Kinetics of Pore Formation by an Antimicrobial Peptide, Magainin 2, in Phospholipid Bilayers

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ABSTRACT: The kinetics of the pore formation by magainin 2, an antimicrobial peptide from *Xenopus laevis*, in lipid vesicles was investigated. The pore formation was estimated by the efflux of a fluorescent dye, calcein, from large unilamellar vesicles composed of egg yolk phosphatidylglycerol. The time courses of the dye release were well-described by a novel model in which the peptide molecules translocate from the outer to the inner monolayer by forming a pore. The concentration dependence of the leakage rate suggested that the pore consists of pentameric magainin. The obtained kinetic parameters estimate that, at a lipid-to-peptide molar ratio of 117, 9 pores with a lifetime of 40 μs open per second per vesicle in the initial phase. The apparent deactivation of the pore with increasing time can be ascribed to the reduced peptide density in the outer leaflet due to the translocation. Incorporation of phosphatidylcholine destabilized the pore, indicating the importance of anionic lipids in the stable pore formation.

A number of cytolytic peptides comprising 15−40 amino acids, such as defensins, magainins, melittin, and alamethicin, are known to be ubiquitously distributed among mammalians, amphibians, insects, and fungi (Cornut et al., 1993; Saberwal & Nagaraj, 1994; Sansom, 1991). They act as offensive or defensive weapons by disturbing the barrier property of the cell membranes of the target cells. The mechanisms of the membrane permeabilization are considered to involve "pores" formed in the membranes; these peptides form ion channels in planar lipid bilayers and cause the efflux of water-soluble contents entrapped within lipid vesicles. However, the detailed molecular machinery of the pore formation is not yet clarified in spite of extensive studies. One of the approaches to the elucidation of the mechanisms is kinetic studies of the pore formation (Grant et al., 1992; Parente et al., 1990; Schwarz et al., 1987, 1992; Williams et al., 1990); the observed time courses of the pore formation under various experimental conditions are curve-fitted to model equations to obtain insight into the underlying mechanisms. Although these analyses provide valuable information, the phenomenologically inferred reaction schemes are sometimes difficult to apply in visualizing the physical picture of the pore formation.

Magainin 2 is a 23-residue helical peptide isolated from the skin of the African frog, *Xenopus laevis* (Zasloff, 1987) (NH_2-Gly-Ile-Gly-Lys-Phe^5-Leu-His-Ser-Ala-Lys^{10}-Lys-Phe-Gly-Lys-Ala^{15}-Phe-Val-Gly-Glu-Ile^{20}-Met-Asn-Ser-COOH). It possesses potent antimicrobial (Zasloff, 1987; Zasloff et al., 1988) and antitumor (Cruciani et al., 1991) activities. Although there is a consensus that the peptide primarily acts by permeabilizing the cell membranes, the molecular details of the mechanism have been debated, i.e., the ion channel formation (Cruciani et al., 1992; Duclohier et al., 1989; Juretić et al., 1994; VazGomes et al., 1993; Westerhoff et al., 1989a,b) versus the disruption of the lipid organization in the membrane (Bechinger et al., 1993; Grant et al., 1992; Williams et al., 1990). The former comes from the observations that magainins form ion channels in planar lipid bilayers and that the membrane permeabilization supralinearly depends on the peptide concentration. The latter was deduced from the experimental evidence that the peptide helix lies on the membrane surface and does not span the bilayer. This kind of argument is not limited to magainins but is applicable to other amphiphilic peptides, e.g., melittin (Dempsey, 1990). We have recently proposed and proved a novel model that would terminate this debate [see Figure 1 of Matsuzaki et al. (1995b)]. (a) The peptide molecules first bind to the outer surface of the bilayer membrane with the aid of electrostatic interactions between the positive charges of the peptide and the negative charges of the lipid (Matsuzaki et al., 1989, 1991b, 1995a; VazGomes et al., 1993) to form an amphiphilic helix, which lies parallel to the surface (Bechinger et al., 1993; Matsuzaki et al., 1994) in a monomer−dimer equilibrium (Matsuzaki et al., 1994). (b) They form transient pores (channels) comprised of a multimeric bundle of membrane-spanning helices, as observed in planar membrane experiments (Cruciani et al., 1992; Duclohier et al., 1989). (c) Upon closure of the pore, the peptides can translocate across the membrane. A multimer of helices arranged in bundles with the polar side chains oriented toward the bundle center can effectively translocate across the membrane without unfavorably exposing the polar moieties to the hydrophobic interior of the lipid bilayer. Reduction in the peptide density on the outer surface greatly slows the channel formation, because it is a highly cooperative process. The translocation could also relieve the bilayer from the unfavorable strain due to the asymmetrical peptide binding and resultant membrane expansion. The translocated peptides are again in a monomer−dimer equilibrium in the inner leaflet.

In this article, we consider the kinetics of the pore formation based on the above model. The pore formation was estimated by the leakage of a water-soluble fluorescent

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dye, calcein, from large unilamellar vesicles (LUVs)[1] containing egg yolk phosphatidylglycerol. The time course of the leakage could be well-explained by our translocation model, assuming a pentameric pore.

