Translocation of a Channel-Forming Antimicrobial Peptide, Magainin 2, across Lipid Bilayers by Forming a Pore

Katsumi Matsuzaki,* Osamu Murase, Nobutaka Fujii, and Koichiro Miyajima

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

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ABSTRACT: A channel-forming antimicrobial peptide, magainin 2, has been shown to translocate across phospholipid bilayers by forming a pore comprising multimeric peptides. The translocation was demonstrated by four sets of experiments by use of resonance energy transfer from tryptophan introduced into the peptide to a dansyl chromophore incorporated into the lipid membrane. The translocation was coupled to pore formation, as detected by the dye efflux from the lipid vesicles; about 30% of the total peptide molecules translocated into the inner leaflets over 10 min, while 80% of the dye molecules leaked out at a lipid to peptide ratio of 57. This novel model can explain the problems debated so far, i.e., the peptide forms an ion channel whereas the magainin helix essentially lies parallel to the membrane surface. Channel (pore) formation in the vesicles is a transient process observable mainly during the early stage of the peptide membrane interactions.

A number of channel-forming cytotoxic peptides of 20−40 amino acids have been isolated and characterized for the last 3 decades, such as melittin from bee venom and alamethicin from *Trichoderma viride*. Although there is consensus that they act on membranes, mainly the lipid matrices of the target cells, the details of the action mechanisms have not yet been clarified (Cornut et al., 1993; Dempsey, 1990; Saberwal & Nagaraj, 1994; Sansom, 1991). Magainins, which are found in the skin of the African frog, *Xenopus laevis*, are a member of this family. They kill various microorganisms as well as tumor cells by permeabilizing the cell membranes (Cruciani et al., 1991; Juretić et al., 1989; Westerhoff et al., 1989b; Zasloff, 1987; Zasloff et al., 1988), thus being a candidate compound for therapeutic use. The peptides also act on mitochondrial membranes (Westerhoff et al., 1989a,b) and liposomes (Grant et al., 1992; Juretić et al., 1989, 1994; Matsuzaki et al., 1989, 1991, 1994; Vaz Gomes et al., 1993). It is reported (Matsuzaki et al., 1991; Vaz Gomes et al., 1993) that magainin 2, composed of 23 amino acid residues, is positively charged at physiological pH and binds to negatively charged phospholipid membranes with the aid of electrostatic interactions, forming an amphiphilic helix and permeabilizing the bilayers even in the absence of the transmembrane potential.

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

The observations that magainins form ion channels in planar lipid bilayers (Cruciani et al., 1992; Duclohier et al., 1989) and that the membrane permeabilization supralinearly depends on the peptide concentration (Duclohier et al., 1989; Juretić et al., 1994; Matsuzaki et al., 1991, 1994; Westerhoff et al., 1989a,b) lead to the following picture: a bundle of at least three or four membrane-spanning helices form an ion channel with the polar side chains oriented toward the bundle center. In this model, the helices should be present as a multimer, taking an orientation perpendicular to the membrane surface. However, there is a paradox in that available experimental evidence indicates that the helices, which are in a monomer−*dimer* equilibrium (Matsuzaki et al., 1994), essentially lie *parallel* to the membrane surface, even at lipid to peptide ratios (15−30) (Bechinger et al., 1993; Matsuzaki et al., 1994) lower than the ratios at which membrane permeabilization occurs (ca. 100). This problem is not limited to magainins but is applicable to other amphiphilic peptides (Altenbach & Hubbell, 1988; Chung et al., 1992). Thus, some groups have considered that the fraction of peptides involved in channel formation is too small to be detected at least in the absence of the transmembrane potential and that the potential facilitates the formation of the membrane-spanning pore (Altenbach & Hubbell, 1988; Chung et al., 1992; Juretić et al., 1994). Others have proposed that surface-lying peptides perturb lipid organization, enhancing the membrane permeability instead of forming a channel (Grant et al., 1992; Hermetter & Lakowicz, 1986; Williams et al., 1990). The purpose of this article is to propose and prove a novel model that can clarify the preceding problems.

