Fusion of Phospholipid Vesicles Induced by an Amphiphilic Model Peptide: Close Correlation between Fusogenicity and Hydrophobicity of the Peptide in an α-Helix†

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ABSTRACT: A model peptide with 51 amino acid residues consisting of tandem repeats of a Lys-Lys-Leu-Leu sequence and a turn sequence of Asn-Pro-Gly at the center of the molecule has a random conformation at neutral pH but adopts an amphiphilic α-helical form in the presence of various salts or nucleotides [Goto, Y., & Aimoto, S. (1991) *J. Mol. Biol. 218*, 387–396; Goto, Y., Okamura, N., & Aimoto, S. (1991) *J. Biochem. (Tokyo) 109*, 746–750]. The interaction of this model peptide with liposome membranes and the resulting α-helical conformational transition and membrane fusion as well as the effect of the nucleotide ATP on these events were examined at neutral pH. The peptide associated stoichiometrically with liposome membranes composed of phosphatidylserine (PS) and phosphatidylcholine (PC) in a molar ratio of 2:1, resulting in formation of an amphiphilic α-helix and induction of fusion of the liposomes. However, the final fusion level was not correlated with the amount of binding or the helix content and was found to increase on an increase in hydrophobicity of the peptide in the α-helical form by neutralization of its positive charges by the negative charges of PS. In contrast, in the presence of ATP, the peptide bound completely to the PS/PC membranes at a lower concentration of liposome and concomitantly induced membrane fusion, indicating that ATP cooperates with PS to neutralize the charges of the peptide. When increasing amounts of ATP were added to a mixture of the peptide and low amounts of PS/PC vesicles that caused a low level of fusion, the helix content increased monotonously, while the binding of the peptide to the membranes and the extent of fusion increased transitionally at an ATP concentration at which the net charge of the peptide was greatly reduced by neutralization of its positive charges by the negative charges of both PS and ATP. The model peptide did not associate with liposome membranes composed of PC alone, and, therefore, did not acquire any activity for helix formation or membrane fusion. However, the peptide interacted with PC liposome membranes and triggered fusion when it became fairly hydrophobic by binding of ATP. These results provide evidence that the fusogenicity of the peptide is correlated with its hydrophobicity rather than amphiphilicity in an α-helix.

An amphipathic α-helix is defined as an α-helix in which the distribution of amino acid residues results in opposing polar and nonpolar faces. It is an important structural unit in various proteins and peptides, such as several peptide hormones (Kaiser & Kezdy, 1984), cytolytic peptides (Bernheimer & Rudy, 1986), virus fusion proteins (White, 1990), mitochondrial precursor proteins (Lemire et al., 1989), and apolipoproteins (Anantharamaiah, 1986), and is responsible for interaction with biological membranes to elicit their biological functions. One of these functions is membrane fusion activity. Melittin (Morgan et al., 1983), an albumin fragment (Garcia et al., 1984), and various synthetic peptides (Parente et al., 1988; Suenaga et al., 1989; Kono et al., 1990) can form amphipathic α-helices in relation to induction of membrane fusion. Most viral spike proteins, such as orthomyxovirus hemagglutinin, paramyxovirus fusion proteins, and envelope glycoproteins of human immunodeficiency virus, contain amino-terminal fusion peptides, which are likely to adopt an α-helical configuration during the fusion reaction (White, 1990). Synthetic fusion peptides with the same amino acid sequence as the N-terminal region of influenza virus hemagglutinin have activities for both helix formation and membrane fusion (Murata et al., 1987; Takahashi, 1990). However, the relationships between the amphiphilicity, helicity, and fusogenicity of these proteins and peptides and their molecular mechanism of induction of membrane fusion are still unknown.

For elucidation of the molecular mechanism of protein-induced membrane fusion, we have studied fusion of phospholipid vesicles induced by the protein clathrin and have almost completely elucidated its mechanism (Hong et al., 1985; Yoshimura et al., 1987; Maezawa et al., 1989; Maezawa & Yoshimura, 1990a,b, 1991). On the other hand, Goto and co-workers studied the mechanism of salt- and acid-dependent formation of molten globules of various proteins (Goto & Fink, 1989, 1990; Goto et al., 1990a,b). In their series of studies, they synthesized a model peptide with 51 amino acid residues, which consists of tandem repeats of a Lys-Lys-Leu-Leu sequence and a turn sequence of Asn-Pro-Gly at the center of the molecule, assuming that it would form two amphiphilic α-helices interacting with each other intramolecularly through hydrophobic leucine residues,[1] and found that it indeed adopts

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[1] Strictly speaking, this peptide is not in the category of an amphipathic α-helix, but the lysine and leucine residues form somewhat twisted opposing polar and nonpolar faces. We name this type of α-helix an "amphiphilic" α-helix.

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an amphiphilic α-helical conformation in the presence of various salts (Goto & Aimoto, 1991) and nucleotides (Goto et al., 1991). In the present study, we examined the interaction of this model peptide with liposome membranes and the resulting α-helical conformational transition and membrane fusion as well as the effect of the nucleotide ATP on these events at neutral pH. Our results showed that the hydrophobicity rather than the amphiphilicity of the peptide in an α-helical form has a significant role in induction of membrane fusion.

