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Membrane Fusion Activity of Succinylated Melittin Is Triggered by Protonation of Its Carboxyl Groups

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ABSTRACT: The membrane fusion activity of melittin and its succinylated derivative was studied as a function of pH by the transfer of spin-labeled phosphatidylcholine as well as by internal content mixing and electron microscopy. The protonation process of the carboxyl groups introduced into melittin was studied by ^{13}C NMR spectroscopy using the derivative prepared with [1,4-^{13}C]succinic anhydride. Melittin causes fusion of sonicated phosphatidylcholine vesicles in a wide range of pH. In marked contrast, melittin with all four amino groups succinylated induces fusion only at acidic pH lower than 5.2, with the maximum at pH 5.1. The fusion reactions are very rapid, reaching a saturation level within 1 min. The fusion efficiency depends on the peptide-to-phospholipid ratio in the reaction mixture. Trypsinized succinylated melittin, which has lost the four positively charged C-terminal residues, causes aggregation of vesicles at acidic pH but cannot induce fusion. The ^{13}C NMR peaks for the carboxyl and carbonyl groups of succinylated melittin shifted to higher field as the pH was lowered. The pK_a value of the four carboxyl groups was obtained as 5.19 and 4.83 in the presence and absence of vesicles, respectively. The pK_a value in the presence of vesicles agrees quite well with the half-maximal pH for fusion of 5.15, indicating that the fusion activity is triggered by protonation of the carboxyl groups in the hydrophobic segment of the peptide. The higher shift of pK_a value in the presence of vesicles can be due to stabilization of the protonated form by entrance into lipid bilayer hydrocarbon layer. Only a single peak was observed for each carboxyl and carbonyl group at various pH values, indicating fast exchange between the protonated and deprotonated forms of the segment, faster than the NMR time scale of 3 ms. If the protonated segment entered the lipid bilayer, the entrance and return to the surface of bilayer membrane should also be fast.

Membrane fusion plays an essential role in the intracellular sorted transport of materials in endocytic and exocytic processings of cells (Goldstein et al., 1986). It also provides an essential mechanism for enveloped viruses to transfer their genome into the target cell cytoplasm, a crucial initial step in infection. Elucidation of the mechanism of membrane fusions and their control is important for understanding the cellular processes.

Virus membrane fusions have been extensively studied, and

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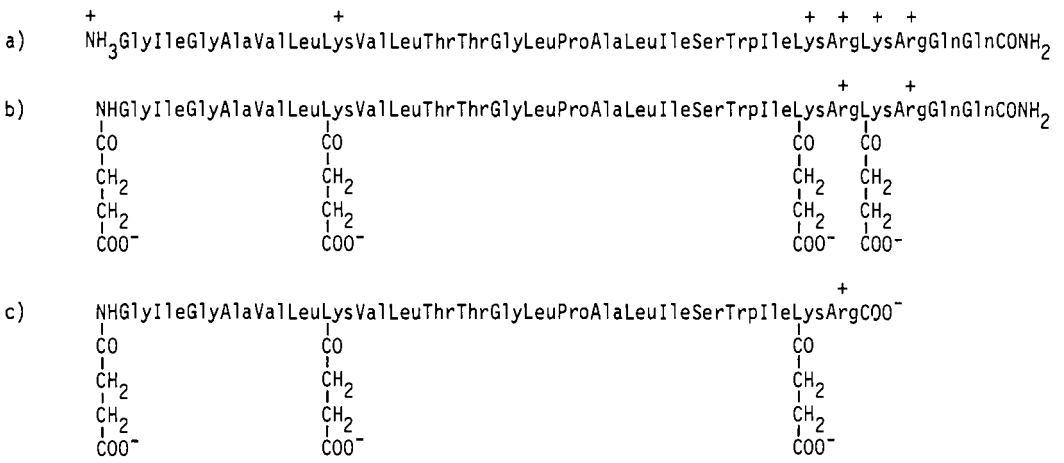

FIGURE 1: Intact (a), succinylated (b), and trypsinized succinylated (c) melittins.

a wealth of informations is available [for reviews, see White et al. (1983) and Ohnishi (1985, 1987)]. The membrane fusion is mediated by a specific glycoprotein in the viral envelope, e.g., F in hemagglutinating virus of Japan, HA in influenza virus, E in Semliki Forest virus, etc. These fusion proteins have a stretch of around 20 hydrophobic amino acids at the amino terminus or internally, which is assumed to interact with the target membane lipid bilayer to induce fusion. The hydrophobic segments in fusion proteins, which are active only at acidic pH, often contain a few acidic residues, while those active at neutral pH do not contain such acidic residues. The amino-terminal hydrophobic segment in the HA2 subunit of influenza virus HA contains two or three Asp or Glu residues. The low pH for these viruses is required to induce conformational change in the fusion protein, resulting in exposure of the hydrophobic segment to be able to approach the target membrane (Skehel et al., 1982; Sato et al., 1983) and then probably to protonate the acidic residues so that the neutralized hydrophobic segment could interact with the target lipid bilayer more easily (Maeda & Ohnishi, 1980; Ohnishi, 1985, 1987).

