Magainin Oligomers Reversibly Dissipate $\Delta\mu_{H^+}$ in Cytochrome Oxidase Liposomes[†]

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ABSTRACT: Magainin peptides present in the skin of *Xenopus laevis* and identified as antimicrobial agents
are shown to decrease the membrane potential in cytochrome oxidase liposomes. They also released respiratory
control with a third or higher order concentration dependence. Respiratory control was restored by proteolytic
digestion of the added magainin. The amount of magainin required for half-maximal stimulation of respiration
was proportional to lipid concentration. At appreciably higher concentrations magainins inhibited uncoupled
respiration. The results are discussed in terms of a model in which most of the added magainin adsorbs
as a monomer to the membranes but equilibrates with a multimeric pore that causes rather general permeability
of membranes. The ensuing ion permeation dissipates membrane potential and stimulates respiration.

The skin of amphibia and reptiles has been a rich source
of biologically active compounds (Witkop & Goessinger,
1983). This was again stressed by the observation that the
skin of *Xenopus laevis* actively secretes numerous peptides
(Gibson et al., 1986; Giovannini et al., 1987) and the
demonstration (Zasloff, 1987; Soravia et al., 1988; De Waal
et al., 1991; Baker et al., 1993) that at least three of these,
i.e., magainin 1, magainin 2,[1] and PGLa, to which we shall
collectively refer as magainins, are cytotoxic for microbial
and higher eukaryotic cells. Synthetic analogues of these
magainins have been shown to be even more active in
antimicrobial assays than the parent compounds (Chen et al.,
1988; Juretić et al., 1989). Magainins may be part of a
structurally heterogeneous, but functionally definable, class
of oligopeptides (Boman & Hultmark, 1987; Dimarcq et al.,
1988; Lehrer et al, 1988; Nakajima et al., 1987; Bevins &
Zasloff, 1990; Boman, 1991) that provide a secondary immune
system for organisms as diverse as insects, amphibia, and man.

At pH 7, magainins are positively charged, amphiphilic
oligopeptides consisting of some 23 amino acids (Zasloff, 1987;

Gibson et al., 1986; Soravia et al., 1988). In 5% trifluoro-
ethanol, magainin 2 takes on an amphipathic α-helical
conformation (Marion et al., 1988), suggesting, as confirmed
by Raman spectroscopy (Williams et al., 1990), that it may
also do so when present in the hydrophobic environment of
the membrane. Magainins have been proposed to line an ion
channel (Guy & Raghunatan, 1988; Duclohier et al., 1989;
Cruciani et al., 1992; Vaz Gomes et al., 1993) or to lie in the
plane of the membrane–solution interface (Bechinger et al.,
1991) and perturb membrane impermeability (Cruciani et
al., 1992; Grant et al., 1992). To decide between these
possibilities, information on whether the magainin effects are
reversible should be pertinent. Most experimental systems
have not allowed addressing that question.

That increase of the ion permeability of biological mem-
branes may be the mechanism of their lethal action was
suggested by the finding that magainins rapidly depolarize
the plasma membrane of *Escherichia coli* (Westerhoff et al.,
1989b). The killing was proposed to be caused by the
interference of the magainins with the free-energy metabolism
of the victim cell (Mitchell, 1961; Westerhoff & Van Dam,
1987).

Studies of the interaction of magainins with the energy
metabolism and membranes of *E. coli* (Westerhoff et al.,
1989b), rat liver mitochondria (Westerhoff et al., 1989a),
and hamster spermatozoa (De Waal et al., 1991) were
complicated by the multitude of processes occurring in those
systems, as well as by the abundance of proteases (Westerhoff
et al., 1989a,b). On the other hand, studies with pure lipid
membranes without active free-energy-transducing enzymes
(Cruciani et al., 1992; Duclohier et al., 1989; Matsuzaki et
al., 1989, 1990; Vaz Gomes et al., 1993), though they have
confirmed the proposed (Westerhoff et al., 1989b) membrane
permeabilization, cannot demonstrate the interference of
magainins with free-energy transduction. Consequently we
chose the system of cytochrome *c* oxidase, reconstituted into
small unilamellar liposomes [Hinkle et al. (1972), as modified
by Wrigglesworth et al. (1987)], as the model system to study
fundamental characteristics of the action of magainins on
proton-mediated free-energy transduction. We report that
the magainins reversibly interfere with proton-mediated free-

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[1] Abbreviations: magainin 2, H$_2$N-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-
Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-
CONH$_2$; PGLa, H$_2$N-Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-
Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-CONH$_2$; Z-peptide, the
stereoisomer of magainin 2 that has all Lys and Phe residues in the D
configuration; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhy-
drazone; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-
morpholino)propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piper-
azineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TMPD,
N,N,N′,N′-tetramethyl-*p*-phenylenediamine; TPP$^+$, tetraphenylphos-
phonium ion; w/w, mass/mass; PL, phospholipid; ALSK, (alanine-leucine-
serine-lysine)$_6$ amide.

energy transduction from redox free energy to proton electrochemical potential difference.

MATERIALS AND METHODS

Materials. Cytochrome oxidase was purified according to Yoshikawa et al. (1977). The preparation contained 0.9–1.6 mM heme A (10–11 nmol of heme A per milligram of protein) in 0.01 M sodium phosphate at pH 7.4, and it was stored aerobically at −80 °C in small aliquots (Hendler & Sidhu, 1988). Asolectin was purified as described by Darley-Usmar et al. (1987) and stored under Ar or N_2 below −19 °C. The purified asolectin contained 0.88 mmol of phosphorus per gram.