Experimental Procedures

Materials. A model peptide with the sequence H-(Acm-Cys)-(Lys-Lys-Leu-Leu)$_5$-Lys-Lys-Asn-Pro-Gly-(Leu-Leu-Lys-Lys)$_6$-Tyr-NH_2 was synthesized with an ABI 430A peptide synthesizer by the Boc-amino acid anhydride method (Goto & Aimoto, 1991). The resulting peptide mixture was fractionated on a preparative C_{18} HPLC column, and the main fraction eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid was collected. The amino acid composition of the preparation was consistent with that expected from the designed sequence. The polypeptide concentration was determined by amino acid analysis. Bovine brain PS,[2] egg PC, NBD-PE, and Rh-PE were purchased from Avanti Polar Lipids (Birmingham, AL). ATP was obtained from Kohjin (Tokyo, Japan). All other reagents were commercial products of reagent grade.

Liposome Preparation. LUV and LUV labeled with both NBD-PE and Rh-PE at 1 or 0.1 mol % each were prepared in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) with the modifications described by Wilschut et al. (1980) and then filtered through polycarbonate membranes of 0.1-μm pore size (Szoka et al., 1980).

MLV were prepared by vortex mixing of the lipid film in the same buffer at 30 °C for 1 min. The resulting vesicles were passed through polycarbonate membranes of 0.4-μm pore size and washed 4–5 times by centrifugation at 13000g for 4.5 min to remove small multilamellar and unilamellar vesicles.

The liposome concentration was determined by measuring total lipid phosphorus by the method of Bartlett (1959).

Fusion Assay. Membrane fusion was measured at 25 °C in 10 mM Tris-HCl (pH 7.5) as described by Maezawa et al. (1989). Fusion was initiated by addition of the model peptide to a mixture of LUV labeled with both NBD-PE and Rh-PE at 1 mol % each, and unlabeled LUV, at a molar ratio of 1:9. The extent of fusion was calibrated taking the fluorescence of the labeled and unlabeled LUV mixture before addition of the peptide as zero fusion and the fluorescence of LUV containing the two fluorescence probes at 0.1 mol % each as the 100% fusion level.

Binding Assay. The binding of the model peptide to liposome membranes was determined by a centrifugation procedure essentially as described by Yoshimura and Sone (1987). The peptide was incubated at 25 °C for 30 min in 10 mM Tris-HCl (pH 7.5) in the absence and presence of MLV or LUV. The mixtures were then centrifuged at 13000g for 5 min for MLV or 180000g for 5 h for LUV, and the amount of peptide in the supernatant was determined by the method of Bradford (1976). The amount of membrane-bound peptide was expressed relative to the amount of peptide in the supernatant in the absence of MLV or LUV.

Peptide Aggregation Assay. The aggregation of the peptide induced by ATP was measured essentially as described by Yoshimura et al. (1987). The peptide was incubated with ATP at 25 °C for 30 min in 10 mM Tris-HCl (pH 7.5). The mixture was then centrifuged at 13000g for 5 min, unless otherwise specified, and the amount of peptide in the supernatant was determined by the method of Bradford (1976). The amount of aggregated peptide was expressed relative to the amount of peptide in the absence of ATP.

CD Measurement. CD spectra were measured from 195 to 250 nm at 20 °C in 10 mM Tris-HCl (pH 7.5) in a cell of 1- or 2-mm light path with a Jasco spectropolarimeter, model J-500A, equipped with an interface and a personal computer. The data were expressed as mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100\theta_{obsd}/(lc)$ where θ_{obsd} is the observed ellipticity in degrees, c is the concentration in residual moles per liter, and l is the length of the light path in centimeters.

Results

Interaction of the Model Peptide with ATP. Previously, Goto et al. (1991) found that a synthetic model peptide with tandem repeats of a Lys-Lys-Leu-Leu sequence forms an amphiphilic α-helix at neutral pH in the presence of ATP. The effect of ATP on helix formation of this model peptide was examined in more detail at the peptide concentration used in later experiments. As shown in Figure 1A, CD spectra were initially changed with an isosbestic wavelength of about 204 nm, but above an ATP concentration of 25 μM the CD curves did not pass through this isosbestic point, and the depth of the trough around 222 nm was greater than that around 208 nm. Two phases were observed in the plots of the mean residue ellipticity at 222 nm and the ratio of the residue ellipticity at 222 nm to that at 208 nm (hereafter referred to as the CD ratio) against the ATP concentration (Figure 1B,C), and the CD ratio was reversed above an ATP concentration of 20–25 μM.

On the other hand, the peptide was aggregated above an ATP concentration of about 20 μM, and the ATP concentration at the midpoint transition was close to its concentration at which the CD ratio was reversed (Figure 1D). In addition, the critical concentration of ATP for reversal of the CD ratio and peptide aggregation was found to depend on the ratio of ATP to peptide: for instance, at a peptide concentration of 10 μM, the CD ratio was reversed and the peptide tended to aggregate above an ATP concentration of 45 μM (Goto et al., 1991), indicating that ATP molecules bind stoichiometrically to the peptide. At a peptide concentration of 6 μM, complete aggregation was observed at an ATP concentration of 30 μM (Figure 1D). Since ATP has four negatively charged groups at neutral pH (Taqui Khan & Martell, 1962) and the model peptide has 24 lysine residues, this result indicated that approximately 83% of the positive charges were neutralized by binding of negatively charged ATP, that aggregation occurred when the peptide became fairly hydrophobic, and consequently that the CD ratio was reversed: in other words, the charge neutralization, that is, the hydrophobicity of the peptide, can be evaluated by the CD ratio (see Discussion).

Interaction of the Model Peptide with Liposome Membranes. The above results and previous studies (Goto et al., 1991) show that, at neutral pH, ATP molecules bind to the model peptide and induce its helical structure. This model peptide probably associates with membranes and consequently forms an amphiphilic α-helix. Therefore, we investigated the

[2] Abbreviations: LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, phosphatidylcholine; PS, phosphatidylserine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine.