MATERIALS AND METHODS

Materials. Magainin 2 and its equipotent Trp-substituted analog (12-Trp magainin 2) were synthesized by a standard fluoren-9-ylmethoxycarbonyl-based solid phase method. The crude peptides were purified by HPLC and gel filtration (Sephadex G-15, 2.5 × 35 cm column, 0.02 N HCl being used as an eluent), as described previously (Matsuzaki et al., 1991b, 1994). The purities of the synthesized peptides were ascertained by quantitative amino acid analysis. Egg PG enzymatically converted from egg PC was a kind gift from Nippon Fine Chemical Co. (Takasago, Japan). Egg PC and NBD-PE were purchased from Sigma (St. Louis, MO) and Molecular Probes (Eugene, OR), respectively. Calcein and spectrograde organic solvents were supplied by Dojindo (Kumamoto, Japan). All other chemicals from Wako (Tokyo, Japan) were of special grade. A Tris-HCl buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.0) was prepared from water twice distilled in a glass still.

Vesicle Preparation. LUVs were prepared and characterized, as described elsewhere (Matsuzaki et al., 1991a,c). Briefly, a lipid film, after being dried under vacuum overnight, was hydrated with a 70 mM calcein solution (pH was adjusted to 7.0 with 1 N NaOH) and vortex mixed. The suspension was freeze−thawed for five cycles and then successively extruded through polycarbonate filters (a 0.6 μm pore size filter, 5 times; two stacked 0.1 μm pore size filters, 10 times). The calcein-entrapped vesicles were separated from free calcein on a Sephadex G-50 column. The lipid concentration was determined in triplicate by phosphorus analysis (Bartlett, 1959).

Leakage. The release of calcein from LUVs was monitored by fluorescence at an excitation wavelength of 490 nm and an emission wavelength of 520 nm on a Shimadzu RF-5000 spectrofluorometer whose cuvette holder was thermostated at 30 ± 0.5 °C. To ensure the rapid mixing of the peptide with the vesicles, which is essential for kinetic experiments (Polozov et al., 1994), an RX-1000 stopped flow apparatus (Applied Photophysics Ltd., U.K.) was used, and the leakage time course was recorded at 0.1 s intervals. The mixing dead time was less than 20 ms, according to the manufacturer. The maximum fluorescence intensity corresponding to 100% leakage was determined by the addition of 10% Triton X-100 (20 μL) to 2.5 mL of the sample. The reported time courses of the leakage were the average of 6−8 experiments, and the standard deviations were smaller than 1.2 absolute percent leakage.

Dithionite Ion Permeability. The permeability of the membranes to dithionite ions was examined in the presence of peptide on either one side or both sides of the bilayer. In the former case, LUVs or MLVs composed of egg PG/egg PC/NBD-PE (50:50:0.25, moles/moles) were prepared as described earlier. Small aliquots of the liposome suspension were injected into a 12-Trp magainin 2−sodium dithionite buffer solution. In the latter experiments, the lipid film of the same composition was hydrated with a 12-Trp magainin 2 buffer solution and vortex mixed. The suspension was freeze−thawed for five cycles to equilibrate the peptide molecules across the lipid bilayers. Sizing was not carried out because of the plausible loss of peptide. A 1 M sodium dithionite solution (20 μL) was added to 2 mL of the liposome suspension in a cuvette. The fluorescence intensity of NBD at 530 nm (excited at 450 nm) was monitored at 30 °C. The final concentrations of peptide, lipid, and dithionite were 3 μM, 170 μM, and 10 mM, respectively.

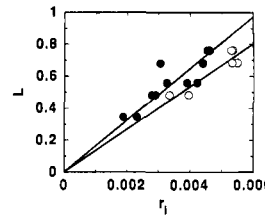

FIGURE 1: Relationship between pore formation and peptide translocation. The pore formation was estimated on the basis of the leakage of a water-soluble fluorescent dye, calcein, from egg PG/egg PC (1:1, moles/moles) LUVs. The extent of leakage is denoted by *L*. The translocation was evaluated by two methods and expressed as r_i, the moles of the translocated peptide in the inner leaflet per mole of the lipid in the bilayer. Results from 2−3 experiments were plotted. [peptide] = 3 μM, [lipid] = 170 μM, and temperature = 30 °C. ○: 12-Trp magainin 2 remaining in the outer monolayer was digested by externally added trypsin [Figure 3 of Matsuzaki et al. (1995b)]. ●: The untranslocated peptide was extracted by addition of the second population of vesicles [Figure 2 of Matsuzaki et al. (1995b)]. The lines are the regression lines.