Magainin 2, PGLa, (tyrosine[5])magainin 2, "Z-peptide" (the stereoisomer of magainin 2 that has all the lysine and phenylalanine residues in the D configuration) and (Ala-Leu-Ser-Lys)$_6$, or "ALSK" peptide, were the synthetic carboxy-amidated derivatives (Soravia et al., 1988). These peptides were purified by HPLC as described (Zasloff et al., 1988). Purity exceeded 95%, as determined from the amino acid composition. Melittin, calcein, some FCCP (carbonyl cyanide *p*-trifluoromethoxy)phenylhydrazone), and some valinomycin were obtained from Sigma, while asolectin, some FCCP, and some valinomycin were obtained from Fluka. Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid) was obtained from Kodak. Peptide "concentrations" given in molar units refer to added amounts of peptide per unit total volume.

Preparation of Cytochrome Oxidase Liposomes. Cytochrome oxidase liposomes were prepared according to Hinkle et al. (1972), with modifications described by Wrigglesworth et al. (1987). One hundred milligrams of purified asolectin was suspended under nitrogen or argon in 2 mL of 50 mM potassium phosphate, pH 7.4, by vortexing for 30 s and then stored overnight at 4 °C. The following morning the preparation was vortexed for 1 min, followed by the addition of 40 mg of sodium cholate and further vortexing on ice. The suspension was clarified by sonication (four or five times for 15 s (with 45 s between)) on ice, at power 3 of a Heat Systems sonifier. To 1.5 mL of this suspension was added 1.6 mg (0.022 mL) of cytochrome oxidase. The suspension was vortexed for 1–5 s and put into Spectrapor (M_r cutoff 12 000–14 000) dialysis membrane (which had been soaked overnight in water plus a pinch of Na_2EDTA). The suspension was dialyzed at 4 °C against 50 mM potassium phosphate at pH 7.4 four times: twice for 3–4 h, overnight, and once more for 3–4 h the next morning. The resulting liposome suspension was stored at 4 °C for up to two weeks, the respiratory control ratio (i.e., the respiratory rate in the presence of excess FCCP plus valinomycin divided by the rate in their absence) remaining above 10.

"Pyranine liposomes" (Clement & Gould, 1981) were prepared as described above except that 0.5 mM pyranine was also present throughout dialysis and the resulting liposomal suspension was passed through a Sephadex G25 column (equilibrated with 50 mM potassium phosphate, pH 7.4). These liposomes exhibited the same respiratory control ratios as the liposomes without internal pyranine. "Calcein liposomes" (Oku et al., 1982) were prepared in 0.1 mM calcein plus "buffer 600" (75 mM MES, 33 mM MOPS, 11.5 mM HEPES, 65 mM Tris, 64 mM boric acid, and 50 mM K_2SO_4, pH 7.4). The respiratory control ratio in this buffer was considerably lower (approximately 2) than in phosphate buffer.

Respiratory Titrations. Respiratory rates were measured in an oxygraph (Clark electrode, T = 298 K, 1.8-mL volume). To 50 mM potassium phosphate (pH 7.4) were added 0.56 mM Na_2EDTA, 6 mM ascorbic acid, 0.083 mM TMPD, and
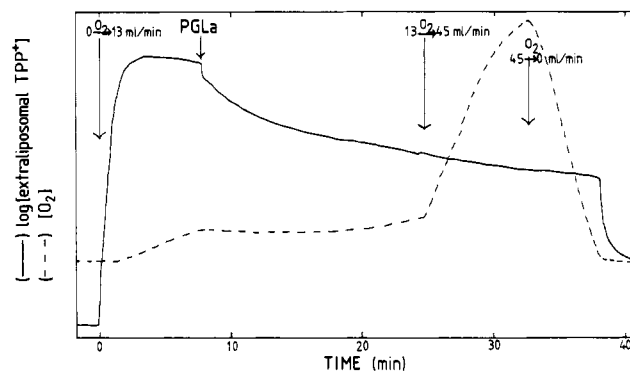

FIGURE 1: Dissipation by PGLa of the membrane potential in cytochrome oxidase liposomes. Aeration of a suspension of cytochrome oxidase liposomes (the pO₂ is indicated by the dashed line) leads to uptake of TPP⁺ (i.e., decrease in [TPP⁺] as indicated by the upward deflection of the solid line). Neglecting binding, this uptake would be calculated to correspond to a membrane potential of 0.19 V. At the indicated time PGLa was added to an overall concentration of 2.2 μM. The argon flow rate (33 mL/min) was maintained throughout the experiment. O₂ flow rate was increased from 0 to 13 and 45 mL/min and then decreased to 0 at the indicated times.

0.022 mM cytochrome *c* (horse heart type VI, Sigma), and the background (i.e., nonenzymatic) respiratory rate was recorded. After addition of the indicated amounts of cytochrome oxidase liposomes and a 5-min incubation, the coupled respiratory rate (J_c) was recorded. Subsequently, peptide was added and the respiratory rate was redetermined after 1 min, after which more peptide was added. All reported respiratory rates were first corrected for the background respiratory rate. Then relative respiratory rates were calculated as the ratio of the actual respiratory rate in the presence of peptide (or protonophore) to the coupled respiratory rate.

Calcein Fluorescence Measurements. Calcein fluorescence was monitored in an SLM 4000 spectrofluorimeter (SLM Instruments, Inc.). Excitation and emission wavelengths were 490 (bandwidth, 4 nm) and 520 (bandwidth, 4 nm) nm, respectively. Experiments were done at 25 °C by circulating water through the water jacket in the sample chamber.