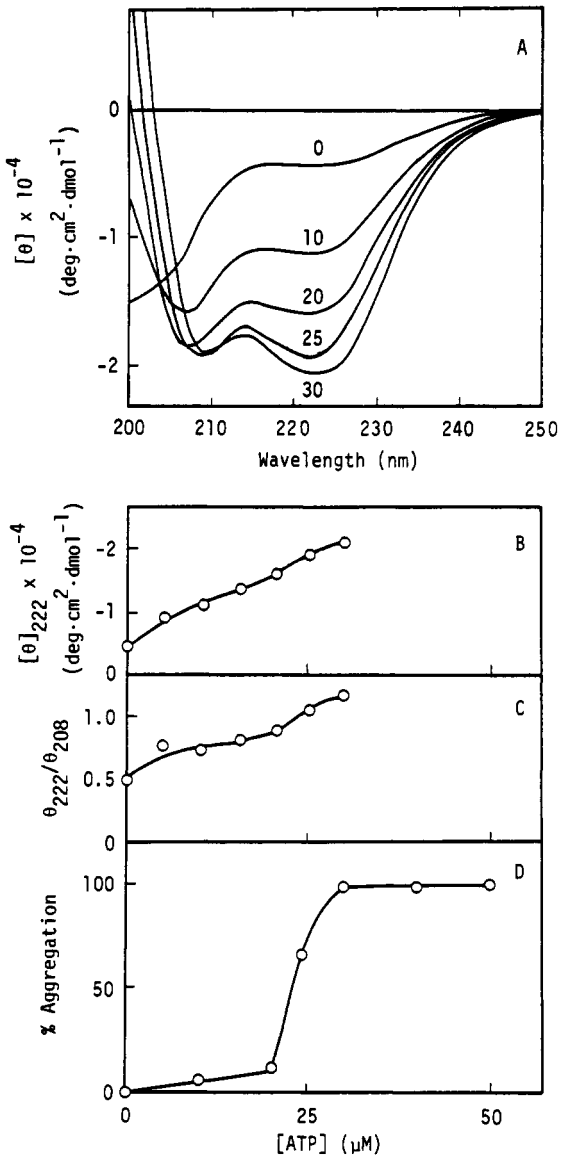

FIGURE 1: ATP-induced formation of α-helix and aggregation of the synthetic model peptide. CD spectra and the amount of aggregated peptides were measured at a peptide concentration of 6 μM in 10 mM Tris-HCl (pH 7.5). Typical CD spectra of the peptide are illustrated at the indicated micromolar ATP concentrations (A). The mean residue ellipticity at 222 nm (B), the ratio of ellipticity at 222 nm to that at 208 nm (CD ratio) (C), and the percentage of aggregated peptide (D) are plotted as functions of ATP concentration.

interaction of this peptide with PS/PC (2:1) and PC vesicles and its helix formation at pH 7.5 in the presence of these liposomes.

Figure 2 shows the CD spectra of the model peptide in the presence of PS/PC (2:1) and PC LUV. The CD spectrum of the peptide alone indicated a random conformation, but on addition of PS/PC (2:1) LUV, the ellipticities increased (Figure 2A). The mean residue ellipticities at 222 nm were plotted as functions of the PS/PC (2:1) liposome concentration at two peptide concentrations, 6 and 12 μM, and were found to change depending on the ratio of peptide to liposomes (Figure 3A). In contrast, little change in the CD spectrum was observed with PC LUV (Figures 2B and 3C).

The associations of the peptide with PS/PC (2:1) MLV and LUV and PC MLV are shown in Figure 3, panels B and D, respectively. The peptide associated with the former two vesicles, but scarcely with the latter, and although MLV are known to have two or three times less surface area than LUV containing the same amount of lipid, the dependence of the amount of binding on the liposome concentration for PS/PC (2:1) LUV was quite similar to that for PS/PC (2:1) MLV. This seemed to be due to the difference in surface areas of the outer layers of the two types of liposomes, because the average diameters of MLV and LUV were about 0.4 and 0.1 μm, respectively (see Experimental Procedures). The results indicates that MLV can be used instead of LUV in binding assays. Moreover, the amount of binding also depended on the ratio of peptide to liposomes and was well correlated with the helix content. These results indicate that the peptide binds stoichiometrically to liposome membranes containing PS and as a result changes in conformation to an amphiphilic α-helix.

Fusion of Liposomes Induced by the Model Peptide. Next, we examined whether the model peptide induces fusion at neutral pH. Addition of increasing amounts of peptide to PS/PC (2:1) LUV resulted in dequenching of the NBD fluorescence, indicating fusogenicity of this peptide. Figure 4A shows the final levels of fusion induced by the peptide. At a peptide concentration of 6 μM, the final fusion level was only 3% at a liposome concentration of 50 μM (expressed as phospholipid concentration), but, at higher liposome concentrations of 100 μM, steep increase to 72% and then a decrease were observed, the decrease probably being due to excess of liposomes to peptide. The rate of fusion was also dependent on the liposome concentration, being 3% s^{-1} and 14% s^{-1} at 50 and 150 μM, respectively (data not shown). At a peptide concentration of 3 μM, the fusion profile was similar to that at a concentration of 6 μM, but a steep increase in the fusion level was observed above a liposome concentration of about 50 μM, that is, above the same peptide-to-liposome ratio as in the case with 6 μM peptide. At this concentration, the association of the peptide with PS/PC (2:1) MLV was complete at half the liposome concentration at which 6 μM peptide bound completely (Figure 4B), the same tendency as observed in Figure 3B. However, at both peptide concentrations, the final fusion level was not correlated with the binding and appeared to increase when the peptide bound almost completely to liposome membranes. Figures 3A and 4A also show that the extent of fusion is unrelated to the helix content of the peptide.