The clue to solving this problem is the observation (Grant et al., 1992; Matsuzaki et al., 1991; Schwarz et al., 1992) that membrane permeabilization, estimated by the efflux of encapsulated water-soluble fluorescent dyes from lipid vesicles, ceases within ca. 10 min (see also Figure 5). This phenomenon suggests the following two points: First, the membrane permeabilization reflects a relaxation process. Second, when one studies the orientation and aggregation of peptides in membranes, one discusses the *equilibrium* state after the permeabilization has practically stopped, that is, the state where most of the channels, or more generally pores, no longer exist. Thus, the channel formation is a transient process observable mainly during the early stage of the peptide membrane interactions. We propose a new model

* Author to whom correspondence should be addressed. Telephone: 81-75-753-4574. Fax: 81-75-761-2698.

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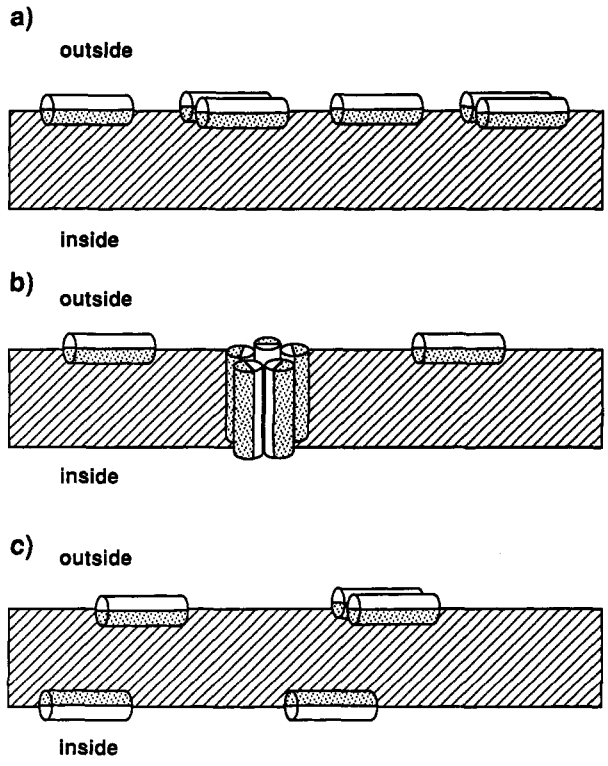

FIGURE 1: Model for pore formation induced by magainin 2 in lipid bilayers. The magainin helices are represented by rods. The dotted and shaded areas represent the hydrophobic parts of the amphiphilic helices and the bilayers, respectively. (a) The peptide molecules first bind to the outer surface of the bilayer membrane, lying parallel to the surface in a monomer−dimer equilibrium. (b) They form transient pores (channels) comprising a multimeric bundle of membrane-spanning helices. (c) Upon their closure, the peptides translocate across the membrane and are again in the monomer−dimer equilibrium. The pore density is greatly reduced compared to (b). See text for more details.

(Figure 1): (a) The peptide molecules first bind to the outer leaflet of the bilayer membrane, lying parallel to the membrane surface (Bechinger et al., 1993; Matsuzaki et al., 1994). The hydrophobic face (dotted area) of the amphiphilic helix shallowly penetrates into the apolar core (shaded area) of the bilayer. (b) They form pores (channels), as observed in the planar bilayer experiments (Cruciani et al., 1992; Duclohier et al., 1989). (c) Upon their closure, it is reasonable to imagine that the peptides can also go to the less populated *inner* leaflet; the peptides translocate across the membrane. Reduction in the peptide density on the outer surface greatly slows down the channel formation, leading to the deactivation of the pore, because it is a highly cooperative process.[1] This model can well explain the observation (Grant et al., 1992) that further addition of the peptide to pore-deactivated vesicles again induces the fast phase of leakage. Translocation could relieve the bilayer from the unfavorable strain due to asymmetrical peptide binding and resultant membrane expansion. In this article, we first will demonstrate the translocation by four sets of experiments, and second, we will show that the translocation is actually associated with pore formation.