Membrane Potential Measurements. We used the uptake of TPP⁺ as monitored by a macroscopic TPP⁺ electrode to probe the electric potential developed across the membranes of the cytochrome oxidase liposomes. This was done in a quartz vessel equipped with a reference electrode, a light guide leading to a photomultiplier allowing measurement of pyranine fluorescence (Hendler, 1991), a Davies-type rapid-response oxygen electrode (Reynafarje et al, 1982), a reference electrode, a glass pH electrode, and a TPP⁺ electrode (Hendler, 1991). The inner chamber of the reaction vessel was surrounded by a water jacket maintained at 25 °C by circulating water. Mixtures of O_2 and Ar could be blown over the surface of the suspension, typically at rates of 45 mL/min. In the experiment reported in Figure 1, the vessel contained 5.5 mL of 50 mM KCl and 10 mM HEPES (pH 7.4), supplemented with 0.50 mL of pyranine liposomes (34 g of phospholipid/liter of cytochrome oxidase liposomes in potassium phosphate at pH 7.4). The TPP⁺ electrode was then calibrated with (in total) 0.05 mM TPP⁺. Subsequently, 0.016 mM cytochrome *c*, 8.3 mM ascorbate, and 0.025 mM TMPD were added. While O_2 (12 mL/min) and Ar (33 mL/min) were led over the suspension, the liposomes were allowed to develop a membrane potential. Subsequently, the O_2 flow was interrupted and Ar (33 mL/min) was led over the suspension, causing a substantial decrease in that potential. After this preincubation period, the experiment shown in

Figure 1 was begun by again leading O_2 (13 mL/min) and Ar (33 mL/min) over the suspension.

RESULTS

Magainin Dissipation of Membrane Potential. We used tetraphenylphosphonium ion (TPP^+) as a qualitative indicator of the electric potential across the liposomal membranes. The solid line in Figure 1 represents the signal monitored by a TPP^+-sensitive electrode in a suspension of cytochrome oxidase liposomes in the presence of ascorbate, cytochrome *c*, and TMPD (a redox mediator between ascorbate and cytochrome *c*). A decrease in external TPP^+ concentration is indicated by an upward deflection of the solid line in Figure 1. During the preincubation period, argon had been blown over the suspension, maintaining it anaerobic (as indicated by the dashed line at the start of the trace). When an approximately 1:3 (v/v) mixture of O_2 and Ar was blown over the suspension, much of the TPP^+ rapidly disappeared from the medium (cf. Figure 1), indicating that a transmembrane electric potential, negative inside, was formed. Addition of PGLa then caused a change in the TPP^+ electrode signal indicative of a gradual drop in membrane potential. That drop could not be reversed by increasing the O_2/Ar ratio to 1.4 in the gas mixture flowing over the suspension: as confirmed by an oxygen electrode in the suspension, the suggestive drop in membrane potential was not due to anaerobiosis. That the addition of this amount of PGLa did not completely dissipate the membrane potential is demonstrated by the further increase in extraliposomal [TPP^+] (i.e., downward deflection in the trace) occurring when only argon was flushed over the suspension (Figure 1).

Magainin 2 was also capable of decreasing the membrane potential (not shown). Magainin 2 and PGLa have both been reported to be active antimicrobial agents, whereas the stereoisomer of magainin 2 that has all the lysine and phenylalanine residues in the D configuration (peptide Z) had little or none of this capability (Soravia et al., 1988). Indeed, at the same concentration, peptide Z had no significant effect (not shown) on the membrane potential, whereas magainin 2 and PGLa had a large effect.

The liposomes used in the experiment of Figure 1 contained 0.5 mM pyranine, which exhibits a pH-dependent fluorescence. The pyranine fluorescence (not shown) indicated that, upon the initial aeration, the pH gradient across the liposomal membranes did not rise by more than 0.11 pH units (inside alkaline). The addition of PGLa appeared to have little effect on this pH gradient.

Magainins Uncoupling Respiratory Rate. The observed reduction in membrane potential could be due (i) to an inhibitory effect of the magainin-like compounds on the electron transfer catalyzed by cytochrome oxidase, (ii) to decoupling of respiration from proton translocation (i.e., to an induction of slip), or (iii) to uncoupling (i.e., to a decrease in electrochemical proton gradient caused by enhanced ion permeability). In the first case the addition of the magainin should lead to a reduction in respiratory rate; in the third case, to an increase in respiratory rate. Figure 2A shows that in the concentration range where they began to reduce the membrane potential (Figure 1), magainin 2 and PGLa stimulated respiration by cytochrome oxidase liposomes. It is only at 10-fold higher concentrations that these compounds began to inhibit respiration in cytochrome oxidase liposomes (Figure 3).

The amount of magainin 2 needed to stimulate respiration to 50% of the maximum rate was about 3 times that of PGLa (Figure 2A). We also tested two analogues of magainin 2.
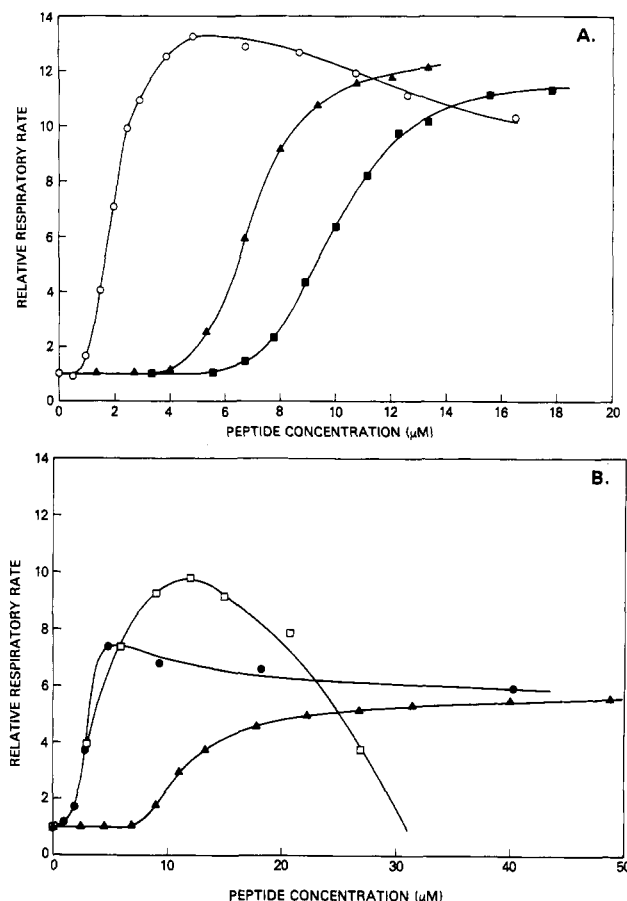

FIGURE 2: The effect on respiration of increasing concentrations of magainin-like peptides. (A) (O) PGLa, (▲) magainin 2, (■) ($tyrosine^5$)magainin 2. (B) (●) ALSK, (□) melittin, (▲) Z peptide. Except for the melittin titration (0.4 g of phospholipid/L) and the ALSK titration (0.25 g of phospholipid/L), the concentration of cytochrome oxidase vesicles was 0.2 g of phospholipid/L. For the melittin titration the TMPD concentration was 0.042 mM. Relative respiratory rates were calculated as described under Materials and Methods.