Membrane-active peptides often contain a stretch of hydrophobic amino acids and may be potentially active in fusion. Bee venom melittin, a polypeptide of 26 amino acids with 20 hydrophobic amino acid residues at the N-terminus and 5 charged residues at the C-terminus (Figure 1a) (Habermann & Jentsch, 1967), attracted our attention for the possibility of fusion activity. The molecular structure has been extensively studied by using various physicochemical techniques. Moreover, melittin is known to cause lysis of model lipid as well as cell membranes (Habermann, 1972; Sessa et al., 1969) and, during the present study, has been shown to actually cause fusion of lipid vesicles (Morgan et al., 1983; Eytan & Almary, 1983). We are interested not only in the fusion activity but also in the conversion of its pH characteristics on the introduction of acidic groups in it. For this purpose, we prepared succinylated melittin and found that the derivative does have fusion activity shifted to acidic pH, in marked contrast to that of intact melittin. We have also shown, using ^{13}C NMR spectroscopy, that the fusion activity is triggered by protonation of the carboxyl groups introduced into the peptide.

Materials and Methods

Succinylation of Melittin and Trypsinization of Succinylated Melittin. Bee venom melittin was purchased from Sigma and purified by a slightly modified method of Quay and Tronson (1983). Succinylation was carried out by reacting melittin (2 mg) with succinic anhydride (6 mg) for 2 h at room temperature in 2 mL of 20 mM NaP_i[1] buffer at pH 7.2 followed by purification. All four amino groups were succinylated by the reaction (see Figure 1b). Free amino groups were not detectable in the product with assays using fluorescamine and ninhydrin. Succinylation of the α-amino group was confirmed by the lack of dinitrophenylation as follows. Succinylated melittin was reacted with 1-fluoro-2,4-dinitrobenzene (Wako Chemicals) according to Conrat et al. (1955) and then throughly hydrolyzed in 6 N HCl. Only a small amount (less than 10%) of dinitrophenylated glycine was detected in the product by a thin-layer chromatogram analysis. For preparation of ^{13}C-labeled succinylated melittin, succinic anhydride synthesized from [1,4-^{13}C]succinic acid (Stohler Isotope Chemicals) was used for the reaction.

Trypsinization of succinylated melittin was carried out as follows. A lyophilized sample was dissolved in 20 mM NaP_i, and the pH was adjusted to 8.0. Trypsin (Sigma type II, TPCK treated, crystallized) was added at a trypsin-to-peptide molar ratio of 1:50 and reacted at room temperature for 15 h. The digested material was fractionated by reversed-phase HPLC using Cosmo sil 10C18-P (Nakarai). It was eluted on a gradient of acetonitrile of 0–40% for 20 min, 40–70% for 80 min, and 70–100% for 20 min in 0.1% trifluoroacetic acid in water. Two major fractions were obtained. One had an amino acid composition (molar ratio) of Thr 1.97, Ser 0.99, Gly 3.06, Ala 1.86, Val 1.88, Ile 2.45, Leu 3.71, Lys 1.83, and Arg 1.00, which is consistent with the N-terminal 1–22 segment of succinylated melittin (Figure 1c). This fraction was used as trypsinized succinylated melittin in the following experiments. Another fraction contained Lys 1.00 and Arg 1.00, which is consistent with the C-terminal 23–24 segment.

Concentration of melittin and its derivatives was estimated with the molar extinction coefficient of 5570 M^{-1} cm^{-1} at 280 nm (Bello et al., 1982).

Phospholipids. Egg yolk PC was extracted and purified according to Singleton et al. (1965). Spin-labeled PC was synthesized by the method of Hubbell and McConnell (1971). Sonicated vesicles were prepared by the method of Bangham et al. (1974). Phospholipid concentration was determined according to the method of Bartlett (1959).

Assay of Fusion of Vesicles. Fusion assay was carried out by using spin-labeled PC as described previously (Maeda et al., 1975; Ohnishi, 1985; Kuroda et al., 1986). Egg PC vesicles