MATERIALS AND METHODS

Materials. A magainin 2 mutant (12-Trp magainin 2), in which a Phe residue at the 12th position was substituted by Trp, was synthesized by a standard fluoren-9-ylmethoxycarbonyl (Fmoc)[2]-based solid phase method. The crude peptide was 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., 1991, 1994). The purity of the synthesized peptide was ascertained by quantitative amino acid analysis. 12-Trp magainin 2 has been confirmed to be equipotent to the parent peptide (Matsuzaki et al., 1994). L-α-Phosphatidyl-DL-glycerol (egg PG) enzymatically converted from egg yolk L-α-phosphatidylcholine (egg PC) was the kind gift of Nippon Fine Chemical Co. (Takasago, Japan). Egg PC and trypsin inhibitor were purchased from Sigma (St. Louis, MO). *N*-[[5-(Dimethylamino)naphthyl]-1-sulfonyl]dipalmitoyl-L-α-phosphatidylethanolamine (DNS-PE) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) were products of Molecular Probes (Eugene, OR). 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. Large unilamellar vesicles (LUVs), a model membrane system, were generated from a suspension of a lipid mixture by extrusion through polycarbonate filters (Matsuzaki et al., 1994). Briefly, a lipid film, after being dried under vacuum overnight, was hydrated 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 lipid concentration was determined by phosphorus analysis (Bartlett, 1959).

Dye Release. Dye-entrapped LUVs were prepared with a lipid mixture composed of egg PC and egg PG in a molar ratio of 5:5 by use of a 70 mM calcein solution (pH was adjusted to 7.0 with 1 N NaOH) as hydrating solution. Calcein-entrapped vesicles were separated from free calcein on a Sephadex G-50 column. 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. 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.

Resonance Energy Transfer (RET). Symmetrically labeled LUVs were prepared by hydrating the lipid film composed of egg PC, egg PG, and DNS-PE in a molar ratio of 5:4:1. Asymmetric labeling was carried out by adding 68 μL of a 5 mM DNS-PE/tetrahydrofuran solution to a 3.4 mM dansyl-free LUV suspension (2 mL). The flip-flop rate of phos-

[1] Note that in ion channel experiments, by use of planar lipid bilayers, the number of peptide molecules is usually in great excess over the amount of lipid, that is, most of the peptides are free in solution. Therefore, the peptide density in the monolayer facing the peptide-added compartment is kept constant and the pore deactivation is barely observed.

[2] Abbreviations: egg PC, egg yolk L-α-phosphatidylcholine; egg PG, L-α-phosphatidyl-DL-glycerol enzymatically converted from egg PC; DNS-PE, *N*-[[(5-(dimethylamino)naphthyl]-1-sulfonyl]dipalmitoyl-L-α-phosphatidylethanolamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine; LUVs, large unilamellar vesicles; RET, resonance energy transfer; Fmoc, fluoren-9-ylmethoxycarbonyl.