As seen in Figure 2A, in the presence of higher amounts of PS/PC (2:1) LUV, the CD curves of the peptide did not pass through an isosbestic point of about 204 nm, and the depth of the trough around 222 nm was greater than that around 208 nm, as in the CD spectra with ATP. As shown in Figure 4C, at a peptide concentration of 6 μM, an additional phase was observed in the plot of the CD ratio as a function of the liposome concentration (also seen in Figure 3A), and the CD ratio was reversed above a liposome concentration of about 100 μM, the concentration at which the final fusion level increased. Thus these results suggest that the extent of fusion increases when the positive charges of the peptide are considerably neutralized by the negative charges of PS, that is, when the peptide becomes fairly hydrophobic.

Effect of ATP on Fusion of Liposomes Induced by the Model Peptide. To clarify the relationship between hydrophobicity and fusogenicity of the amphiphilic peptide, we examined the effect of ATP on various events induced by this peptide at pH 7.5.

First, we examined fusion of PS/PC (2:1) LUV, association with the same type of MLV and LUV, helix formation, and the CD ratio in the presence of 20 μM ATP. The results in Figure 5 show that although the extent of fusion increased at a liposome concentration of 100 μM in the absence of ATP, in its presence, extensive fusion occurred at a much lower

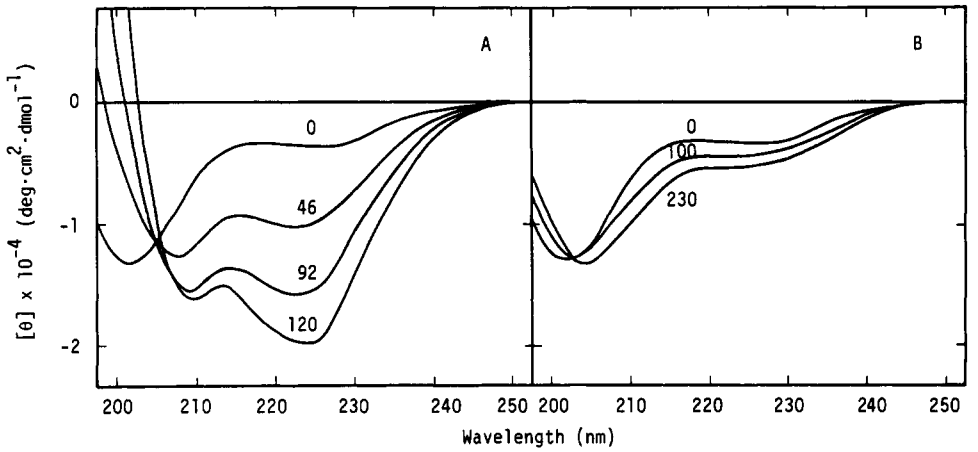

FIGURE 2: CD spectra of the synthetic model peptide in the presence of PS/PC (2:1) LUV (A) and PC LUV (B). Measurements were carried out at a peptide concentration of 6 μM in 10 mM Tris-HCl (pH 7.5). The numbers indicate liposome concentrations in micromolar phospholipid.

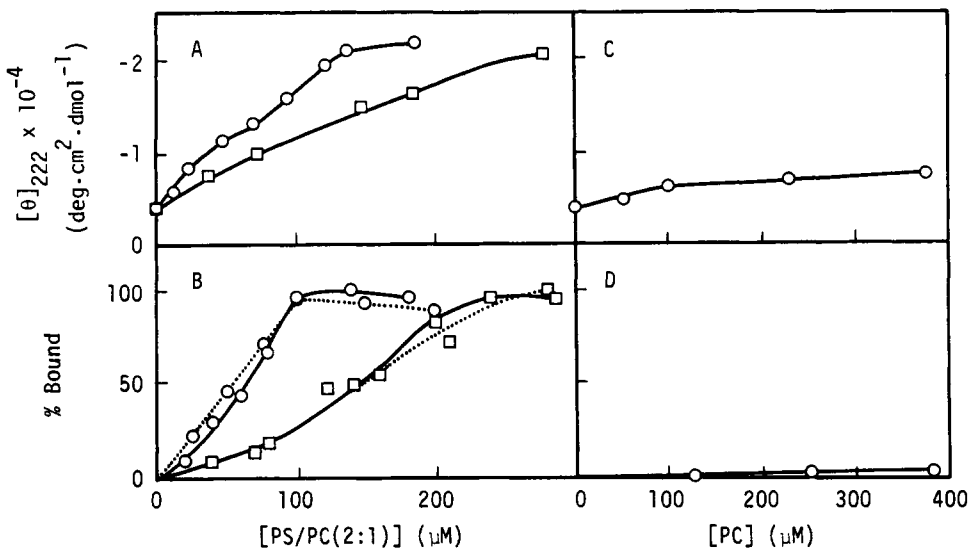

FIGURE 3: Formation of α-helix of the synthetic model peptide in the presence of PS/PC (2:1) LUV (A) and PC LUV (C) and its association with PS/PC (2:1) MLV and LUV (B) and PC MLV (D). Bindings and CD spectra were measured at peptide concentrations of 6 (O) and 12 (□) μM in 10 mM Tris-HCl (pH 7.5). Solid and dotted lines in B indicate bindings to MLV and LUV, respectively.

concentration of liposomes (Figure 5A), and binding of the peptide increased steeply, resulting in complete binding at a liposome concentration of 20–30 μM (Figure 5B). In the presence of ATP, the mean residue ellipticity increased in parallel with that in the absence of ATP, although helix formation occurred in the absence of liposomes (Figure 5C). In contrast, the CD ratio was reversed above a liposome concentration of about 20 μM, being much lower than that in the absence of ATP (Figure 5D). These results indicate that the positive charges of the peptide are neutralized by the negative charges of both PS and ATP, which make the peptide hydrophobic and concomitantly trigger fusion, and that ATP is cooperative, not competitive, with PS.