Taking the half-maximal dose as a measure, the magainin 2 analogue with tyrosine replacing phenylalanine at the 5 position was some 25% less active (Figure 2A) than magainin 2. Z peptide was some 2.5 times less active than magainin 2 (Figure 2B). Other positively charged peptides also stimulated respiration (Figure 2B). Melittin was a little less active than PGLa in stimulating respiration at low concentrations (less than 0.01 mM), but much more powerful in inhibiting respiration at higher concentrations (Figure 3). To see whether the requirement for respiratory stimulation by the oligopeptides resided mainly in their being highly positively charged and amphiphilic, we also tried the effect of the peptide (Ala-Leu-Ser-Lys)$_6$. In line with this proposition, this peptide turned out to be as active as PGLa, hence even more active than magainin 2 itself (Figure 2A,B).

Magainin action in protein-free liposomes appears to depend on electrostatic interactions (Vaz Gomes et al., 1993; Vaz Gomez et al., in preparation). Hence in the cytochrome oxidase liposomes of this paper with somewhat negatively charged lipids, magainin activity might be influenced by membrane charge screening by divalent cations. However, in phosphate medium, the presence or absence of 0.5 mM EDTA (not shown) or the additional presence of 2 mM $MgCl_2$ had little effect on the activity of PGLa (Figure 4) and magainin 2 (not shown). Five millimolar quinine increased the activity of PGLa at low concentrations of the peptide (Figure 4).
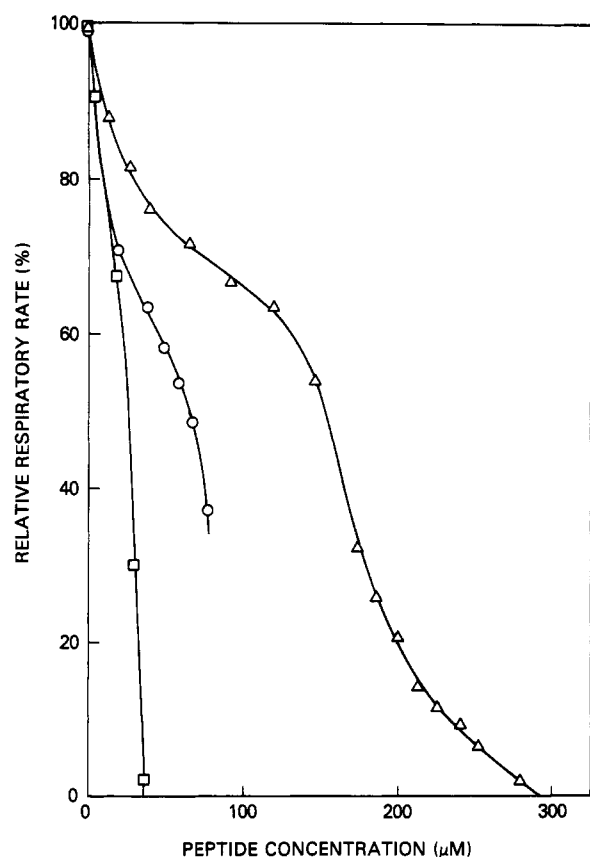
FIGURE 3: Inhibition of uncoupled respiration by large concentrations of magainin 2 (△), PGLa (O), and melittin (□) . To the standard incubation mixture with 0.20 g of phospholipid/L of cytochrome oxidase liposomes were added 0.5 μM valinomycin and 0.56 μM FCCP, leading to an 11-fold enhancement of (background corrected) respiration. Then magainin 2, PGLa, or melittin was added in increasing concentrations. Respiratory rates are expressed as percentage of the respiratory rate in the presence of valinomycin and FCCP, but in the absence of peptides.
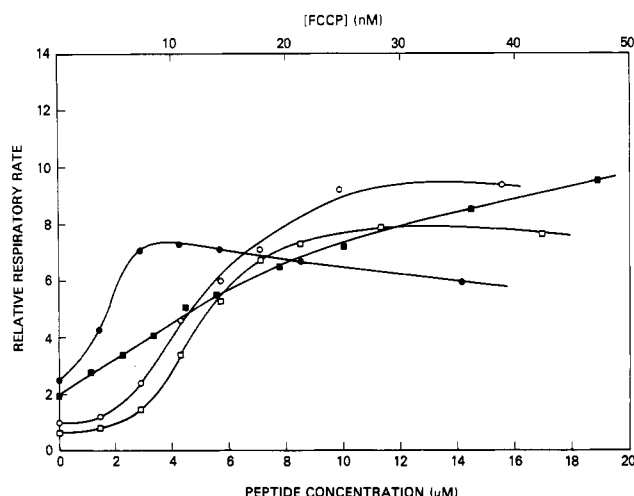

FIGURE 4: Nearly linear dependence of respiration on added FCCP and the effects of Mg^{2+} and quinine on the uncoupling action of PGLa. Standard titrations were performed except that the cytochrome oxidase liposomal concentration was 0.2 g of phospholipid/L and [TMPD] was 0.33 mM. (■) FCCP titration (in the presence of 0.5 μM valinomycin; in the absence of added valinomycin, FCCP stimulated respiration only up to 2.5-fold); (O) PGLa titration; (●) PGLa titration in the presence of 5 mM quinine; (□) PGLa titration in the presence of 2 mM $MgCl_2$ (at the same pH).