[1] Abbreviations: PC, phosphatidylcholine; NaP_i, sodium inorganic phosphate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

containing 5% spin-labeled PC and those not containing spin-labeled PC were mixed at a ratio of 1:3 in 100 mM NaCl, 10 mM EDTA buffered with 40 mM NaP_i (pH 6–8), sodium acetate (pH 4–6), or sodium borate (pH >8) (fusion assay medium). EDTA was included in the medium to avoid any effects due to phospholipase A, which might contaminate. A stock solution of peptide was added to the mixture, and the ESR spectrum was measured repeatedly with a JEOL FE2 spectrometer at room temperature (22–23 °C). The ESR peak height increases on fusion owing to dilution of spin-labeled PC by mixing with nonlabeled phospholipid. The central peak height divided by the double integrated area of the whole spectrum (normalized peak height) was used for the quantitative estimation of fusion (Kuroda et al., 1985). For reference, model spectra of egg PC vesicles containing various concentrations of spin-labeled PC (1.25–5%) were measured, and a graph of the normalized peak height vs. the concentration was made and used for the estimation. When the normalized peak height of spectra for the assay mixtures reached the value for the model spectrum of 1.25% spin-labeled PC, 100% fusion was assumed. In some experiments, vesicles consisting only of spin-labeled PC were used to mix with unlabeled PC vesicles at a molar ratio of 1:40 to obtain qualitative information on fusion. In this case, 100% fusion was assumed when the normalized peak height becomes equal to that of the model spectrum for 2.5% spin-label concentration. Therefore, 100% fusion in these estimations corresponds to complete mixing of vesicle phospholipids.

Fusion was also assayed by the method of internal content mixing. Egg PC vesicles were loaded with Tb^{3+} by sonication in 15 mM $TbCl_3$, 150 mM sodium citrate, 2 mM TES, and 2 mM histidine, pH 7.3. Another population of vesicles was loaded with dipicolinic acid in 150 mM sodium dipicolinate, 2 mM TES, and 2 mM histidine, pH 7.3. These vesicles were mixed in the fusion assay medium, and the fluorescence intensity at 546 nm with excitation at 276 nm was measured (Wilschut et al., 1980). Leakage of the vesicle contents was assayed by dequenching of calcein preloaded in egg PC vesicles by sonication in 50 mM calcein (Dotite), 90 mM NaCl, 2 mM TES, and 2 mM histidine, pH 7.3. Calcein was used since its fluorescence intensity was independent of pH in the range of the present experiments.

Fusion of vesicles was also observed by a negative stain electron microscopy. After incubation, the samples were stained with 2% uranyl acetate and viewed under a JEOL 100B electron microscope.

Light scattering of vesicles was measured at 395 nm to obtain qualitative information on aggregation and, if it occurred, fusion of vesicles. The fluorescence spectrum was measured with a Hitachi 850S fluorescence spectrometer.

NMR Measurements. NMR spectra of ^{13}C-labeled succinylated melittin in the presence and absence of vesicles were measured at 25 °C by using a JEOL GX400 spectrometer equipped with a ^{13}C probe in a 5-mm NMR tube (G40C5) operating at 100.4 MHz. Chemical shifts were referenced to 2,2,4,4-tetradeuterio-3-(trimethylsilyl)propanesulfonic acid as an external standard. Free induction decays of 256–2048 scans were added with a repetition cycle of 2.4 s. A spectral resolution of 3.05 Hz was employed to record spectra. For the source of lock signal, a small amount of D_2O (~10%) was added to the sample.

RESULTS

Low-pH-Induced Fusion of Vesicles by Succinylated Melittin. Succinylated melittin cannot induce fusion at neutral pH but causes a rapid and efficient fusion at acidic pH. The ESR peak height increased rapidly to reach a saturation level before the first peak height measurement, ~1 min (Figure 2). The degree of fusion at saturation was dependent on the amount of melittin, 85% when the lipid-to-protein molar ratio (L/P) was 60 and 45% for L/P = 90. In the absence of succinylated melittin, there was only a negligibly small increase in the ESR peak height (Figure 2c). The fusion was very sensitive to pH, being activated in a very narrow range of acidic pH, the threshold at pH 5.2 and the maximum at pH 5.1 (Figure 3A). Succinylated melittin did not cause aggregation of vesicles in the pH range where it did not cause fusion (Figure 3B).

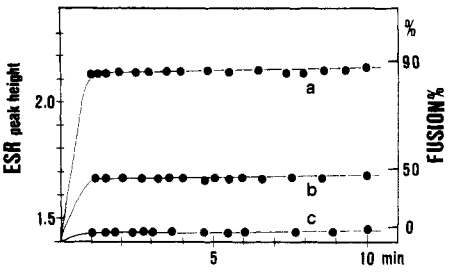

FIGURE 2: Fusion of PC vesicles induced by succinylated melittin at acidic pH. Succinylated melittin at 52 μM (a), 7.9 μM (b), or none (c) was added to a mixture of egg PC vesicles (3.6 mM) and those containing 5% spin-labeled PC (1.2 mM) in the fusion assay medium at pH 5.0. The normalized ESR peak heigh was plotted against time after the addition. The degree of fusion was estimated from the normalized ESR peak height as described under Materials and Methods.