Next, we examined these events in PS/PC (2:1) vesicles by varying the ATP concentration in the presence of 50 μM liposomes, in which the final fusion and binding levels were 5% and 37%, respectively, in the absence of ATP. As shown in Figure 6A, the final fusion level was increased by addition of more than 10 μM ATP. The association of the peptide with the membranes also increased steeply up to 100% above an ATP concentration of about 10 μM (Figure 6B). In contrast, the helix content increased monotonously in a similar manner to that in the absence of PS/PC LUV, although partial helix formation was observed without ATP (Figure 6C). Nevertheless, the CD ratio was reversed above an ATP concentration of about 10 μM (Figure 6D). These results support the idea that fusion occurs when the peptide becomes fairly hydrophobic by neutralization of its positive charges by the negative charges of both PS and ATP.

As shown in Figure 3D, the model peptide scarcely bound to PC MLV and consequently did not induce fusion of PC LUV (data not shown). But as it is quite likely that fusion occurs when the peptide becomes hydrophobic, we examined the effect of ATP on the four events at a PC liposome concentration of 50 μM. As expected, fusion was triggered, and the association was increased steeply by addition of more than 20 μM ATP (Figure 7A,B). However, the peptide aggregated above an ATP concentration of about 20 μM (Figure 1D), and thus self-aggregated peptide could not be distinguished from the membrane-associated form by binding assay using MLV. Therefore, we attempted to separate the two species using LUV and low- and high-speed centrifugation procedures. As shown in Table I, at an ATP concentration of 40 μM, centrifugation at 180g for 5 min, which did not precipitate PC LUV, precipitated about 80% of the peptide in the absence of PC LUV but only about 20% of the peptide in the presence of PC LUV, while centrifugation at 180000g for 5 h, which precipitated the LUV, precipitated both species completely. These data indicate that the peptide associates with PC membranes before or after self-aggregation and that the data in Figure 7B show

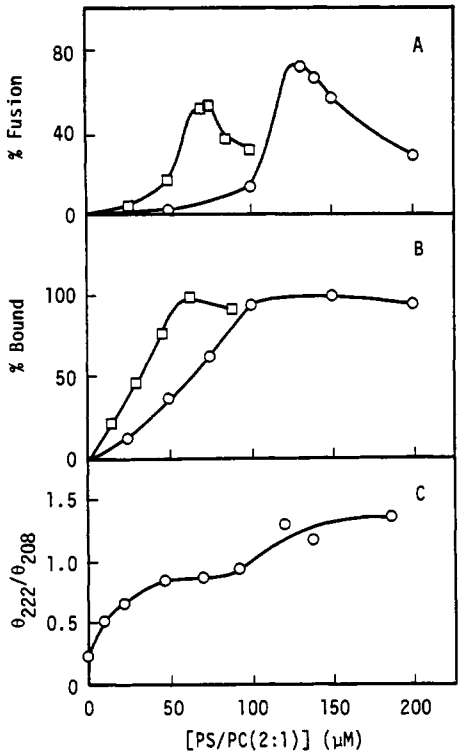

FIGURE 4: Fusion of PS/PC (2:1) LUV induced by the synthetic model peptide (A), its association with the same type of MLV (B), and its CD ratio ($\theta_{222}/\theta_{208}$) in the presence of the same LUV (C). Measurements were made at peptide concentrations of 6 (○) and 3 (□) μM in 10 mM Tris-HCl (pH 7.5).

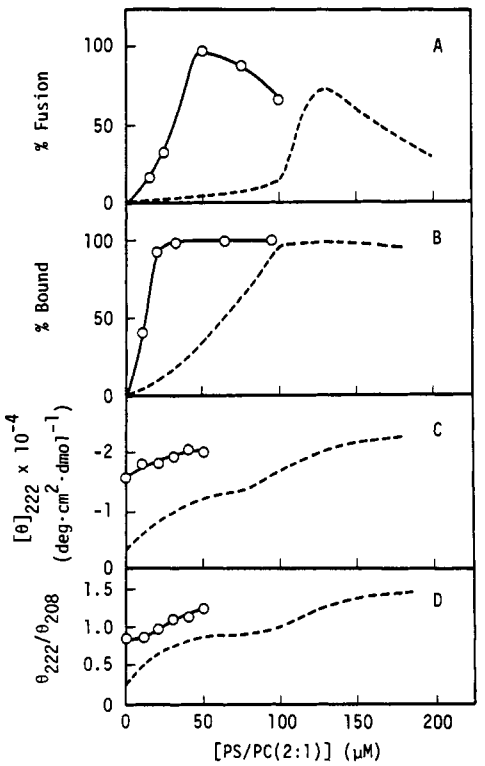

FIGURE 5: Effect of ATP on fusion of PS/PC (2:1) LUV induced by the synthetic model peptide (A), its association with the same type of MLV and LUV (B), and its helix formation (C) and CD ratio (D) in the presence of the same LUV. Measurements were performed at a peptide concentration of 6 μM in 10 mM Tris-HCl (pH 7.5) in the presence of 20 μM ATP. Solid and dotted lines in B show bindings to MLV and LUV, respectively. Dashed lines indicate these four profiles in the absence of ATP as shown in Figures 3A,B and 4A,C.