THEORY

We have demonstrated (Matsuzaki et al., 1995b) that the pore formation induced by magainin 2 is coupled to the translocation of the peptide across the lipid bilayer. The pore formation was estimated on the basis of the leakage of calcein from LUVs. The extent of translocation was estimated by two experiments by use of resonance energy transfer from the Trp residue of 12-Trp magainin 2 to DNS-PE in the membrane. The derivatized peptide had been confirmed to be equipotent to magainin 2 (Matsuzaki et al., 1994). One method was based on the extraction of the peptide remaining on the outer surface of the vesicles by the addition of the second population of DNS-PE free vesicles. The time course of the peptide transfer was biphasic: an initial, fast, large extent of transfer followed by a slow, minor extent of transfer [see Figure 2 of Matsuzaki et al. (1995b)]. The former is considered to reflect the fraction of untranslocated peptide, the untranslocated peptide directly removed from the outer surface of the first population of vesicles to the second population of vesicles. The latter slower phase can be attributed to the back-translocation of the already translocated peptide; the peptide which had reached the inner leaflet moved first to the outer leaflet and then to the second population of vesicles. Closed circles in Figure 1 show the relationship between the fraction of the leaked dye, *L*, and the peptide density in the inner leaflet (the density of the translocated peptide), r_i, expressed as moles of peptide in the inner leaflet per mole of lipid in the bilayer. The *L* value

[1] Abbreviations: egg PC, egg yolk L-α-phosphatidylcholine; egg PG, L-α-phosphatidyl-DL-glycerol enzymatically converted from egg PC; NBD-PE, *N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl]-L-α-phosphatidylethanolamine; DNS-PE, *N*-[[[5-(dimethylamino)naphthyl]-1-sulfonyl]dipalmitoyl]-L-α-phosphatidylethanolamine; LUVs, large unilamellar vesicles; SUVs, small unilamellar vesicles; MLVs, multilamellar vesicles; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

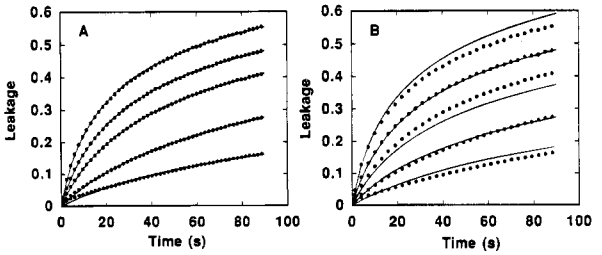

FIGURE 2: Time course of dye leakage. The leakage of calcein from egg PG LUVs ([lipid] = 129 μM) induced by magainin 2 was measured by use of a stopped flow apparatus at 30 °C and plotted against time with circles. The original data were collected at 0.1 s intervals, but every third point is shown for the sake of visual simplicity. The peptide concentrations were 0.7, 0.8, 0.9, 1.0, and 1.1 μM, respectively, from the bottom. (A) The curves are least-square fits by eq 7, assuming $n = 5$. The parameters (m_1 and m_2) are depicted in Figure 3. (B) The curves are the extent of leakage simulated by use of eqs 7−9 with the average parameters, $\alpha = 151$ and $k_+/(1 + g) = 1.11 \times 10^6$ s^{-1}.

was proportional to r_i; that is, the leakage was coupled to the translocation. The value of α was estimated to be 162 ± 7 ($R = 0.834$).

$$L = \alpha r_i \tag{1}$$

The other method utilized the digestion of the untranslocated peptide by externally added trypsin. Refer to Figure 3A of our previous paper (Matsuzaki et al., 1995b). Open circles in Figure 1 indicate that the L value was again proportional to r_i with a slope of $\alpha = 134 \pm 4$ ($R = 0.928$). The latter method appears to underestimate the r_i value, because the trypsin digestion takes 2 min, during which the slow back-translocation occurs. We calculated the extent of the underestimation to be 20−30% from Figure 2 of Matsuzaki et al. (1995b); the true α value evaluated from the trypsin digestion experiment is around 100. Therefore, we conclude that the slope in eq 1 is in the range 100−160, depending on the method of estimation. The reason for the discrepancy between the two methods has been discussed (Matsuzaki et al., 1995b).

We have reported (Matsuzaki et al., 1994) that a pore deactivation process exists; the rate of dye release decreases with increasing time (see also Figure 2). Therefore, the time course of leakage appears to reflect a relaxation process toward the equilibrium. Let us assume the following simplest scheme.

$$nr_{1o} \underset{k_-'}{\overset{k_+}{\rightleftharpoons}} r_n \text{ (pore)} \underset{k_+'}{\overset{k_-}{\rightleftharpoons}} nr_{1i} \tag{I}$$

Here, r_{1o} and r_{1i} are the densities of the monomeric peptides in the outer and inner leaflets, respectively. The density of the n-meric pore is expressed as r_n. The peptide molecules adsorbed on the outer surface form the pore with the rate constant k_+. Upon its closure, the peptides either translocate into the inner leaflet with k_- or return to the outer monolayer with k_-'. The translocated magainin can reform the pore with k_+'. It should be noted that, in the kinetic experiments (Figure 2), we employed the experimental conditions [a peptide-to-lipid (egg PG) molar ratio of ca. 100 and a lipid concentration of 129 μM] to ensure that (1) the binding equilibrium of the peptide to the membrane is established very fast (within 1 s) and (2) the binding isotherm predicts that almost all (>90%) of the added peptides are in the membrane-bound form and monomeric (Matsuzaki et al., 1994). Thus, we can set $r_{1o} \approx r_o$ and $r_{1i} \approx r_i$. Here, r_o is the density of the peptide on the outer monolayer. All the peptide densities refer to the moles of the lipid in the bilayer. The overall density within the bilayer, $r \approx$ [peptide]/[lipid], is the sum of r_o and r_i.