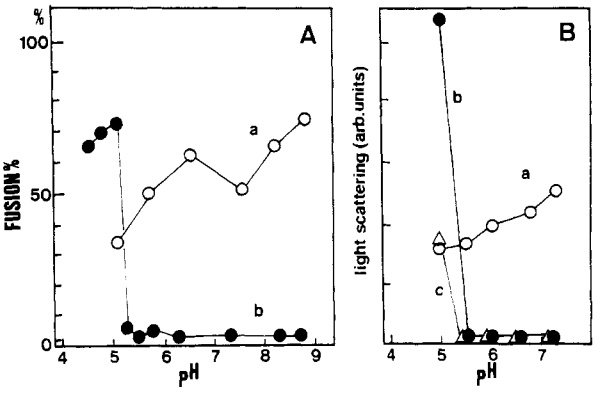

FIGURE 3: (A) pH dependence of fusion of vesicles induced by intact (a) and succinylated (b) melittin. Fusion was assayed in a system of egg PC vesicles containing 5% spin-labeled PC (1.2 mM), egg PC vesicles (3.6 mM), and melittin or succinylated melittin (31 μM). The degree of fusion at saturation was estimated from the normalized ESR peak height. (B) pH dependence of light scattering of egg PC vesicles (0.2 mM) in the presence of intact (6.7 μM) (a), succinylated (6.7 μM) (b), or trypsinized succinylated (4 μM) (c) melittin in the fusion assay medium.

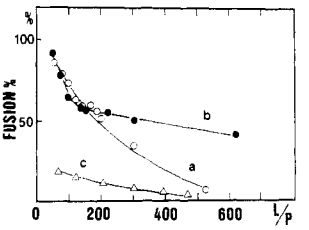

FIGURE 4: Dose dependence of fusion induced by intact melittin at neutral pH (a) and by succinylated melittin at acidic pH (b). Data for trypsinized succinylated melittin at acidic pH are also shown (c). The degree of fusion at saturation was estimated by ESR in a system of egg PC vesicles containing 5% spin-labeled PC (1.2 mM), egg PC vesicles (3.6 mM), and various concentrations of peptide. L/P: molar ratio of lipid to peptide.

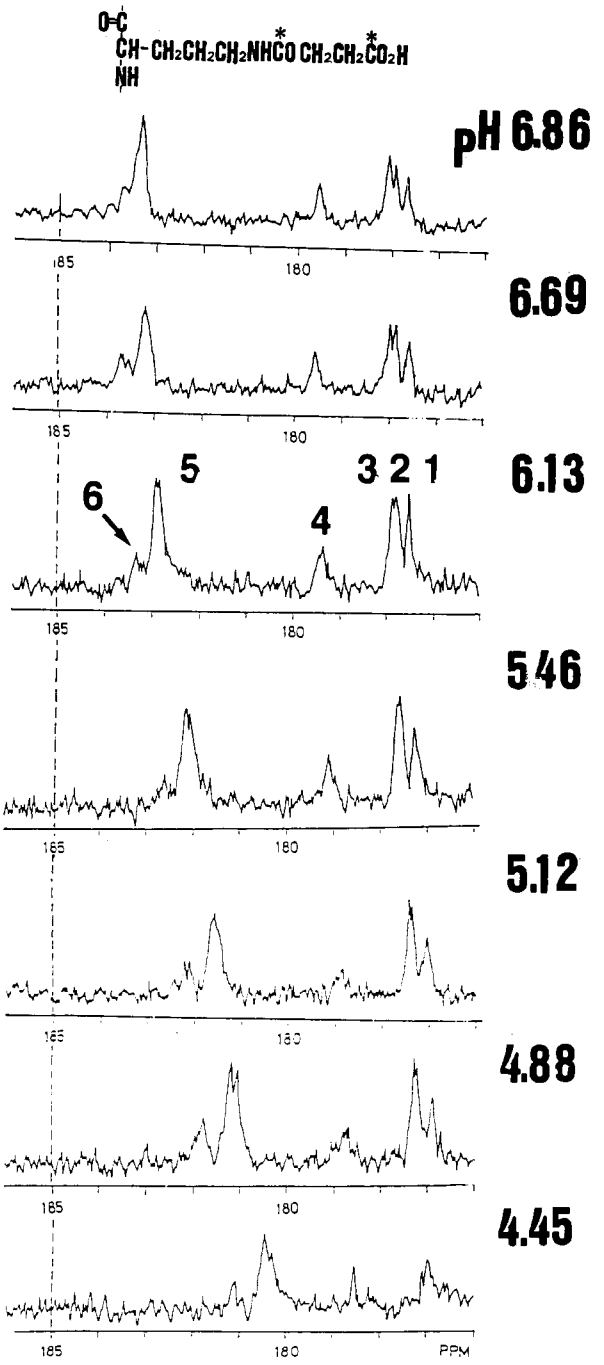

FIGURE 5: ^{13}C NMR spectra of ^{13}C-labeled succinylated melittin at various pH values. The ^{13}C labels are on the carboxyl and carbonyl carbons indicated by an asterisk at the top. Spectra of ^{13}C-labeled succinylated melittin (1.7 mM) in the presence of egg PC vesicles (10 mM) were measured in 20 mM NaP_i at the indicated pHs adjusted by adding 1 N DCl or NaOD. The chemical shift is referred to 2,2,4,4-tetradeuterio-3-(trimethylsilyl)propanesulfonic acid.