the actual association of the peptide with the PC membranes. At an ATP concentration of 10 μM, which rarely induced

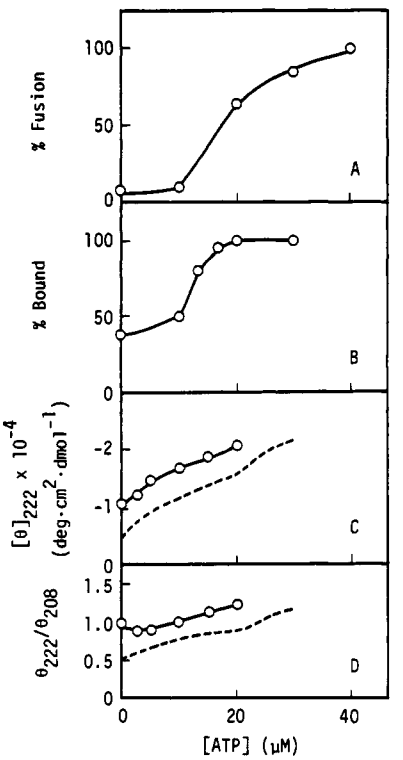

FIGURE 6: Fusion of PS/PC (2:1) LUV induced by the synthetic model peptide (A), its association with the same type of MLV (B), and its helix formation (C) and CD ratio (D) with LUV of the same lipid composition in the presence of various concentrations of ATP at pH 7.5. The peptide and liposomes at concentrations of 6 and 50 μM, respectively, were used. The dashed lines indicate the latter two profiles in the absence of liposomes as shown in Figure 1B,C.

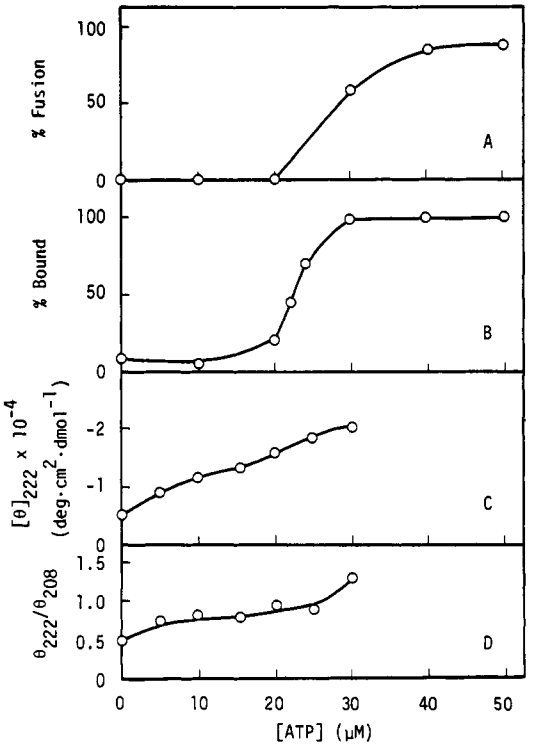

FIGURE 7: Fusion of PC LUV induced by the synthetic model peptide (A), its association with PC MLV (B), and its helix formation (C) and CD ratio (D) with PC LUV in the presence of various concentrations of ATP at pH 7.5. The peptide and liposomes were used at concentrations of 6 and 50 μM, respectively.

self-aggregation of the peptide, there was no change in the amount of peptide in the supernatant after either low- or high-speed centrifugation in the absence and presence of PC

Table I: Distinction of Self-Aggregated and PC Membrane-Associated Peptides by Low- and High-Speed Centrifugation

sample	ATP (μM)	centrifugation	further addition	% supernatant
liposome[a]	0	low[c]	none	98
		high[d]	none	4
peptide[b]	0	low	none	100
			liposome	100
		high	none	100
			liposome	95
	10	low	none	91
			liposome	85
		high	none	72
			liposome	78
	40	low	none	19
			liposome	77
		high	none	0
			liposome	3

[a] PC LUV were incubated at 25 °C for 30 min in 10 mM Tris-HCl (pH 7.5), and the amount of lipid in the supernatant of the liposome solution after centrifugation was determined by the method of Bartlett (1959) and expressed as a percentage of the amount of lipid in the solution before centrifugation. [b] The peptide was incubated at 25 °C for 30 min in 10 mM Tris-HCl (pH 7.5) in the absence and presence of PC LUV, and the amount of peptide in the supernatant of this mixture after centrifugation was determined by the method of Bradford (1976) and expressed as a percentage of the amount of peptide in the mixture before centrifugation. [c] Centrifuged at 180*g* for 5 min. [d] Centrifuged at 180000*g* for 5 h.

LUV, indicating no association of the peptide with the membranes. The effects of ATP on helix formation and the CD ratio were identical to those in the absence of PC LUV: the CD ratio was reversed above an ATP concentration of about 25 μM (Figure 7C,D). These results show that the hydrophobic interaction between the amphiphilic peptide and PC liposomes results in induction of fusion.

Discussion

Previously, Goto and Aimoto (1991) synthesized a model peptide consisting of tandem repeats of a Lys-Lys-Leu-Leu sequence with the turn sequence Asn-Pro-Gly in the center of the molecule and found that it adopts an amphiphilic α-helix in the presence of various salts (Goto & Aimoto, 1991) and nucleotides (Goto et al., 1991), that is, anions. In the present study, we examined the ability of the peptide to bind to membranes and consequently to adopt an α-helical conformation and induce membrane fusion at neutral pH, using liposome membrane systems.