$$r = r_o + r_i \tag{2}$$

Because $r_i^n \ll r_o^n$ (vide infra), the rate of translocation can be written as

$$dr_i/dt = nk_-r_n \tag{3}$$

Assuming a fast equilibrium for the pore formation,

$$k_+r_o^n = (k_- + k_-')r_n \tag{4}$$

$$dr_i/dt = nk_+(r - r_i)^n/(1 + g) \tag{5}$$

The ratio of k_-' to $k_-$was set to g. Equation 5 can be easily integrated under the initial condition of $r_i = 0$ at $t = 0$.

$$r_i = r[1 - (n(n - 1)k_+r^{n-1}t/(1 + g) + 1)^{-1/(n-1)}] \tag{6}$$

Equations 1 and 6 yield

$$L = m_1\{1 - (m_2t + 1)^{-1/(n-1)}\} \tag{7}$$

$$m_1 = \alpha r \tag{8}$$

$$m_2 = n(n - 1)k_+r^{n-1}/(1 + g) \tag{9}$$

RESULTS

Calcein Permeability during the Relaxation Process. Calculation of L from the observed fluorescence intensity, F, depends on the mode of leakage (Weinstein et al., 1984). If the dye leaks in the "all-or-none" manner, that is, some of the vesicles completely lose their contents while the others remain intact, the fluorescence from highly quenched calcein remaining within the vesicles is negligible. On the other hand, if the leakage occurs through the graded mode, where all of the vesicles equally release some of their contents, the fluorescence contribution from diluted intravesicular calcein should be taken into account. We have reported (Matsuzaki et al., 1994) that the dye leakage from LUVs induced by magainin 2 occurs through the latter mode; that is, the life span of a single pore is too short to exhaust all of the vesicular contents. Therefore, L at time t was calculated by use of eq 10 (Weinstein et al., 1984).

$$L(t) = 1 - (1 - F(t)/F_t)/Q_v \tag{10}$$

F_t is the fluorescence intensity after addition of Triton X-100, corresponding to 100% leakage. Q_v is the quenching coefficient of intravesicular calcein, which had been determined as a function of calcein concentration (Matsuzaki et al., 1993).

The closed circles in Figure 2A depict the time courses of the leakage at various peptide concentrations. The lipid concentration was fixed at 129 μM. The solid curves were calculated by use of a least-square curve-fitting program (KaleidaGraph, Synergy Software, Pennsylvania) according to eq 7 with $n = 5$. The adjustable parameters, aside from

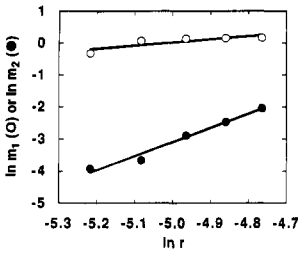

FIGURE 3: Dependencies of the leakage parameters on the peptide-to-lipid ratio. The logarithms of the curve-fitting parameters, m_1 and m_2, obtained from Figure 2A are plotted against ln r, the logarithm of the amount of the membrane-bound peptide per lipid (see eqs 8 and 9). The slopes of the linear regression were 0.98 and 4.38, respectively.

n, for each data set are m_1 and m_2. The fitting was excellent ($R \geq 0.997$). The fitting quality was also good for $n = 4$ or 6 (data not shown). Equations 8 and 9 predict that m_1 and m_2 should depend on r and r^{n-1}, respectively. In Figure 3, the logarithm of m_1 or m_2 is plotted against the logarithm of r. The slopes of the linear regressions for $n = 5$ were 0.98 $\pm$ 0.35 (R = 0.850) and 4.38 $\pm$ 0.38 (R = 0.989), respectively, in good agreement with the theoretical prediction. However, the slopes were also around 1 and 4, respectively, when n = 4 or 6 is assumed, and are incompatible with the theory (data not shown). Thus, we can conclude that the pentameric pore is the most plausible.

Equation 8 indicates that the proportional coefficient of the m_1 versus r plot should be α. The α value estimated from Figure 3 is 151 $\pm$ 16, which falls within the range 100–160 evaluated in Figure 1. This marvelous agreement strongly supports the validity of our model. According to eq 9 and Figure 3, the $k_+/(1 + g)$ value was calculated to be $(1.11 \pm 0.14) \times 10^6$ s^{-1}. The leakage curves were simulated by use of α = 151 and $k_+/(1 + g) = 1.11 \times 10^6$ s^{-1}, assuming a pentameric pore (Figure 2B). It is striking that the five sets of time courses could be well-reproduced with only two adjustable parameters, $k_+/(1 + g)$ and n.[2]

In the translocation experiments (Figure 1), 50% egg PC was incorporated to enhance the rate of peptide desorption from the membrane, enabling the peptide to be easily digested by trypsin or extracted by the second population of vesicles. To examine the effect of PC incorporation on kinetics, stopped flow experiments were also carried out by use of egg PG/egg PC (1:1) LUVs. The time course of dye release was again well-described by eq 7 with $n = 5$ ($R >$ 0.978, data not shown). The α and $k_+/(1 + g)$ values were 92 $\pm$ 7 and (3.64 $\pm$ 0.94) $\times 10^5$ s^{-1}, respectively. The former value was comparable to the range of value (100–160) estimated in Figure 1. Both parameters of the egg PG/egg PC mixed system were smaller than those of the egg PG system.