Intact melittin causes fusion of vesicles at neutral as well as acidic pHs. The pH dependence was very broad, in marked contrast to that of succinylated melittin (Figure 3A). The fusion reaction was as rapid as that induced by succinylated melittin. The degree of fusion at saturation was also dependent on the amount of peptide. Figure 4 compares the efficiency of fusion by intact and succinylated melittins. In the presence of larger amounts of peptide ($L/P < 200$), both peptides had nearly the same high efficiency. In the presence of smaller amounts of peptide ($L/P > 300$), however, succinylated melittin showed higher efficiency.

Trypsinized succinylated melittin did not cause significant fusion at acidic nor at neutral pH. In the presence of larger amounts of peptide ($L/P < 200$), the ESR peak height increased to some extent at acidic pH but the increase was very much smaller that those for intact and succinylated melittins. The fusion extent was calculated at 22% in the presence of the largest amount of peptide used, $L/P = 80$ (Figure 4c). The trypsinized derivative did not cause aggregation of vesicles at neutral pH but did at acidic pH (Figure 3B). However, the degree of aggregation was much smaller than that caused by succinylated melittin.

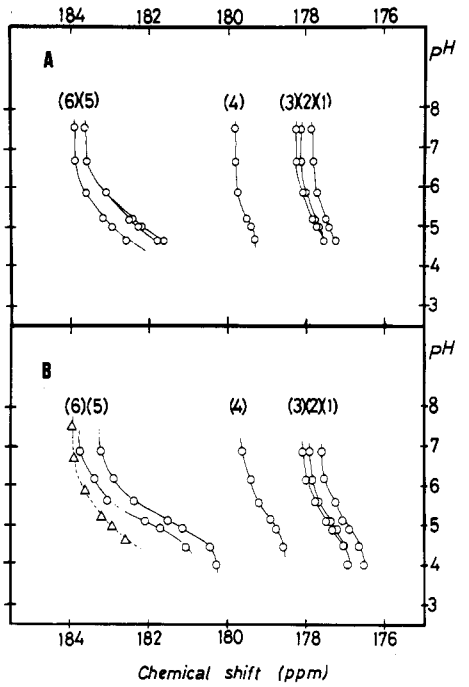

FIGURE 6: Change with pH in the chemical shift of six ^{13}C peaks of succinylated melittin in the absence (A) or presence (B) of egg PC vesicles. The curve for peak 6 in (A) is redrawn in (B) as a dotted curve to show the difference in the absence and presence of vesicles.

Assay of fusion based on the internal content mixing also showed fusion of vesicles by intact and succinylated melittins in qualitatively the same way as that observed by the spin-label assay (data not shown). Rather extensive leakage of vesicle contents accompanied the fusion: 70–80% of the content in succinylated melittin-induced fusion at acidic pH and 28% in intact melittin-induced fusion at neutral pH after 5 min.

Electron micrographs showed formation of much larger vesicles (5 μm and larger) after incubation of egg PC vesicles with succinylated melittin at pH 5.2 for 30 min at room temperature, but, in its absence, the vesicles remained small (20–30 nm) (data not shown).

Low-pH-Induced Fusion Is Triggered by Protonation of Carboxyl Groups of Succinylated Melittin. ^{13}C NMR spectra of succinylated melittin were measured at various pH values in the presence and absence of vesicles to trace the protonation process of the carboxyl groups. Carbon-13 was labeled at the carboxyl and carbonyl groups of succinylated residues (see Figure 5). As a general trend, the NMR peaks in the presence of vesicles were much narrower than those in their absence. Six peaks were observed (Figure 5). Peaks 5 and 6 with the larger chemical shift values and with an approximate intensity ratio of 3:1 can be assigned to the carboxyl carbons attached to the three lysyl amino residues and to the N-terminal amino group, respectively. Peaks 1–4 with smaller chemical shift values can be assigned to the carbonyl carbons.

All the carboxyl and carbonyl peaks shifted to higher field as the pH was lowered, both in the presence and absence of vesicles (Figure 5). Figure 6 plots the peak position vs. pH.