First, we found that this model peptide interacts electrostatically with liposome membranes containing PS, resulting in formation of an amphiphilic α-helix. This has also been shown to be the case on its interaction with ATP (Goto et al., 1991). The association of the peptide with membrane PS or ATP and its helix formation were stoichiometric, although there was no significant dependence of the formation of an α-helix induced by sodium perchlorate upon the peptide concentration (Goto & Aimoto, 1991). This difference may be due to much higher and lower binding affinities of PS or ATP and sodium perchlorate, respectively, for the peptide, as the former membraneous PS group and ATP are polyvalent but the latter is a monovalent anion. In addition, we found that extensive binding of ATP to the peptide makes it hydrophobic and then aggregative (Figure 1). A similar increase in hydrophobicity was observed when the model peptide was bound extensively by excess phospholipids (Figures 3B and 4C).

However, one may wonder whether ATP becomes hydrophobic when its negative charges are neutralized. Chloride anions also induce helix formation of the model peptide (Goto & Aimoto, 1991). We have found that the helical form of the peptide induced by extensive binding of this anion, which probably makes the peptide hydrophobic, has an ability to induce fusion of PS/PC liposomes and that the fluorescence maximum of 1-anilinonaphthalene-8-sulfonate (ANS) is shifted to a shorter wavelength and its fluorescence intensity increases in the presence of the peptide and excess NaCl (unpublished results). Similar changes in the fluorescence properties of ANS were observed in the mixture of the peptide and excess ATP, suggesting that the peptide becomes faily hydrophobic when its positive charges are neutralized by the negative charges of ATP.

The hydrophobicity of the peptide could be evaluated by the ratio of residue ellipticity at 222 nm to that at 208 nm (CD ratio) because extensive binding of ATP or PS to the peptide resulted in reversal of the CD ratio. This evaluation is supported by the following two reports. Lau et al. (1984) measured CD spectra of various polyheptapeptides as repeating sequences of tropomyosin and observed lesser and greater ellipticities at 222 nm than at 208 nm with monomer and hydrophobically interacting dimer, respectively. Cooper and Woody (1990) theoretically estimated the effect of coiled-coil formation induced by hydrophobic interactions of two helices on the backbone CD spectrum of the α-helix and showed that the ellipticities decrease and increase at 208 and 222 nm, respectively.

Second, we demonstrated that an increase in hydrophobicity in the α-helix facilitates binding of the model peptide to membranes and unexpectedly that ATP cooperated with PS in this increase. These facts were demonstrated by the following results: (1) the association of the peptide with PS/PC liposome membranes was complete at a lower concentration of liposomes in the presence of ATP than in its absence by partial binding of ATP to the peptide that reversed the CD ratio (Figure 5B,D), (2) although only 37% of the peptide associated with liposome membranes in the presence of 50 μM PS/PC vesicles, its association with the membranes increased transitionally up to 100% by binding an amount of ATP that reversed the CD ratio (Figure 6B,D) and (3) the peptide was able to associate with liposome membranes containing PC alone when the CD ratio was reversed, that is, the peptide became fairly hydrophobic by binding of ATP to its amphipathic α-helix (Figure 7B,D). The increase in the hydrophobicity of the peptide did not enhance helix formation; in these three events, the helix content increased in parallel with that in the absence of ATP or liposomes (Figures 5C, 6C, and 7C).

Third, we found that the final level of fusion was not correlated with the amount of association of the model peptide with the PS/PC membranes or its helix content and that the extent of fusion increased on a steep increase in association of the peptide with the membranes under the conditions as mentioned above, when the CD ratio was reversed and the peptide became fairly hydrophobic on neutralization of its positive charges by the negative charges of PS or ATP (Figures 4A, 5A, 6A, and 7A).

These three findings indicate that, at neutral pH, the model peptide interacts electrostatically with negatively charged phospholipid membranes, inducing its formation of an amphiphilic α-helix, and hydrophobically with acidic as well as neutral phospholipid membranes when the amphiphilic α-helix becomes fairly hydrophobic by extensive binding of ATP or phospholipids to the peptide. The latter hydrophobic interaction is responsible for induction of membrane fusion. In other words, the fusogenicity of the peptide is correlated with

its hydrophobicity rather than amphiphilicity in an α-helix.

Parente et al. (1990a) synthesized two peptides of 30 amino acids with repeating sequences of Glu-Ala-Leu-Ala (GALA) and Leu-Ala-Glu-Ala (LAGA), respectively. They found that GALA, but not LAGA, forms an amphipathic α-helix and that it partitions into membranes to a greater extent than LAGA and, unlike the latter, induces membrane fusion. They concluded that formation of an amphipathic α-helix is required for membrane association of GALA. Takahashi (1990) synthesized several amphiphilic 20-residue peptides and found that, in the presence of phospholipid vesicles, peptides with an α-helical conformation showed fusion activity, whereas those with a β-structure did not. Thus he concluded that helix formation is a prerequisite for membrane fusion. He also suggested that the fusion activity is correlated with exhaustive neutralization of electric charges on the α-helix. Our findings are consistent with these conclusions and Takahashi's suggestion.

The fusogenic model peptide used in this study has 51 amino acid residues. The minimum peptide length for interaction of an amphiphilic α-helical peptide with membranes is estimated to be about 20 amino acid residues (Stegmann et al., 1989; McLean et al., 1991), which may be related to the length of helix formation through the lipid bilayer. Thus a partial α-helix of this model peptide of critical length and hydrophobicity may be enough to induce membrane fusion. However, it is likely that this peptide forms two amphiphilic α-helices which either interact with each other in the bilayer or each penetrate into a different bilayer to form a bridge between two adjacent membranes. The relationship between the helix content and/or helical length and fusogenicity requires study.