Dithionite Permeability in the Equilibrium State. The above experiments examined the membrane permeability prior to reaching the equilibrium, i.e., $r_o > r_i$. To determine if the membrane is still permeable at the equilibrium ($r_o = r_i$), the permeability of the membrane to $S_2O_4^{2-}$ ions was determined by use of NBD-PE-labeled MLVs equilibrated with 12-Trp magainin 2. The ion permeability was monitored on the basis of the quenching of the NBD fluorophore because of the reduction of the chromophore (McIntyre & Sleight, 1991). First, to confirm the validity of this method, NBD-PE-doped LUVs were examined. Figure 4A depicts that the addition of the dithionite ion in the absence of the peptide quenched 51% of the NBD fluorescence within 4 min, indicating the unilamellarity (curve 1). Curve 2 shows that the presence of the peptide in the external medium completely reduced the NBD group within 2 min, indicating that the ions passed through the magainin pore to reach the inner aqueous phase. It should be noted that the leakage rate of this small ion is larger than that of calcein under identical conditions [Figure 5 of Matsuzaki et al. (1995b)]. The L value of calcein at 2 min was 0.6, reflecting the slower diffusion of the larger molecule through the pore.

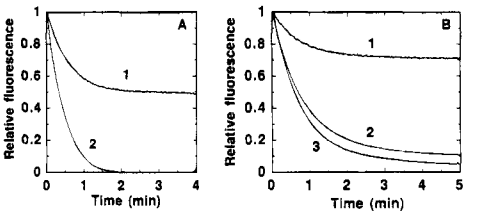

FIGURE 4: Membrane permeability to dithionite ion. The permeability of vesicles composed of egg PG/egg PC/NBD-PE (50:50:0.25) to the $S_2O_4^{2-}$ ion in the absence or presence of 12-Trp magainin 2 was measured on the basis of the quenching (reduction) of the NBD group. The fluorescence at 530 nm (excitation at 450 nm) was monitored as a function of time at 30 °C. [lipid] = 170 μM, [peptide] = 3 μM, and [$Na_2S_2O_4$] = 10 mM. (A) Small aliquots of the LUV suspension were injected into a buffer (curve 1) or a peptide solution (curve 2). (B) Small aliquots of the freeze–thawed MLV suspension were injected into a buffer (curve 1) or a peptide solution (curve 3). Curve 2: freeze–thawed MLVs were prepared in the presence of 12-Trp magainin 2. Small aliquots of a $Na_2S_2O_4$ solution were added at time zero.

Next, this method was applied to MLVs (Figure 4B). In the absence of the peptide (curve 1), the extent of quenching was 29% because of the oligolamellarity. The external addition of the magainin peptide (curve 3) allowed the reducing ion to react with almost all NBD groups within 5 min; the peptide molecules translocated across the outermost bilayers to form pores in the inner bilayers, additional evidence for translocation. However, the rate of quenching was smaller than that of the LUVs (curve 2 in Figure 4A) because of the larger lamellarity. In the case of the peptide-equilibrated MLVs (curve 2 in Figure 4B), about 90% of the NBD groups were accessible to the $S_2O_4^{2-}$ ion within 5 min, clearly indicating that the membrane is still permeable to this small ion in the equilibrium state. The rate of quenching was somewhat smaller than that of curve 3, because of the smaller pore density in the equilibrium peptide distribution.

DISCUSSION

The kinetics of pore formation in lipid vesicles by cytolytic peptides have been studied by several groups. One of the major problems is how to account for the presence of the "pore deactivation process". That is, the rate of pore formation gradually decreases with increasing time. Williams et al. (1990) investigated the kinetics of dye efflux from SUVs induced by magainin 2 amide and a related peptide (PGLa). The data were empirically curve-fitted by use of a biexponential equation to take into consideration

[2] Each observed leakage profile in Figure 2A can be empirically fitted by a biexponential equation; three independent parameters are needed to describe the leakage curve. Therefore, it is possible that any theory by use of more than two parameters would simulate the results.

the slower pore-deactivating phase, although they provided no explanation of the underlying mechanism. The pore formation by magainin 2 amide in SUVs was also studied by Grant, Jr., et al. (1992), who analyzed their data with a triexponential equation based on a transient membrane destabilization model. Namely, they attributed the fast leakage phase to a transient peptide-induced unstable bilayer structure, which is relaxed into a more stable proteoliposome.