Because it had been speculated that magainin 2 might form a chloride channel, our initial experiments were performed with 130 mM KCl and 20 mM potassium HEPES at pH 7.4
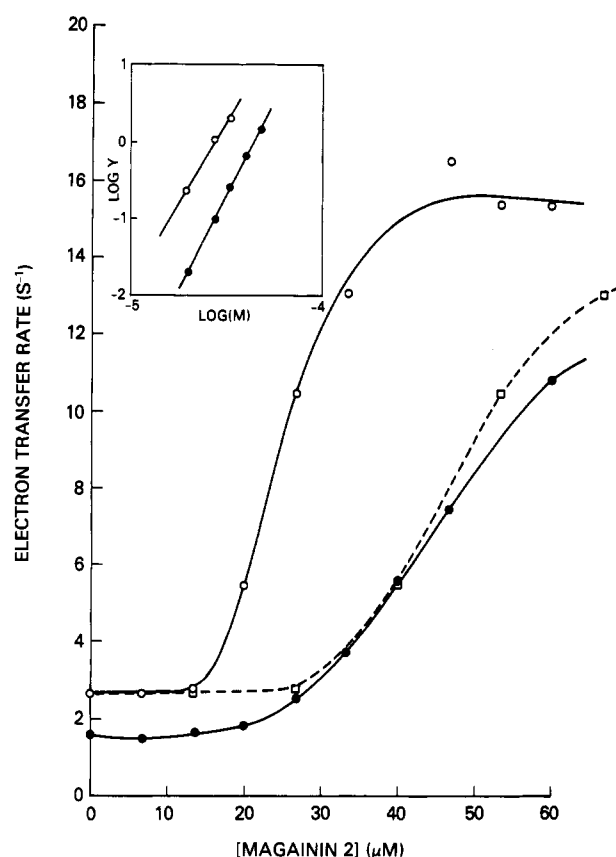

FIGURE 5: The concentration per unit mass of lipid, rather than the concentration per unit volume of water, determines the activity of magainin 2. Cytochrome oxidase vesicles containing 0.8 g of phospholipid/L (O) and 1.6 g of phospholipid/L (●) were exposed to increasing concentrations of magainin 2 (standard conditions except that TMPD concentration was 0.21 mM). Also shown is a replot (□) of the 0.8 g/L data (O) obtained by doubling the added magainin concentrations to correct for the 2-fold lower concentration of lipid. Initial oxygen concentration was 0.22 mM O_2. Shown is the turnover rate of cytochrome oxidase (J_e, in moles of electrons per mole of cytochrome a per second) versus the concentration of added magainin 2. Inset: $\log(y) = \log[\{J_e - J_e(0)\}/\{J_e^{max} - J_e\}]$ versus the logarithm of the added magainin concentration for the same cytochrome oxidase vesicles samples, 0.8 g of phospholipid/L (O) and 1.6 g of phospholipid/L (●). J_e^{max} was taken to be 10% higher than the highest oxygen consumption rate attained in the titration (to account for the inhibition of respiration; cf. Figure 3). In the inset, the peptide concentration needed to achieve half-maximal uncoupling is found from the intercept with the x-axis.

both inside and outside the liposomal membranes. It turned out, however, that magainin 2 was equally active in stimulating respiration when this medium was replaced with 50 mM potassium phosphate at pH 7.4. Since in the latter medium the respiratory control (both by FCCP + valinomycin and by the magainins) was some 3-fold higher, we turned to the latter medium for all the results reported in this paper.

There was a pronounced dependence of the activity of magainin 2, PGLa, and (tyrosine[5])magainin 2 on the concentration of liposomes. Figure 5 shows the dependence of the specific respiratory rate (i.e., the rate normalized for the concentration of cytochrome oxidase) on the concentration of added magainin 2 for concentrations of 0.8 (O) and 1.6 g of phospholipid/L (●) . The squares connected by the dashed line replot the results of the former experiment, after the magainin concentration is normalized with respect to the concentration of vesicles (i.e., after multiplying by 1.6/0.8). Apparently, at the concentrations used in our experiments, the action of magainin 2 (and of PGLa and (tyrosine[5])magainin

Table 1: Temperature Dependence of Half-Uncoupling Dose $D_{1/2}$ and Cooperativity Parameter n^a

		temp (°C)		
		5	25	37
magainin	$D_{1/2}$ (mg/g of PL)	67	92	103
	n	3	5	8
PGLa	$D_{1/2}$ (mg/g of PL)	20	28	18
	n	3	3	3
$(ALSK)_6$	$D_{1/2}$ (mg/g of PL)	25	32	22
	n	4	3	4

^{a} See Discussion for definitions of $D_{1/2}$ and n; see Table 2 for error estimates. Cytochrome oxidase liposomes: 0.25 g of phospholipid/L (at 5 and 25 °C) and 0.08 g of phospholipid/L (at 37 °C). PL: phospholipid.
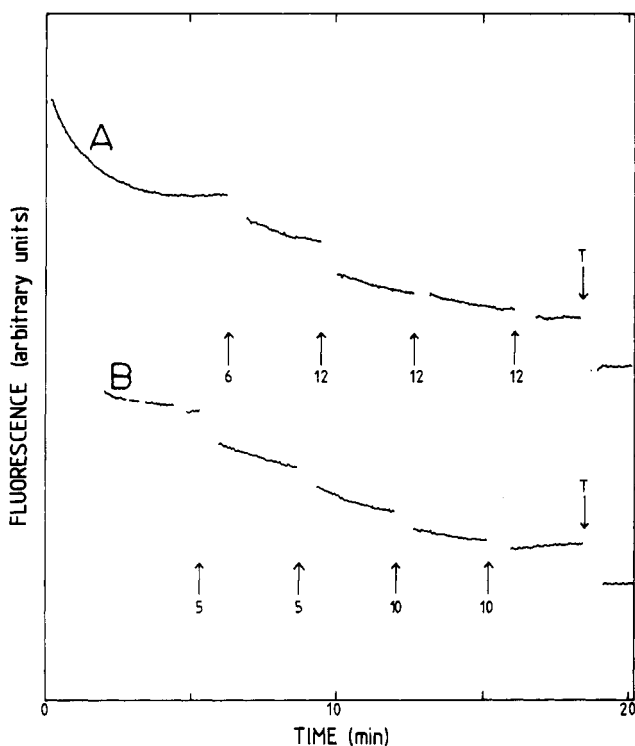