When the positive charges of the model peptide were considerably neutralized by the negative charges of ATP, the CD ratio was reversed and the peptide aggregated. Lau et al (1984) and Cooper and Woody (1990) have also reported that the CD ratio increases on coiled-coil (dimer) formation as well as on hydrophobic interaction of two model helices. Thus molecules of the model peptide may assemble with each other on the liposome membranes by hydrophobic interaction to induce membrane fusion, because the CD ratio was reversed when the peptide became fusogenic. Indeed, the assembly or clustering of fusogenic proteins such as viral spike proteins (Morris et al., 1989; Ellens et al., 1990) and clathrin (Maezawa & Yoshimura, 1990b) has been suggested to be involved in membrane fusion induced by these proteins. Moreover, a model has been proposed for influenza virus hemagglutinin-mediated membrane fusion, in which the association of several hemagglutinin molecules creates a small pore (Bentz et al., 1990; Stegmann et al., 1990, 1991; White, 1990). Formation of a transbilayer channel or pore composed of several molecules of peptide is also reported as a mechanism of release of vesicle contents induced by the peptide GALA (Parente et al., 1990b). Thus further studies on the assembly and pore formation of the model peptide used in the present study seem important for elucidation of the mechanism of peptide-induced membrane fusion.

So far, various peptides such as melittin (Morgan et al., 1983), an albumin fragment (Garcia et al., 1984), synthetic peptides (Parente et al., 1988; Suenaga et al., 1989; Kono et al., 1990), amino-terminal fusion peptides of viral fusion spike proteins (White, 1990), and their synthetic peptides (Murata et al., 1987) have been reported to form amphipathic α-helices associated with their fusion activities. As suggested by Parente et al. (1990a), the amphipathic α-helical conformation of a peptide may be linked to its interaction with membranes. The present study indicated that increase in hydrophobicity of an amphiphilic α-helix of a peptide is directly correlated with its fusogenicity. Thus, either penetration of more hydrophobic α-helices of peptides, which could cause perturbation of lipid bilayers (Takahashi, 1990), or assembly of these helices, which could result in fusion-pore formation of strong lipid–peptide interaction, may facilitate triggering of membrane fusion by these peptides.

The amphiphilic α-helix is an important structural unit present in various proteins and peptides such as several peptide hormones, cytolytic peptides, mitochondrial precursor proteins, and apolipoproteins as well as virus spike proteins. The interactions of peptide hormones such as glucagon and calcitonin, cytolytic peptides such as melittin and δ-toxin, and apolipoproteins with membranes or receptor sites are known to occur as a result of formation of an amphipathic α-helix (Kaiser & Kezdy, 1984). Mitochondrial targeting sequences of precursor proteins often form positively charged amphiphilic α-helices (Lemire et al., 1989), but their precise role in protein transport into mitochondria is still unknown. A molten globule state is proposed to be involved in translocation of proteins across membranes (Bychkova et al., 1988), and Goto and Aimoto (1991) reported that this helical state of the model peptide used in the present study is equivalent to the molten globule state. Protein transport into mitochondria is believed to be ATP-dependent. Thus increase in hydrophobicity in an amphiphilic α-helix caused by the cooperative action of ATP and anionic membrane phospholipids might have a crucial role in perturbation of the membrane structure, although ATP hydrolysis has recently been reported to be unnecessary for the actual translocation step (Pfanner et al., 1990). In summary, amphiphilic α-helices of proteins and peptides may be relevant to their interaction with membranes, and their more hydrophobic forms induced by a specific event(s) may be relevant to expression of their biological functions.

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Autophosphorylation of Skeletal Muscle Myosin Light Chain Kinase†

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ABSTRACT: Ca^{2+}/calmodulin-dependent myosin light chain kinase phosphorylates the regulatory light chain of myosin. Rabbit skeletal muscle myosin light chain kinase also catalyzes a Ca^{2+}/calmodulin-dependent autophosphorylation with a rapid rate of incorporation of 1 mol of ^{32}P/mol of kinase and a slower rate of incorporation up to 1.52 mol of ^{32}P/mol. Autophosphorylation was inhibited by a peptide substrate that has a low K_m value for myosin light chain kinase. Autophosphorylation at both rates was concentration-independent, indicating an intramolecular mechanism. There were no significant changes in catalytic properties toward light chain and MgATP substrates or in calmodulin activation properties upon autophosphorylation. After digestion with V8 protease, phosphopeptides were purified and sequenced. Two phosphorylation sites were identified, Ser 160 and Ser 234, with the former associated with the rapid rate of phosphorylation. Both sites are located amino terminal of the catalytic domain. These results indicate that the extended "tail" region of the enzyme can fold into the active site of the kinase.

Myosin light chain kinases catalyze the Ca^{2+}-dependent phosphorylation of myosin regulatory light chains. This phosphorylation results in potentiation of skeletal muscle contraction (Stull et al., 1986; Sweeney & Stull, 1990), whereas it is responsible for the initiation of smooth muscle contraction (Kamm & Stull, 1985; Hartshorne, 1987). Distinct forms of the kinase exist in smooth and skeletal muscle (Stull et al., 1986).

The complete amino acid sequence of the rabbit skeletal muscle myosin light chain kinase has been deduced by direct amino acid sequencing and cDNA cloning (Takio et al., 1986;

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