More rigorous theoretical treatments have been carried out in the laboratory of Schwarz (Schwarz & Robert, 1990, 1992; Schwarz et al., 1992). They derived a relationship between the experimental dye release rate and the pore formation rate, the latter being analyzed by use of a series of mass action equations. They ascribed the deactivation process to the formation of nonpore aggregates in the case of alamethicin (Schwarz & Robert, 1990) and to the rate-limiting dimer formation after the exhaustion of an initial fast dimer deposit in the case of melittin (Schwarz et al., 1992).

In this study, we used LUVs after we had confirmed the absence of any morphological changes in the vesicles. As we have already pointed out (Matsuzaki et al., 1991b), the use of SUVs is not recommended because of the plausible vesicle aggregation/fusion and inhomogeneity. We clarified that the pore deactivation process observed for magainin is due to the translocation of the peptide across the bilayer and the resultant decrease in the peptide density on the outer monolayer.

Rate-Limiting Step. The rate-limiting step is considered to be the pore formation process by peptide self-assembly within the membrane. The initial binding step is very fast (<1 s), as described elsewhere (Matsuzaki et al., 1994). There is no competition between the peptide self-association in the aqueous phase and the membrane binding, because the peptide is monomeric at a peptide concentration up to 50 μM (K. Matsuzaki and J. Seelig, unpublished work). The dye diffusion through the aqueous pore formed by magainin is also sufficiently fast. The aqueous contents of an LUV (diameter, 100 nm) are depleted through a single pore [area, 0.4 nm^2 for a pentameric pore (Sansom, 1991)] in the time scale of 10 ms (Schwarz & Robert, 1990, 1992). Thus, the observed dye leakage, L, reflects the kinetics of the pore formation in the lipid matrix.

Reversibility. In the previous study (Matsuzaki et al., 1995b), we elucidated that magainin can translocate across the lipid bilayer and that the translocation is coupled to the pore formation (see Figure 1). Taking this observation into account, we considered the simple reaction Scheme 1. The back-translocation, $r_i \rightarrow r_n$, could occur, but we selected the experimental conditions where its rate is negligible; eqs 1 and 2 predict that $(r_o/r_i)^5 \geq 10$ if $L/r \leq 60$, which is the condition fulfilled in Figure 2. This condition is needed to obtain the analytical solution (eq 6) of the differential equation, eq 5. If the driving force of the translocation is the relaxation of the asymmetric expansion of the bilayer, the peptide molecules will be almost unidirectionally transferred to the inner monolayer ($k_-' = k_+' = 0$; thus, $g \approx 0$). On the other hand, if the translocation is merely a result of the stochastic destruction of the membrane-spanning helical aggregate into both monolayers, $k_+ = k_+'$ and $k_- = k_-'$ ($g = 1$).

To distinguish these two cases, the membrane permeability in the equilibrium state was examined. Figure 4B clearly demonstrates that the membrane is still permeable to the $S_2O_4^{2-}$ ion; the pore is present under the condition of $r_o = r_i$. A small ion may permeate through a peptide-induced defect in the lipid organization. However, the perturbation of the lipid acyl chain is minimal as detected by differential-scanning calorimetry (Matsuzaki et al., 1991b), Raman (Williams et al., 1990), and NMR (Matsuzaki & Seelig, 1995) techniques. Therefore, it is reasonable to assume $k_+ = k_+'$ and $k_- = k_-'$ at least under the conditions of $r_o = r_i$, where both monolayers are symmetrically expanded.

Molecularity. The leakage data could be best described by the pentameric pore model based on Scheme 1 (Figure 2B). We have reported (Matsuzaki et al., 1994) that the very initial leakage rate (within 2 s) depends on the 4th power of the peptide concentration, incompatible with our present results. A closer inspection of Figure 2A reveals that this discrepancy mainly arises from the fact that the observed leakage values at lower peptide concentrations in the very early stage are higher than predicted. One explanation is that these low percent leakage values (<3%) suffer from various sources of errors, such as the reproducibility (<1.2%) and the estimation of the base line, including a small extent of vesicle rupture due to the mixing shock. Another possibility is that there is a distribution of the pore size centered at $n = 5$. A minor fraction of tetrameric pores, which are formed faster than the pentamer, contributes more to the observed leakage at lower r values.[3] The estimated molecularity is in keeping with other studies: 3−6 (Duclohier et al., 1989), 3−5 (Westerhoff et al., 1989a), and 4−6 (Juretić et al., 1994; Westerhoff et al., 1989b). The pentameric pore (diameter, ca. 7 Å) might be somewhat small to allow the dye (diameter, ca. 10 Å) to pass through. If the pore is made up of a peptide−lipid complex (Cruciani et al., 1992), the central aqueous channel will be larger. In fact, amphiphilic peptides (Fattal et al., 1994) including magainin 2 (O. Murase et al., unpublished work) cause a pore-mediated rapid flip-flop of membrane lipids, suggesting the involvement of the lipid head groups in the pore lining.