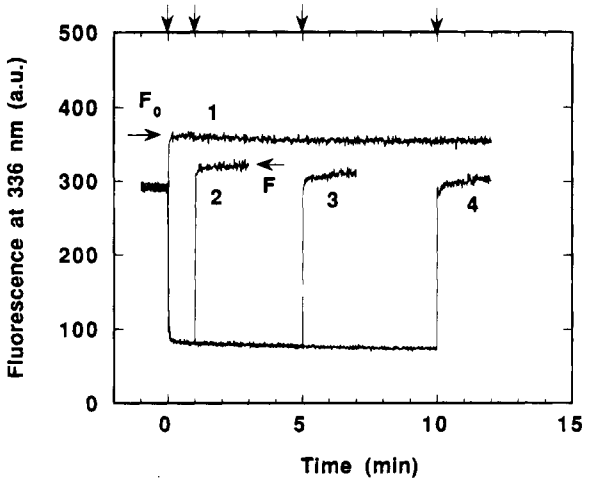

FIGURE 2: Detection of the peptide translocation using RET (I). 12-Trp magainin 2 was mixed with dansyl LUVs (egg PC/egg PG/DNS-PE, 5:4:1, mol/mol) at time zero. The final peptide and lipid concentrations were 3 and 170 μM, respectively. Under these conditions, most (>90%) peptide molecules are in the membrane-bound state. The fluorescence intensity of Trp at 336 nm (excited at 280 nm) was recorded. The binding of the peptide to the vesicle reduced the intensity due to RET from Trp to the dansyl chromophore. At various time intervals of incubation (1, 5, and 10 min, traces 2−4), a large excess (1.27 mM) of the second population of dansyl-free LUVs (egg PC/egg PG, 5:5) was added, as indicated by the arrows. An increase in intensity (F) indicates the relief from RET caused by the redistribution between the two vesicle populations of the peptide molecules that had been bound to the *outer surface* of the first vesicle. The F value decreases with prolonged incubation and is smaller than the fluorescence intensity (F_0) when both populations of vesicles were simultaneously added at time zero (trace 1), thus indicating that some of the peptides translocated into the inner leaflet during incubation. $\Delta F = F_0 - F$ is a measure of the amount of translocated peptide. Each trace is the average of 2−3 experiments. The temperature of all experiments was controlled at 30 ± 0.5 °C.

pholipid is so slow that the added DNS-PE molecules are expected to reside on the outer leaflet during the experiment. The extent of asymmetry was estimated by use of NBD-PE instead of DNS-PE, because the fluorescence of the former in the outer monolayer is easily quenched by reaction with externally added water-soluble dithionite ions. More than 80% of the dye was found to reside in the outer leaflet. RET from the Trp residue of the peptide to the dansyl chromophore in the membrane was monitored by observing the fluorescence intensity of either the Trp residue (336 nm) or the dansyl group (510 nm) upon excitation at 280 nm. The temperature was controlled at 30 ± 0.5 °C.

RESULTS

RET from the Trp residue to the dansyl chromophore (DNS-PE) incorporated into the lipid vesicles was utilized to monitor the peptide membrane association.

Detection of Peptide Translocation (I). In the first two experiments, we demonstrated that the peptide becomes less exposed to the outer membrane surface in a time dependent manner. The addition of symmetrically dansyl-labeled vesicles to a peptide solution at time zero resulted in a significant decrease in Trp fluorescence, indicating RET due to binding of the peptide to the membrane (Figure 2). The binding was very fast and completed within 1 s, as in the case of melittin (Schwarz & Beschiaschvili, 1989) and alamethicin (Schwarz et al., 1987). After a short incubation for 1 min, a second population of dansyl-free vesicles (DNS-PE was substituted by egg PG) was added in large excess (trace 2 in Figure 2). An increase in the fluorescence intensity implies that the peptide molecules, which had been bound to the *outer* leaflet, were redistributed between the two vesicle populations, resulting in relief from RET. The increased fluorescence intensity, F, however, was smaller than F_0, the intensity when both populations of vesicles were added simultaneously to the peptide solution at time zero (trace 1), suggesting that a fraction of the peptides, which is related to $\Delta F = F_0 - F$, became unexposed to the outer surface and therefore untransferrable to the second vesicles. The buried fraction, ΔF, increased with incubation time (traces 2−4).

Detection of Peptide Translocation (II). To confirm this observation, we carried out protease (trypsin) digestion experiments. The enzyme was added externally to a peptide lipid mixture or encapsulated within the internal aqueous phase of the vesicle to selectively digest the peptide located in the outer or inner monolayer, respectively. Trypsin hydrolyzes the peptide bonds on the C-terminal sides of the Lys residues. The hydrolyzed Trp-containing fragment, Trp^{12}-Gly-Lys, will be desorbed from the membrane and can be detected by the RET technique; the desorption results in relief from RET, i.e., a decrease in dansyl fluorescence at 510 nm when excited at 280 nm, where Trp is selectively excited. Figure 3A shows that the magainin peptide is time dependently less sensitive to externally added trypsin. The enzyme was added to a peptide solution that had been incubated with dansyl-labeled vesicles for various times. Simultaneous addition of the protease and the vesicles to the peptide solution digested the peptide molecules and completely desorbed them from the membrane within 2 min (trace 1), whereas prolonged preincubation protected the peptide from enzyme attack (traces 2−4). These results reinforce the conclusion from Figure 2 that the peptide becomes less exposed in a time dependent manner, suggesting the possibility that the peptide molecules translocate into the inner leaflet across the bilayer. They also indicate that the magainin-induced pore is not large enough for the enzyme (MW 24 000) to pass through.