FIGURE 6: Mixing of intraliposomal calcein with extraliposomal Co^{2+}, as induced by (A) magainin 2 or (B) melittin. Shown is the fluorescence of intraliposomal calcein, as it is being quenched by cobalt ion, originating in the extraliposomal phase. Calcein liposomes at a concentration of 1.0 g of phospholipid/L and 0.024 mM $CoCl_2$ were added to 1.0 mL of buffer 600. After the fluorescence stabilized, the indicated amounts (nmol) of magainin 2 or melittin were added. Triton X-100 (indicated with the letter T) was added at the end of each titration at a concentration of 5 g/L. Changes shown are beyond those caused by the dilution accompanying the additions.

2, not shown) is determined by its amount relative to lipid, rather than by its amount per total (largely aqueous) volume.

There was little temperature dependence of the amount of magainin 2 or PGLa required to stimulate respiration to one-half its uncoupled rate ($D_{1/2}$ in Table 1). The effects of magainin 2 on respiration were independent of pH in the range pH 6–8.

Membrane Permeabilization in Cytochrome Oxidase Liposomes. The stimulation of respiration by magainins could be explained by a slip in cytochrome oxidase, or an increased ion permeability of the liposomal membrane. By enclosing calcein in the liposomes and incubating them in the presence of Co^{2+} (a quencher of calcein fluorescence (Oku et al., 1982)), we could monitor membrane permeability by fluorescence [cf. Vaz Gomes et al. (1993)]. Figure 6 shows that magainin 2 allowed calcein and Co^{2+} to come in contact with one another. Part of this mixing occurred rapidly, while another part was
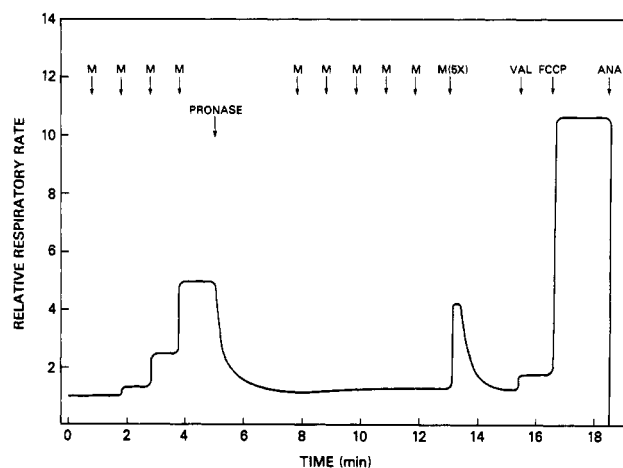

FIGURE 7: Pronase sensitivity of the uncoupling activity of magainin 2. The incubation was as in a standard respiratory titration with 0.15 g of phospholipid/L, except that [cytochrome c] was 0.034 mM and that at the indicated time 83 mg/L pronase was added. One micromolar magainin 2 (M) (except the last addition, which was 5 μM [M(5×)]), 0.5 μM valinomycin (VAL), and 0.56 μM FCCP were added at the indicated times. The titration ended when the anaerobic state (ANA) was reached. Respiratory rates were stable for each magainin 2 concentration except in the presence of pronase.

appreciably slower, but more continuous. In contrast, Triton X-100 caused a single phase of increased contact [cf. Grant et al. (1992)]. The concentrations of the peptides necessary for this mixing were 20, 25, and 50 μg/mL for PGLa, melittin, and magainin 2, respectively. These are similar to the concentrations required in the case of liposomes without cytochrome c oxidase (Vaz Gomes et al., 1993). To investigate further whether the permeabilization by magainins depended on the presence of cytochrome oxidase, we incubated enzyme-free liposomes containing potassium phosphate in a sodium phosphate medium and induced a diffusion potential by the addition of valinomycin. Magainin 2 was able to dissipate this electric potential: it induced efflux of TPP^+ (not shown).

Reversibility of the Magainin Effect. The above observations are consistent with the notion that magainins cause fairly nonspecific permeability changes of the liposomal membranes. These might be due to the formation of a pore or to lysis of the liposomes. In the subsequent experiments, we attempted to distinguish between these two possibilities. Because in the liposome system any active membrane repair mechanism must be absent, lysis should entail an irreversible rupture of the membrane. If our observations were due to any irreversible consequence of the action of magainin, then the respiratory rate should not return to the coupled rate upon removal of magainin 2. Figure 7 shows the results of an experiment in which we added pronase to remove magainin 2 from the liposomal suspension. After respiration was stimulated 2.5-fold by the addition of magainin 2, added pronase quickly returned the respiratory rate to the fully coupled level. Additional magainin was then hardly able to stimulate respiration again, while valinomycin plus FCCP could fully release respiratory control. Trypsin had effects similar to those of pronase, although it seemed to be somewhat less efficient in degrading magainin 2 and more effective in inhibiting the FCCP–valinomycin uncoupled rate of respiration. Uncoupling by PGLa was also sensitive to pronase (not shown). These results suggest that the effects of the magainins on liposomal respiration are reversible and are hence inconsistent with (irreversible) lysis.

