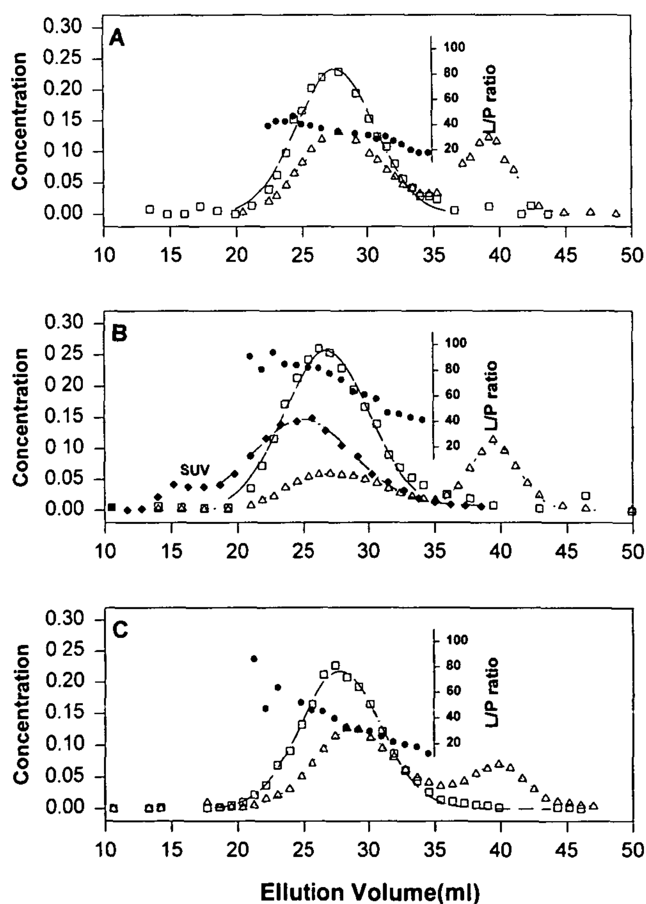

Fig. 9. **Sepharose CL-4B gel filtration chromatography of glucagon-lipid complex.** The complexes of glucagon and DMPC MLV were formed and isolated chromatographically, eluting with (A) pH 7 (40 mM ammonium acetate, 0.5 M NaCl), (B) pH 7 [after Jones *et al.* (*10*)], and (C) pH 3 (20 mM glycine, 0.5 M NaCl) buffer. The glucagon concentration (△) and lipid concentration (□) were determined by modified Lowry assay and phosphate assay. Sonified DMPC SUV were loaded into the same column [◆ in (B)]. The L/P ratios determined are also given.

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complex at pH 3 had a somewhat higher α-helical content than at pH 7. The CD spectrum of the discoidal complex prepared by using the method of Jones *et al.* (*10*) at pH 7 was the same as that shown in Fig. 8 (line d, data not shown).

Braun *et al.* (*26*) determined the conformation of glucagon bound to perdeuteriated dodecylphosphocholine micelles by 2D NMR. They found that the secondary structures of glucagon in the micellar complex are composed of an irregular α-helix of residues 17-29, an extended structure of residues 14-17 and residues 5 to 10 and an α-helix-like turn of residues 10-14. The backbone is located close to and runs roughly parallel to the micelle surface. Their distance geometry analysis suggested the presence of a hydrophobic patch formed by the side-chains of Phe-6, Tyr-10, and Leu-14 and also by the side-chains of Ala-19, Phe-22, Val-23, Trp-25, and Leu-26. The second derivative absorption spectra and the intrinsic fluorescence of Trp obtained here suggest that Tyr-10 and/or Tyr13 and Trp-25 are in contact with the hydrophobic tail part of lipids in the discoidal complex. If the segment of glucagon containing these three residues forms an unbroken α-helix in the discoidal complex, 55% of the amino acids should be in this structure. This α-helical content is much larger than the CD results indicate. It appears, therefore, that smaller segments including Tyr-10 and/or Tyr-13 and Trp-25 may be in contact with the acyl chains in the vesicular complex and the periphery of the discoidal complex. It is also possible that some secondary structures other than α-helix may exist in the discoidal complex.

Recently, Kimura *et al.* (*27*) determined the depth of glucagon penetration into the bilayer using the fluorescence quenching of Trp residue by doxyl-5 and -16 stearic acids incorporated into SUV. They concluded that glucagon is located near the bilayer surface. Somewhat earlier, we did the same experiment with doxyl-7 and -10 stearic acid in addition to doxyl-5 and -16 stearic acids. Figure 10 shows

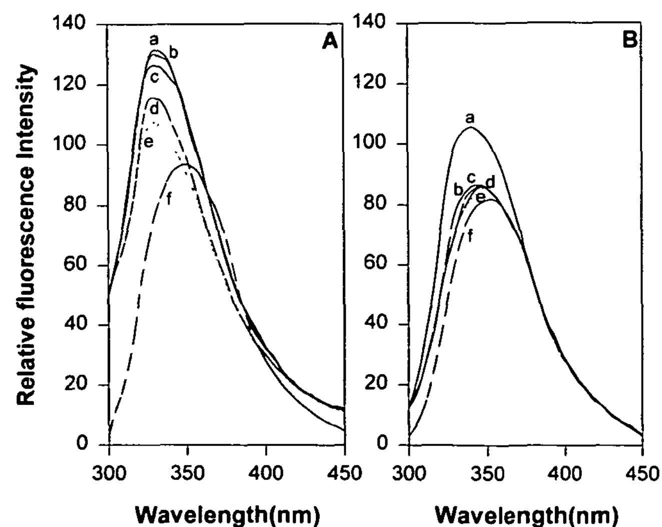

Fig. 10. **Effect of doxyl stearic acid methyl esters on the fluorescence of Trp of glucagon at pH 3.3.** (A) L/P ratio of 175, vesicular complex. (B) L/P ratio of 20, discoidal complex. All samples are consisted of 10 μM glucagon and DMPC with 10% (a) stearic acid, (b) 16-doxyl, (c) 12-doxyl, (d) 7-doxyl, and (e) 5-doxyl stearic acid methyl ester except (f), glucagon alone (10 μM).

the effect of the stearic acid analogs containing a doxyl spin-label at different positions along the chain on the Trp-25 fluorescence in the vesicular glucagon-DMPC complex. SUV was used to minimize the effect of light scattering. It can be seen that the doxyl label at the 5-position quenched the fluorescence of the Trp of glucagon most strongly, and the effect decreased gradually as the doxyl group was moved toward the end of the acyl chain in the vesicular complex (Fig. 10A). This result suggests qualitatively that Trp is located near the interfacial region of the lipid bilayer, confirming the conclusion drawn by Kimura *et al.* (*27*). When glucagon formed discoidal complexes with DMPC SUV containing doxyl stearic acids, the extent of quenching was similar regardless of the position of the doxyl group (Fig. 10B). This means that the topologies of glucagon in vesicular complex and discoidal complex are quite different. At least a major portion of the glucagon should line the periphery of the discoidal complex, segregating the acyl chain from the aqueous solution. This, in turn, implies that the glucagon in the discoidal complex assumes an amphiphilic conformation which is possible when this peptide is mainly in the form of a helix. As to the orientation of glucagon on the periphery of the discoids, it could be either normal to the bilayer surface, parallel to the surface or randomly oriented. The orientation normal to the bilayer surface is inconsistent with the results of Trp fluorescence quenching by doxyl groups. So the glucagon is either oriented parallel to the bilayer surface or it is randomly oriented on the periphery of the discoids.

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