Figure 3B directly indicates that the magainin molecule actually reaches the inner leaflet. Trypsin-entrapped unilamellar vesicles were prepared. The enzyme outside the vesicle was inactivated by addition of the trypsin−chymotrypsin inhibitor. The lower trace shows that the peptide was gradually digested by the enzyme inside the vesicle. The upper trace depicts a control experiment where the inhibitor was present on both sides of the vesicle. No digestion was detectable.

Detection of Peptide Translocation (III). During the third experiment, we directly observed the time course of peptide translocation by use of RET (Figure 4). Selective labeling of the outer monolayer with DNS-PE enabled us to distinguish the peptide in the outer leaflet from that in the inner one.[3] The fluorescence emission of both Trp and dansyl was simultaneously measured as a function of time after peptide addition. An increase in Trp fluorescence with a concomitant decrease in dansyl fluorescence strongly indicates that the translocation of the peptide from the outer to the inner leaflet accompanies the relief from RET.

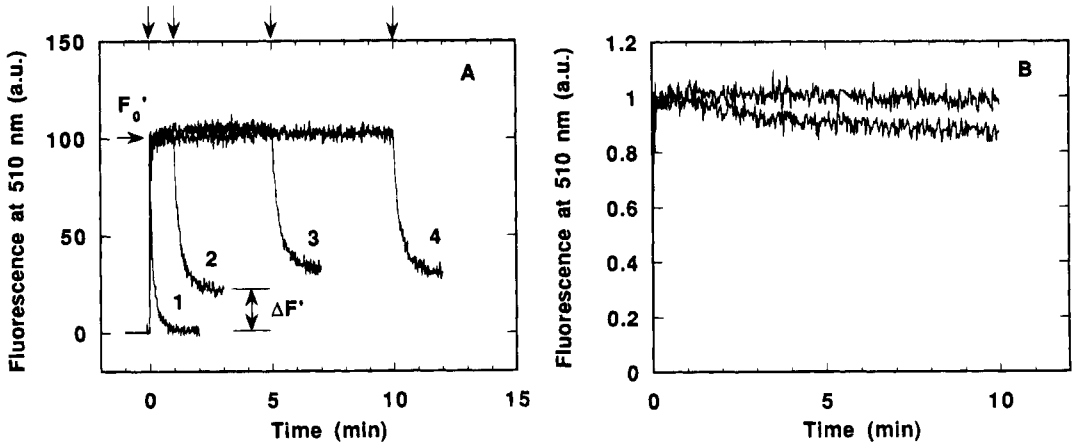

FIGURE 3: Detection of peptide translocation by use of RET (II). (A) Selective digestion of magainin on the outer leaflet. Trypsin (final concentration, 25 μg/mL) was added to a 12-Trp magainin 2 solution that had been incubated with dansyl-labeled vesicles for various times, as indicated by the arrows. The peptide and lipid concentrations were the same as in Figure 2. The fluorescence intensity of the dansyl chromophore at 510 nm (excited at 280 nm) was recorded. The binding of the peptide increases fluorescence due to RET. Simultaneous addition of trypsin and the vesicles to the peptide solution digested the peptide molecules and completely desorbed them from the membrane within 2 min (trace 1), whereas prolonged preincubation (for 1, 5, or 10 min) protected the peptide from enzyme attack (traces 2–4). $\Delta F'/F_0'$ corresponds to the fraction of translocated peptide. Each trace is the average of three experiments. (B) Selective digestion of translocated magainin. RET experiments similar to (A) were carried out. Trypsin-entrapped unilamellar vesicles were prepared by hydration of lipid films with a trypsin (200 μg/mL) containing Tris buffer. The vesicle preparation was carried out at 4 °C to avoid inactivation of the enzyme. Upper trace: trypsin on both sides of the vesicle was inactivated with trypsin–chymotrypsin inhibitor (400 μg/mL) by the addition of the inhibitor to the hydrating solution. No digestion was observed. Lower trace: the inhibitor was added externally to the trypsin-containing vesicle. The extravesicular enzyme was selectively inhibited. Each trace is the average of three experiments.