Linearity between L and r_i. The leakage, L, was experimentally proportional to r_i (Figure 1). According to Schwarz and Robert (1992), the leakage is related to the pore formation rate, $v_+(t)$, i.e., the number of formed pores per vesicle per time, through eq 11.

$$L = 1 - \exp\{-\tau/(\tau_0 + \tau)\int_0^t v_+(t')\,dt'\} \qquad (11)$$

Here, τ is the lifetime of the pore ($= 1/[k_-(1 + g)]$), and τ_0 is the relaxation time of the release of the internal marker in a cell with just one open pore. The latter was calculated to be 10 ms, as described above. The following equations give the relationship between v_+ and r_i according to eqs 3 and 4.

$$v_+ = \nu k_+ r_o^n = \nu(1 + g)(dr_i/dt)/n \qquad (12)$$

The number of lipid molecules per vesicle (9×10^4 for a 100 nm LUV) is denoted by ν. Equations 11 and 12 lead to

$$L = 1 - \exp\{-\tau\nu(1 + g)r_i/n(\tau_0 + \tau)\} \qquad (13)$$

[3] Let us assume an equimolar mixture of the tetrameric and pentameric pores. The fraction of the leakage due to the tetramer, $L_4/(L_4 + L_5)$, at a given t is a monotonously decreasing function of r according to eq 7.

If the exponent is much smaller than unity,[4] we may set

$$L \approx \tau\nu(1 + g)r_i/n(\tau_0 + \tau) = \alpha r_i \quad (14)$$

In our case, $\alpha \approx 150$ and $r_i \leq 0.005$; therefore, $\alpha r_i \leq 0.75$. In a crude approximation,

$$\alpha/\nu \approx \tau(1 + g)/n(\tau_0 + \tau) \approx 2 \times 10^{-3} \quad (15)$$

This implies that τ_0 (10 ms) $\gg \tau$ (40 μs for $g = 1$), in keeping with the graded leakage mode (Matsuzaki et al., 1994). The pore deactivation rate constant k_- is estimated to be

$$k_- = 1/\tau(1 + g) \approx 1 \times 10^4\ s^{-1} \quad (16)$$

It should be noted here that the time constant to reach the pore formation equilibrium (eq 4) is τ, guaranteeing the fast equilibrium approximation.[5]

These analyses give the following concrete example; at a lipid-to-peptide ratio of 117 (the top curve in Figure 2A), 9 pores per vesicle per second with a lifetime of 40 μs are initially formed, whereas the number of the pores decreases to 0.4 at $t = 90$ s.

Incorporation of 50% PC reduced both α and k_+, suggesting that acidic phospholipids stabilize the pore, i.e., increase k_+ and decrease k_- (also see below).

Mode of Leakage. Magainin 2 induces the leakage of calcein from egg PG LUVs in the graded mode (Matsuzaki et al., 1994), whereas magainin 2 amide is reported to cause the all-or-none leakage from bovine brain phosphatidylserine SUVs (Grant et al., 1992). Because τ_0 is proportional to (vesicle diameter)$^{-3}$ (Schwarz & Robert, 1990, 1992), a 20 nm vesicle would have a τ_0 value 100 times shorter than the τ_0 value of a 100 nm LUV (10 ms). Melittin also leaks a fluorescent dye from SUVs in the all-or-none mode (Schwarz et al., 1992). An amphiphilic triacontapeptide GALA is known to induce this type of leakage from 100 nm LUVs (Parente et al., 1990). Its leakage kinetics were expressed as that of formation of aggregates based on the Smoluchowski equation. In the case that the leakage occurs in the all-or-none mode, our treatments need to be modified, because the formation of the very first pore in a vesicle prior to translocation is sufficient to delete the intravesicular contents.

Membrane-Perturbing Activity. We have examined "the membrane-perturbing activity", i.e., the relationship between the r value and the percent leakage for the initial 1 or 5 min after peptide addition for magainins 1 and 2 in SUVs composed of various lipids (Matsuzaki et al., 1991b). In most cases, the leakage was almost linearly dependent on r above a threshold r value, r_c. Figure 5 shows several membrane-perturbing activity curves simulated by use of the parameters listed in Table 1. The curves can be linearly approximated in the range $L > 0.3$, as observed (Matsuzaki et al., 1991b). A change in k_+ does not modify the slope

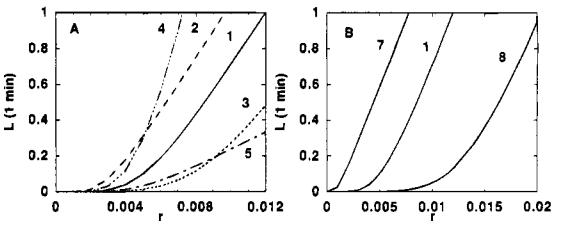

FIGURE 5: Simulation of membrane-perturbing activity. The extent of dye leakage, L, for the initial first minute was calculated as a function of r, the amount of membrane-bound peptide per lipid, by use of eq 7. The parameters used are listed in Table 1.