Table I: Chemical Shift Values of ^{13}C NMR Peaks of Succinylated Melittin in the Presence and Absence of Vesicles and the pK_a Value of Carboxyl Groups[a]

peak	vesicles	chemical shift δ_A[b]	chemical shift δ_{HA}[c]	$\Delta\nu$ (Hz)	pK_a
6	+	183.75	180.75	301.2	5.20
	−	184.00	181.00	301.2	4.83
5	+	183.25	180.01	325.3	5.18

[a] The NMR titration data (Figure 6) are analyzed by eq 1 in the text and the best-fit values for pK_a, δ_A, and δ_{HA} are given. $\Delta\nu$ is $\delta_A - \delta_{HA}$ in hertz. [b] Deprotonated form. [c] Protonated form.

The shift of the carboxyl carbons (peaks 5 and 6) was much larger than those of the carbonyl carbons. The shift in the presence of vesicles occurred at higher pH than that in their absence (see curves for peak 6 in Figure 6B). However, the amplitude of shift, i.e., the difference of the peak positions for the acid and neutral forms, appeared to be similar (see Table I). Only a single peak was observed for each carboxyl and carbonyl carbon at various pH values, indicating fast exchange between the protonated and deprotonated forms.

The NMR titration curve can be satisfactorily analyzed with the equation

$$pH = -pK_a + \log [(\delta - \delta_{HA})/(\delta_A - \delta)] \qquad (1)$$

where δ represents the chemical shift and the subscripts A and HA are for unprotonated and protonated forms, respectively. It is assumed that all four carboxyl groups have the same pK_a value. Analysis of the titration curve for peaks 5 and 6 was made, and the best-fit values for pK_a as well as δ_A and δ_{HA} are given in Table I. The pK_a value was obtained as 4.83 and 5.19 (average) in the absence and presence of vesicles, respectively. The pK_a value in the presence of vesicles was higher (0.36 unit) than that in their absence. The pK_a value 5.19 agrees quite well with the pH value of 5.15 for the half-maximal fusion.

For comparison, proton uptake by succinylated melittin in the absence of vesicles was measured at various pHs. The titration curve was satisfactorily fitted with a pK_a value of 4.63 in 20 mM NaCl and of 4.73 in 140 mM NaCl with 4 mol of proton uptake/mol of peptide. The pK_a value was only slightly dependent on the ionic strength. These values agree rather well with that obtained by the NMR titration.

Other Characteristics of Fusion. The low-pH-induced fusion by succinylated melittin can be stopped immediately by shifting to neutral pH and restarted by adjusting to acidic pH (Figure 7A). Similar on/off switching of the reaction by shifting pH was also observed by light scattering measurements (Figure 7B).

Fusion of vesicles initiated and reached a saturation, but further addition of a small amount of melittin induced further fusion. On the other hand, further addition of vesicles did not elicit further fusions. This is true for fusions induced by both intact and succinylated melittins (Figure 8).

Fluorescence of the Tryptophanyl Residue in Melittin. Melittin and its derivatives have a single Trp residue at position 19. The fluorescence spectrum of the peptides was measured with excitation at 285 nm. In the absence of vesicles, the emission maximum was observed at 350, 343, and 352 nm for intact, succinylated, and trypsinized succinylated melittins, respectively. The wavelength values suggest mostly monomer, tetramer, and monomer for intact, succinylated, and trypsinized succinylated melittins, respectively, according to Talbot et al. (1979) and Quay and Condie (1983). In the presence of vesicles, the emission maximum for intact and succinylated melittins blue-shifted to the same wavelength (333 nm), indicating a very hydrophobic environment. The fluorescence for trypsinized succinylated melittin also blue-shifted but to a much smaller extent (342 nm). All these fluorescence

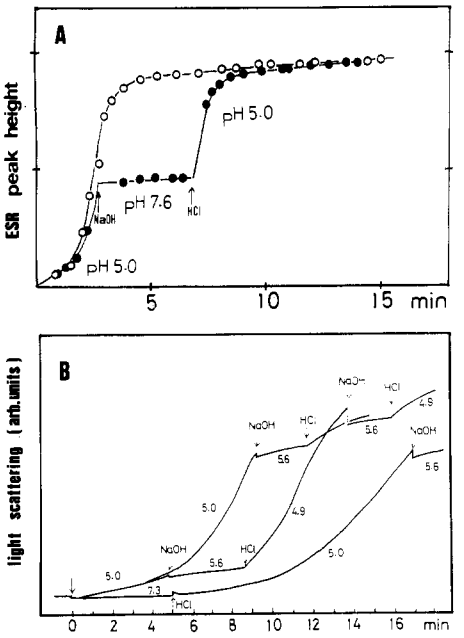

FIGURE 7: On/off switching of succinylated melittin induced fusion by shifting the medium pH to acid and neutral, respectively. (A) Spin-label assay. Succinylated melittin (23 μM) was added to a mixture of spin-labeled PC vesicles (0.11 mM) and egg PC vesicles (4.6 mM) at pH 5.0, and the ESR peak height was measured. After 2.5 min, the pH was shifted to 7.6 by adding 1 N NaOH and then, after 7 min, readjusted to 5.0 by adding 1 N HCl (filled circles). Open circles represent data on keeping the pH always at 5.0. (B) Light scattering measurements. Succinylated melittin (2.3 μM) was added to egg PC vesicles (0.2 mM) at pH 5.0 or 7.3. The pH was shifted by adding 1 N HCl or NaOH.