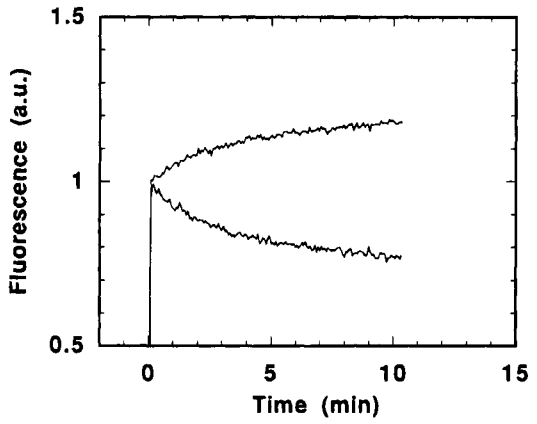

FIGURE 4: Detection of peptide translocation using RET (III). The magainin peptide was mixed with LUVs whose outer leaflets were asymmetrically labeled by DNS-PE. The peptide and lipid concentrations were the same as those in Figure 1. The fluorescence emissions of both Trp at 336 nm (upper trace) and dansyl at 510 nm (lower trace) were simultaneously measured as a function of time after peptide addition by rapidly changing the observation wavelength at 4 s intervals (excited at 280 nm). The intensities are normalized with respect to those at time zero. Each trace is the average of three experiments.

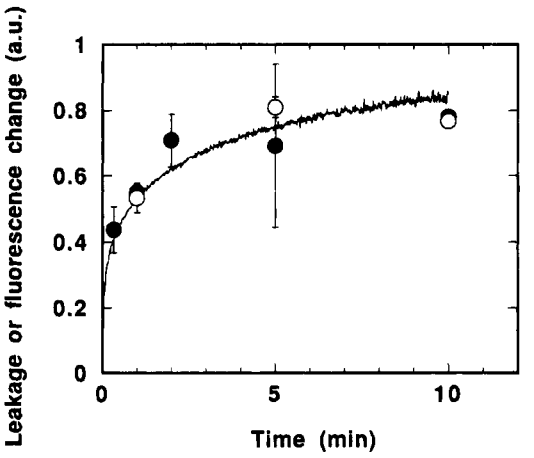

FIGURE 5: Relationship between peptide translocation and pore formation. The former was estimated by ΔF in Figure 2 (●) and $\Delta F'$ in Figure 3A (○). The latter was evaluated on the basis of magainin-induced release of a fluorescent dye, calcein, entrapped within the LUVs. The peptide and the calcein-entrapped vesicles composed of egg PC/egg PG (5:5) were mixed at time zero under the same conditions as in Figure 2. The efflux of the dye from the vesicles was monitored fluorometrically. The fluorescence intensity corresponding to 100% leakage was measured by adding Triton X-100. The time course of the leakage was shown by the solid curve, which is the average of three experiments. The reproducibility was within 3%.

Relationship between Translocation and Pore Formation. These four sets of experiments clearly prove the translocation of the magainin peptide across the bilayer. The next subject is to examine how the translocation process is related to pore formation. A convenient method to evaluate membrane permeabilization is to fluorometrically measure the efflux of a water-soluble fluorescent dye entrapped within lipid vesicles (Weinstein et al., 1977). The solid line in Figure 5 shows the time course of membrane permeabilization, expressed as the extent of leakage of the entrapped dye, calcein. The fluorescence signal was converted to the extent of leakage by taking the graded mode of leakage into consideration (Matsuzaki et al., 1994). The rate of leakage was initially fast, but subsequently slowed down, that is, pore deactivation was observed.[4] The time course of peptide translocation was estimated by ΔF in Figure 2 and $\Delta F'$ in Figure 3A and is plotted in Figure 5. The parallelism

[3] The critical distance between the donor (Trp) and the acceptor (dansyl), above which RET is significantly decreased, was estimated on the basis of Förster's theory (Förster, 1959) to be 20 Å, which is almost equal to the lipid monolayer thickness. Therefore, the Trp residues in the outer and inner leaflets are distinguishable, i.e., the transbilayer RET barely occurs, because both the Trp residue (Matsuzaki et al., 1994) and the dansyl group are located near the membrane surface.

between membrane permeabilization and translocation is striking. Figure 4 also indicates that translocation follows a similar time course. It should be noted that vesicle fusion would cause both leakage and translocation. The absence of any significant fusion was confirmed by measuring the 90° light-scattering intensity at 400 nm, which is sensitive to the change in vesicle diameter. The increase in intensity was less than 3%.