Concentration Dependence of the Action of the Magainins. As evident from Figure 2, the release of respiratory control

Table 2: Half-Uncoupling Doses ($D_{1/2}$) and Cooperativity
Parameters (n) for Various Magainin-Like Peptides at Room
Temperature[a]

peptide	[PL] (g/L)	[TMPD] (μM)	[cyt c] (μM)	$D_{1/2}$ (mg/g of PL)	n
magainin 2	0.4	42	22	83	5
	0.2	42	22	76 ± 2	5 (4.6; 5.8)
	0.4	83	22	70	4
	0.28	83	22	92	5
	0.2	83	22	84 ± 3	5 (4.4; 5.4)
	0.09	83	22	85	4
	0.2	83	35	77	4
	0.15	83	35	83 ± 20	4 (4.2; 4.3)
PGLa	0.4	83	22	22 ± 4	3 (2.8; 2.9; 3.6)
	0.25	83	22	28	3
	0.2	83	22	23	3
	0.2	83	35	35 ● 1	2 (2.2; 2.4)
	0.15	83	35	35 ± 8	3 (2.6; 3.0; 2.9; 2.9)
Z peptide	0.4	83	22	211	2
	0.2	83	22	164	3
(tyrosine[5]) magainin-2	0.3	83	22	123	6
	0.2	83	22	118	6
melittin	0.4	83	22	72	2
	0.2	42	22	66	2

[a] In all experiments the respiratory control induced by FCCP plus
valinomycin exceeded 10. For experiments done more than once, the
largest variation of $D_{1/2}$ and the two measured values for n are given in
parentheses.

by PGLa and magainin 2 and its analogues is not proportional
to their concentration. This could mean that respiration does
not increase linearly with induced membrane permeability,
or that induced membrane permeability does not increase
linearly with added magainin concentration. The closed
squares in Figure 4 show that respiratory rate did increase
almost linearly with added FCCP, suggesting that the latter
explanation holds. We decided to inspect the nonlinear
dependence of permeability on magainin concentration in more
detail. Plotting the logarithm of the increase in respiratory
rate versus the logarithm of the added concentration of
magainin 2, we found a fairly linear relation at the lower
concentrations, which tended to level off to saturation at the
higher concentrations (not shown). To account for this
saturation we plotted the logarithm of $\{J_e - J_e(0)\}/\{J_e - J_e^{max}\}$
versus the logarithm of the added magainin concentration
(see the Discussion section for the rationale). The inset of
Figure 5 shows the results of such a plot for titrations of
magainin 2 (at two concentrations of vesicles). This trans-
formation linearizes the data and allows accurate estimation
of the dose $D_{1/2}$ necessary to stimulate respiration to a level
halfway between the fully coupled and the maximum rate.
The procedure also reveals the cooperativity parameter n (an
indication of the number of magainin monomers required to
form the complex that causes the membrane permeability;
see Discussion) as the slope of the plot.

Table 2 shows that the concentration of neither liposomes,
the redox mediator TMPD, nor cytochrome c affected these
parameters significantly. (It should be noted that $D_{1/2}$ is
given in mass units of peptide per mass unit of lipids).
Magainin 2, PGLa, and melittin uncoupled at peptide to lipid
(w/w) ratios below 10%. The $D_{1/2}$ for melittin was similar
to the concentrations at which melittin was shown to uncouple
mitochondrial oxidative phosphorylation (Das et al., 1985).
For magainin 2 the $D_{1/2}$ was significantly above the amount
required (Westerhoff et al., 1988) to dissipate the membrane
potential in respiring rat liver mitochondria; for PGLa it was
some 3-fold higher than the amount required to dissipate the

membrane potential across bacterial membranes (Westerhoff
et al., 1989b). It is, however, a well-known phenomenon that
uncouplers tend to be less active in reconstituted liposomes
than they are in the physiological free-energy-transducing
systems (Westerhoff & Kell, 1985). The results shown in
Table 2 confirm that PGLa is almost 4-fold more active than
magainin 2, whereas peptide Z is some 3-fold less active. More
strikingly, the cooperativity parameter of magainin 2 (4.7 ±
0.2, SEM) significantly exceeded that of PGLa (2.9 ± 0.1)
and Z peptide (2.3). (Tyrosine[5])magainin 2 seemed to have
a somewhat higher cooperativity parameter than magainin 2
(6.3). Melittin exhibited a very low cooperativity parameter
of approximately 2. The cooperativity parameter for magainin
2 depended on the temperature, in contrast to that for PGLa
(cf. Table 1).

DISCUSSION

Magainins have been proposed to be cytotoxic to prokaryotic
cells because of interference with chemiosmotic free-energy
transduction through the formation of an ion-permeable
membrane pore (Westerhoff et al., 1989b). This paper has
tested this working hypothesis on two counts. First, it has
shown that in one of the best defined model systems of
chemiosmosis, i.e., liposomes reconstituted with a purified
proton pump, magainins dissipate membrane potential, hence
the electrochemical potential difference for protons ($\Delta\bar{\mu}_{H^+}$),
and release respiratory control. Second, it has shown that
this magainin action is reversible, hence not due to irreversible
lysis of the membranes. We shall now discuss some additional
information on magainin action that emanates from the results
in this paper.

*The Active Form of the Peptides Appears To Be an
Oligomer.* The stimulation of respiration by magainin 2
exhibited a striking concentration dependence. At low
concentrations the peptides were virtually without effect until
a critical concentration was reached. Then additional peptide
very strongly affected respiration: respiration could be
increased some 9 times by doubling the magainin 2 concen-
tration. Also in rat liver mitochondria (Westerhoff et al.,
1989a) and in hamster spermatozoa (De Waal et al., 1991)
such supralinear concentration dependence of the magainin
effect has been observed. In those more complex systems it
was unclear, however, whether this was due to a nonlinear
dependence of membrane permeability on magainin concen-
tration or to a nonlinear dependence of the observed effects
on membrane permeability. Because of the simplicity of the
system used in the present paper [see also Vaz Gomes et al.
(1993)] and because we could demonstrate that respiration
varied linearly with membrane permeability (see below), the
latter interpretation now seems most likely: The species
actually causing the increase in respiration may well be a
complex of magainin molecules, whereas the majority of the
magainin molecules are present as monomers.