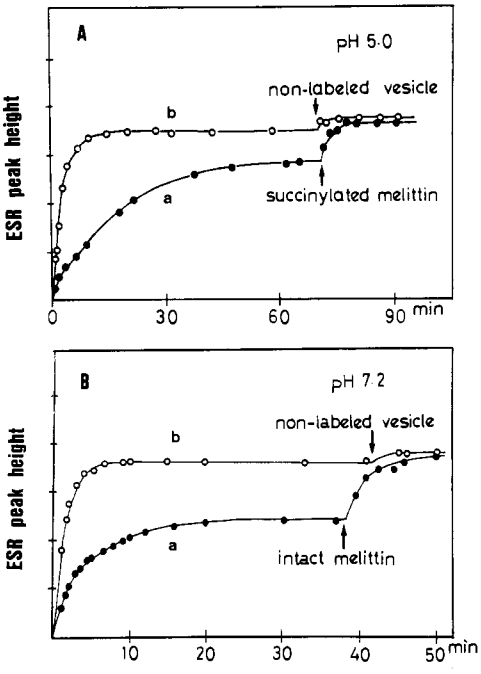

FIGURE 8: Effect of further addition of vesicles or peptide on succinylated melittin induced fusion at acidic pH (A) or intact melittin induced fusion at neutral pH (B). (a) Fusion was initiated by adding peptide (40 μM) to a mixture of spin-labeled PC (0.11 mM) and egg PC (4.7 mM) vesicles and, after 70 or 38 min, more peptide was added to a final concentration of 50 μM. (b) Fusion was started by adding 50 μM peptide to a mixture of spin-labeled PC (0.1 mM) and egg PC (4.1 mM) vesicles and, after 70 or 41 min, more egg PC vesicles were added to a final concentration of 4.8 mM.

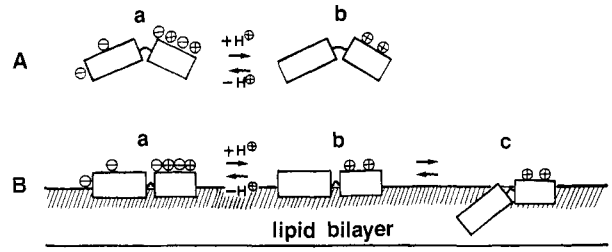

FIGURE 9: Protonation and deprotonation of the carboxyl groups of succinylated melittin in the absence (A) and presence (B) of lipid bilayer. In (B), the protonated hydrophobic segment is assumed to be more stabilized in the bilayer hydrocarbon layer (c). The ^{13}C NMR measurements indicate fast exchange between (a) and (b). In the presence of lipid bilayer (B), exchange of the protonated hydrophobic segment between the two sites (b and c) may also be fast.

spectra were not affected by changing the pH to 7.3 or 5.0.

Discussion

Requirement of Protonation of Acidic Residues for Fusion Activity. This study clearly demonstrates that the fusion activity of a hydrophobic peptide can be made sensitive to pH by introducing carboxyl groups into it. While melittin has fusion activity in a broad pH range from 4 to 9, succinylated melittin shows fusion activity only at acidic pH with the threshold at pH 5.2 and the maximum at pH 5.1. This study also clearly shows that the fusion activity of succinylated melittin is triggered by protonation of the negatively charged carboxyl groups. The half-maximal pH of fusion agrees quite well with the pK_a value of the four carboxyl groups in the peptide in the presence of vesicles. The very narrow range of pH for the fusion activation may be due to the fact that the present system consists simply of fusogenic peptide and vesicles and the four acidic residues have the same pK_a value.

These results therefore support our proposal that the low-pH activation of influenza virus fusion activity involves protonation of the acidic residues in the HA2 amino-terminal hydrophobic segment, in addition to the structural change of HA to expose the segment (Maeda & Ohnishi, 1980; Ohnishi, 1985, 1987). Moreover, we have recently discovered that a 20 amino acid peptide with the same sequence as that of the HA2 N-terminal segment has a low-pH-induced fusion activity similar to that of the parent virus (Murata et al., submitted for publication).

Interaction of the Protonated Hydrophobic Segment with the Lipid Bilayer. The pK_a value of the carboxyl groups in succinylated melittin in the presence of vesicles was higher than that in their absence. The higher shift will occur when the protonated segment is stabilized in the presence of vesicles. Stabilization will cause an apparent shift of the acid–base equilibrium in favor of the basic (protonated) form. A probable model for the stabilization is entrance of the protonated hydrophobic segment into a lipid bilayer hydrocarbon layer (Figure 9Bc).