DISCUSSION

Detection of Translocation. We proposed the novel model (Figure 1) where the magainin peptide translocates across the lipid bilayer by forming a pore and proved it by the RET experiments (Figures 2−4), although the detailed structure of the pore has not yet been revealed. In the first experiment (Figure 2), a major fraction of the peptide could be extracted by the addition of the second population of vesicles, in keeping with the observations that their addition immediately stops dye leakage (Grant et al., 1992) and that an increase in lipid concentration reduces the leakage (Matsuzaki et al., 1989, 1991). However, the remaining unextractable fraction increased in a time-dependent manner. These results coincide with the trypsin digestion experiment (Figure 3A); the peptide became undigestible to the externally added enzyme. The $\Delta F'/F_0'$ value gives a direct measure for the translocated fraction; under our conditions, 30% of the peptides translocated within 10 min (Figure 3A). This value is comparable to the value of 20% roughly estimated from Figure 2 as follows. If we assume that the affinity of 12-Trp magainin 2 to the dansyl-labeled vesicles is identical to that to the unlabeled vesicles, the F value can be expressed as

$$F = k_{DNS} f [P] + (1 - f) F_0$$

The translocated fraction, the fluorescence coefficient of the Trp residue in the labeled vesicle, and the total peptide concentration are denoted by f, k_{DNS}, and [P], respectively. The k_{DNS}[P] value can be estimated from the quenched fluorescence intensity (the lowest trace in Figure 2A). Thus, the f value can be evaluated for the observed F and F_0 values. Figure 3B indicates that about 11% (ca. 170 molecules per vesicle) of the peptides was hydrolyzed by entrapped trypsin within 10 min. The reason why this value is smaller than that (20−30%) estimated earlier is the fact that the average number of trypsin molecules per vesicle was less than 3. Entrapment of a higher concentration of the enzyme was difficult because of the high viscosity of the solution.

It should be noted that at 10 min the system had not yet reached the true equilibrium where 50% of the peptides should translocate; 30% translocation reduces the pore formation rate to one-sixth of (0.7^5) of the initial value, assuming a pentameric pore (vide infra), leading to the pseudoplateau. Indeed, a prolonged incubation (4.5 h) increased the extent of translocation by another 10% (data not shown).

Pore Model. The following observations argue for pore formation as the action mechanism of magainin. (1) Ion channel activities in planar bilayers have been reported (Cruciani et al., 1992; Duclohier et al., 1989). (2) The pore serves as a molecular sieve (Figures 3A and 5); sucrose (MW 300, Westerhoff et al., 1989a) and calcein (MW 600) are permeable but trypsin (MW 24 000) is not. (3) The magainin-induced perturbation of lipid organization is minimal (Matsuzaki et al., 1991; Williams et al., 1990; K. Matsuzaki and J. Seelig, unpublished work). (4) The peptide does not fuse (this study) nor micellize (Juretić et al., 1994; Westerhoff et al., 1989a,b; K. Matsuzaki and J. Seelig, unpublished work) the membrane. The pore seems to be a multimer of peptides. Our previous study suggested a tetrameric pore (Matsuzaki et al., 1994); however, our recent kinetic study (Matsuzaki et al., unpublished work) indicates that a pentameric pore is more plausible, in keeping with other studies: 3−6-mer (Duclohier et al., 1989), 3−5-mer (Westerhoff et al., 1989a), and 4−6-mer (Juretić et al., 1994; Westerhoff et al., 1989b). An ion channel comprising a pentameric bundle of helices has been proposed for the nicotinic acetylcholine receptor (Hucho & Hilgenfeld, 1988). 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. The pentameric pore might be somewhat small to allow the dye to pass through. If the pore is made up of a peptide−lipid complex, the central aqueous channel will be larger or the dye can leak out through a temporary defect in the lipid organization upon translocation. A recent study demonstrated (Fattal et al., 1994) that amphiphilic peptides cause a rapid flip-flop of membrane lipids.