Table 1: Kinetic Parameters Used for the Calculation of the Curves in Figure 5

figure	curve	α	$k_+/(1 + g)$ (s^{-1})	n
5A	1	150	1×10^6	5
	2	150	1×10^7	5
	3	150	1×10^5	5
	4	300	1×10^6	5
	5	75	1×10^6	5
5B	1	150	1×10^6	5
	7	150	1×10^6	4
	8	150	1×10^6	6

(curves 1−3), whereas a variation in α never affects the r_c value (curves 1, 4, and 5). An increase in the n value raises the r_c value without changing the slope (curves 1, 7, and 8). These results can be explained as follows. If the r value is large, i.e., $n(n - 1)k_+r^{n-1}t/(1 + g) \gg 1$, eq 7 can be expressed as

$$L \approx \alpha(r - r_c) \quad (17)$$

$$r_c = \{n(n - 1)k_+t/(1 + g)\}^{1/(n-1)} \quad (18)$$

Therefore, the α value only influences the slope while the parameters k_+ and n change the r_c value.[6]

More Realistic Scheme. Scheme 1 assumes the implausible simultaneous assembly of five monomers. Our previous work suggests (Matsuzaki et al., 1994) that the peptide molecules lying in the plane of the membrane are in a monomer−dimer equilibrium. A more realistic scheme would involve the trimer and/or the tetramer as intermediates. The process may be described by multiple equilibria. For example,

$$2r_{1o} \overset{K_2}{\rightleftharpoons} r_{2o},\ 2r_{2o} \overset{K_4}{\rightleftharpoons} r_{4o},$$

$$r_{4o} + r_{1o} \overset{k_5}{\underset{k_{-'}}{\rightleftharpoons}} r_5 \rightarrow \text{translocation} \quad \text{(II)}$$

Assuming a fast establishment of the preequilibria, one can write

$$k_+ = k_5K_4K_2^2 \quad (19)$$

In other possible schemes, k_+ can also be expressed as a product of relevant equilibrium constants and k_5. One can

[4] The linearity in eq 1 indicates that the observed leakage at higher r_i values is larger than that predicted by eq 13. One explanation is that only monomeric unquenched dye molecules can pass through the magainin pore. The fraction of the monomer increases at lower intravesicular calcein concentrations.

[5] Let us consider the very early stage of the pore formation, where $r_o \approx r$. The pore formation rate is expressed as $dr_n/dt = k_+r^n - k_-(1 + g)r_n$. This differential equation can be easily integrated, yielding $r_n = [1 - \exp\{-k_-(1 + g)t\}]k_+r^n/k_-(1 + g)$.

[6] We did not evaluate the kinetic parameters for the previous results for SUVs because (1) small vesicles are susceptible to the vesicular aggregation and/or fusion (Matsuzaki et al., 1991b), (2) a broader size distribution of the vesicles with different packing strains has to be taken into consideration, and (3) neither the mode of leakage nor the extent of translocation has been determined.

hardly imagine that a magainin aggregate larger than a dimer stably lies parallel to the membrane surface, because such a situation would create a large cavity in the hydrophobic core of the membrane. Therefore, once the trimer or the tetramer is formed, the peptide-induced membrane deformation might trigger the concerted formation of the pentameric pore. The translocated peptides are also in the monomer−dimer equilibrium and mostly in the monomeric form at low r_i values.

We will discuss the pentamer formation constant by use of the obtained parameters, assuming $g = 1$. The constant is estimated to be $k_+/k_- = 1.8 \times 10^2$ for the egg PG system and 0.37×10^2 for the egg PG/egg PC system. The former value implies that $r = 0.12$ is needed for 10% of the total peptide molecules to form the pentamer. The binding isotherm reaches a plateau at $r \approx 0.12$ (Matsuzaki et al., 1994), suggesting that the pentamer can hardly be detected even at the highest available r value, in keeping with our previous observation (Matsuzaki et al., 1994) that the magainin helix lies parallel to the membrane surface irrespective of the r value.

Ludtke et al. (1994) reported that the magainin helix could take an orientation perpendicular to the membrane surface at high r values around 0.1 by the oriented circular dichroism technique.[7] Alamethicin (Huang & Wu, 1991) and melittin (Frey & Tam, 1991) also take the two orientations, i.e., parallel and perpendicular, according to the r value, the extent of membrane hydration, and the membrane fluidity. A channel-forming peptide, M2δ, was found to span the bilayer with the oriented NMR method (Bechinger et al., 1991). Thus, it seems that (1) amphiphilic pore-forming peptides can assume the two orientations to stably accommodate their amphiphilic helices into the amphiphilic lipid matrix, avoiding unfavorable exposure of the polar residues to the hydrophobic region of the lipid, and (2) their relative stability depends on the overall hydrophobicity of the peptide and the spacial segregation of the polar and nonpolar residues [hydrophobic moment (Eisenberg et al., 1982)] as well as the ambient conditions. In the case of magainin, its large hydrophilicity and hydrophobic moment favor the surface orientation. The population of the membrane-spanning pore is too small to be detected and the pore is unstable, in keeping with its very short lifetime (40 μs).

In summary, magainin translocates into the inner leaflet by forming a short-lived pentameric pore to reach the equilibrium distribution across the lipid bilayer. The apparent deactivation of the pore with increasing time can be ascribed to the reduced peptide density in the outer leaflet.

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[7] It cannot be said that their results are incompatible with the above arguments because their experimental conditions were quite different from ours; they examined magainin 1 in oriented dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (3:1) membranes hydrated without salts.

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