Succinylated melittin binds to vesicles at neutral as well as acidic pHs, as suggested by the Trp fluorescence. At neutral pH, the hydrophobic segment 1–20 may stay on the bilayer surface, owing to the negative charges on the four carboxyl groups (Figure 9Ba), and the two membranes cannot come close. Vesicles do not aggregate under such conditions. At acidic pH, when the charges are neutralized by protonation, the two membranes may be brought into close contact owing to the increased surface hydrophobicity, which could overcome the surface dehydration energy (Ohki, 1987). Moreover, the protonated hydrophobic segment may enter the lipid bilayer with the C-terminal positively charged residues 21–24 remaining bound on the surface (Figure 9Bc). The charged residues should be neutralized to enter the bilayer because of the high energy needed to bring a charge into hydrocarbon layer (Parsegian, 1969). The protonation occurs spontaneously at acidic pH but requires energy at neutral pH. Therefore, the free energy value for transfer of the segment into lipid bilayer is more negative at acidic pH than at neutral pH (Ohnishi, 1985, 1987). Although we have not yet had conclusive evidence for the entrance, similar models have been presented for intact melittin by several authors (see below).

Dynamic Nature of the Interaction. ^{13}C NMR spectra of succinylated melittin at various pH values always consisted of a single peak for each carboxyl and carbonyl carbon, whose position shifted to higher field as the protonation proceeded. The observation of single peak indicates fast exchange between the protonated and the deprotonated forms to average out the two different peak positions. The exchange rate should be faster than the peak separation of 300 Hz or 3 ms (Table I). In the absence of vesicles, this simply means fast protonation and deprotonation of the carboxyl groups (Figure 9A). In the presence of vesicles, not only the protonation–deprotonation reaction but also exchange of the protonated hydrophobic segment between the surface and the stabilized location in the bilayer should be faster than 3 ms. If the segment enters the bilayer as assumed above, then exchange between the surface and the inside locations should occur quickly (Figure 9B).

The hydrophobic segment entered into lipid bilayer would compress lipid molecules surrounding it, creating density fluctuations since the interaction is suggested to be dynamic. Another possibility is formation of micelle-like nonbilayer structures by the hydrophobic segment, which have been proposed as the intermediate in membrane fusions (Cullis & de Kruijff, 1979). Its formation and disappearance could also be dynamic. Such dynamic interaction may well explain the rapid on/off switching of the fusion reaction by shifting the medium pH to acid and neutral, respectively (Figure 7).

Interaction of Intact Melittin with the Lipid Bilayer. The molecular structure of melittin in crystals has been determined by X-ray analysis (Terwilliger & Eisenberg, 1982a,b). It has a bent-rod structure involving two helices of the N-terminal 10 residues and the C-terminal 14 residues with an angle of 120°. The molecules associate into a tetramer via the hydrophobic inner surface of helices, with the outside charged hydrophilic surface. As a model for the interaction with lipid bilayer, these authors proposed the N-terminal helix inside the hydrocarbon layer with the C-terminal helix bound on the surface (Terwilliger et al., 1982). Vogel et al. (1983) studied orientation of melittin in lipid multibilayer membranes under a condition of small water content by polarized infrared spectroscopy. Two models were proposed: (a) the bent α-helix spanning lipid bilayer and (b) the wedgelike helices folding back within one monolayer. NMR studies of melittin in the presence of micelles showed that the peptide is highly helical and interacts with the hydrocarbon layer with the C-terminal 21–24 residues located on the surface (Lauterwein et al., 1979; Brown et al., 1982). All these models indicate entrance of the peptide segment, either including or excluding the N-terminus, into lipid bilayer. Model b, assuming entrance of the internal segment with the N-terminus as well as the C-terminal charged residues on the surface, would be more favorable thermodynamically since it does not require energy to neutralize the N-terminus positive charge. In the case of succinylated melittin, the whole segment (1–20) may enter the bilayer stably because there is no charge in the arm when it is protonated.

Intact melittin causes fusion of egg PC vesicles in a wide range of pH. The wide pH range for the activity can be

explained by the lack of acidic groups in the intact melittin. However, it has a positive charge at Lys-4, which will inhibit its entrance into the lipid bilayer. It is not known if the internal hydrophobic segment 5–20 enters the bilayer, rather than the segment 2–20, to avoid neutralization of the Lys charge.

Trypsinized succinylated melittin binds to vesicles and causes aggregation at acidic pH. But the interaction appears weaker than that with succinylated melittin and does not cause fusion. Anchoring to the membrane surface by the C-terminal charged residues may be necessary for stronger interactions.

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