There is the possibility that the dye efflux pore formed in the absence of the transmembrane potential does not necessarily equate with the channel underlying voltage dependent ion conductance, as suggested for melittin and its derivative (Dempsey et al., 1991). However, at least in the case of magainin, both structures may be similar. (1) The molecularity of the pore in the liposome (5) falls within the estimated range (3−6) of the ion channel molecularity (Duclohier et al., 1989). (2) Cruciani et al. (1992) reported that the magainin channel in the acidic phospholipid bilayer shows cation selectivity, but fails to discriminate between different cations (Na^+, K^+, Li^+, and *N*-methyl-*d*-glucamine$^+$) or anions (Cl^-, I^-, and sulfonate). Similar phenomena were also observed in the liposomal system (Matsuzaki et al., unpublished work).

If pore formation accompanies the reorientation of the helix from the parallel state to the perpendicular membrane-spanning state (Figure 1), it will be sensitive to membrane fluidity. Gel state membranes are reported to be less susceptible to magainin than fluid membranes (Matsuzaki et al., 1991). Furthermore, the incorporation of cholesterol, which also rigidifies membranes in the liquid crystalline state, reduces magainin activity (Matsuzaki et al., 1995).

An inside negative transmembrane potential would electrophoretically facilitate the translocation of the positively charged magainin peptide, although helical dipole electric field interactions may also be involved. In fact, the potential has been reported to enhance the permeability of liposomal (Vaz Gomes et al., 1993) and erythrocyte membranes (Matsuzaki et al., 1995).

Comparison with Other Peptides. It is a surprise that such a large (ca. 2.5 kDa), charged (+4) molecule can easily pass

[4] If the number of open pores per vesicle is constant with respect to time, t, the leakage, L, should obey the equation, $\ln(1 - L) = -kt$ (Schwarz & Robert, 1992). However, the $\ln(1 - L)$ versus t plot shows a downward deviation (Matsuzaki et al., 1994), indicating that the pore density decreases with increasing time (pore deactivation).

through the hydrophobic core of the membrane. The group of de Kruijff (De Kroon et al., 1993) investigated the translocation of several peptides across the membrane. A pentapeptide with two positive charges (NH_3^+-Arg^+-Met-Leu-Trp-Ala-$COOCH_3$) does not permeate the bilayer, although the deprotonated form of a hexapeptide (NH_3^+-Ala-Ile-Met-Leu-Trp-Ala-$COOCH_3$) can pass through the membrane. The binding of a longer membrane lytic tetradecapeptide, mastoparan X, is enhanced by the transmembrane potential, which might be attributed to translocation. Recently, Maduke and Roise (1993) demonstrated that a positively charged, amphiphilic, mitochondrial presequence peptide 25 residues long can translocate across phospholipid bilayers in a potential dependent manner. This peptide also permeabilizes and fuses the membrane (Roise et al., 1986). The coupling of translocation and permeabilization unfortunately is unclear, because they used different types of vesicles, both in size and lipid composition, for these studies. Melittin, a hexacosapeptide with +6 charge, in planar lipid bilayers has been suggested to assume a transmembrane configuration and to flip to the transleaflet upon application of trans-negative potential (Kempf et al., 1982). It is possible that the translocation of amphiphilic peptides by forming pores, in some cases with the aid of the transmembrane potential, is a more universal phenomenon. Huang et al. postulated, by use of the oriented circular dichroism technique, that magainin 1 (Ludtke et al., 1994) and alamethicin (Huang & Wu, 1991) switch their orientations from parallel to perpendicular to the membrane surface as the peptide concentration reaches the critical concentration (peptide:lipid = 1:10 for the former and 1:120−300 for the latter). Melittin also assumes the two orientations depending on the conditions (Frey & Tamm, 1991). The pore deactivation was again observed for melittin (Schwarz et al., 1992) and alamethicin (Schwarz & Robert, 1990). If the translocation is universal, the reported interactions of these peptides with proteins on the inner side of the membrane, e.g., G proteins (Mousli et al., 1990), will become biologically significant. Furthermore, our results open up the possibility that magainins may be utilized to selectively incorporate drugs into tumor cells.

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