In the most general case one may envision the existence of
monomers, dimers, trimers, tetramers, etc., all with different
equilibrium dissociation constants and all with quantitatively
different effects on the ion permeability of the liposomal
membranes. From the limited information available, it is
difficult to determine all the dissociation constants and
permeabilities. We may, however, examine whether the
concentration dependence is compatible with a simple case.
We tried the case where the peptide is present in an equilibrium
between monomer and n-mer, where n is a number we attempt
to estimate from the experimental results. It will be assumed

that only the n-mer affects the ion permeability. Such an equilibrium is described by (Vaz Gomes et al., 1993)

$$K \cdot C = M^n = (T - n \cdot C)^n \qquad (1)$$

Here C, M, and T are the concentration of the n-mer ("complex"), the concentration of the monomer, and the total added concentration of the compound, respectively. K is the equilibrium constant for the dissociation of the n-mer. The same equation should apply if the peptide were present only as dimer and 2n-mer.

Respiratory rate (J_e) did not vary linearly with membrane permeability throughout all magnitudes of the latter: At the higher concentrations respiratory rate tended to a maximum. Mosaic nonequilibrium thermodynamics (Westerhoff & Van Dam, 1987) predicts a hyperbolic dependence of respiration on FCCP (the latter added to the endogenous proton permeability)

$$\frac{J_e}{J_e^{max}} = \frac{L_H^l}{L_H^l + (n_H^e)^2 \cdot \gamma_H^e \cdot L_e} \qquad (2)$$

Here L_H^l is the proton permeability of the liposomal membrane, linearly related to the added [FCCP]. The other parameters are constant under these conditions (Van Dam et al., 1980; Westerhoff & Van Dam, 1987). Equation 2 can be rewritten to show that $J_e/(J_e^{max} - J_e)$ should be linearly related to the proton permeability of the liposomal membranes. With the assumption that the complex of magainin 2 molecules increases respiration essentially by causing proton permeability in excess of that already present, this leads to the following combination of eqs 1 and 2:

$$K \frac{y}{\alpha} = M^n = \left(T - n \cdot \frac{y}{\alpha} \right)^n \qquad (3)$$

Here y has been defined as the following function of the actual respiratory rate:

$$y = \frac{J_e - J_e(0)}{(J_e^{max} - J_e)} \qquad (4)$$

α is a measure for the proton (or OH$^-$) permeability induced by the complex. $J_e(0)$ is the respiratory rate in the absence of permeabilizing compounds.

If a small amount of complex already induces substantial proton permeability (i.e., if toward the end of the titration magainin 2 is still largely present as monomer), then α is so large that the y/α term on the right-hand side of eq 3 can be neglected. [For a more general data analysis procedure, see Vaz Gomes et al. (1993)]. In that case a double logarithmic plot of y versus concentration of added magainin 2 (T) should yield a straight line with slope n. If, on the other hand, the y/α term is not insignificant, the plot should show a slope that decreases with T.

For the FCCP titration this procedure yielded a straight line with slope l (not shown), consistent with the notion that FCCP is active as a monomer and indicating that this is a good method to correct for the saturation of respiration at low proton electrochemical gradients. For magainin 2 titrations (cf. Figure 5, inset) this also yielded straight lines. This suggests that most of the magainin 2 is present as the monomer throughout the titration. The linear least-squares fit for the case of magainin 2 in the inset of Figure 5 yielded a slope near 5 (cf. Table 2). This suggests that the complex permeabilizing the membrane consists of some five monomers. From our

experience in fitting the data (cf. the variation in n in Table 2), we would not exclude that the results are also consistent with a tetramer or a hexamer, or with all of these forms having some activity. The change of n with varying temperature supports the latter suggestion for magainin 2.

Not all the peptides exhibited the same cooperativity number. Notably, PGLa, which is the other of the biologically most active peptides from *Xenopus* skin (Soravia et al., 1988), had a cooperativity number close to 3. This is consistent with the active PGLa complex consisting of a trimer. However, it could also mean that most of the PGLa is present as dimer and that the active complex is a hexamer.

For melittin, we observed a cooperativity parameter of only 2. In aqueous solutions, melittin is known to exist predominantly as tetramers and monomers with a dissociation constant of some 1.6×10^{-12} M^3 (DeGrado et al., 1981; Kaiser & Kezdy, 1987). This implies that at the concentrations below 30 mg/L used in our experiments the aqueous melittin mainly existed in monomeric form. If most of the added melittin remained in the aqueous phase, dimeric melittin must have been the active complex. However, under our conditions, it is likely that most of the melittin resides in the membrane rather than in the aqueous phase; doubling the concentration of liposomes doubled the concentration of melittin required to half-maximally stimulate respiration. If melittin in the lipid phase existed mainly as dimer, then our results are consistent with the suggestion (Tosteson et al., 1988) that the ionophoretic activity is associated with a tetrameric form of melittin. If on the other hand, as suggested by fluorescence transfer experiments by Hermetter and Lakowicz [1986], the majority of the membrane-bound mellitin was also monomeric, dimeric melittin must have been responsible for the uncoupling we observed.

Lysis or Just an Increase in Membrane Permeability? The dimer of melittin has been shown to be responsible for the release of hemoglobin from erythrocytes (DeGrado et al., 1981). The latter activity of melittin is generally classified as lysis of the erythrocyte. There is uncertainty as to what the precise mechanistic role of melittin is in this lysis (Bernheimer & Rudy, 1986). In one model melittin forms an aqueous channel, which then causes an ionic imbalance and, secondarily, lysis. In another, melittin itself causes lysis by disrupting the phospholipid structure of the membrane, acting like a detergent. In either interpretation the ultimate lysis is accompanied by irreversible damage to the membranes.

With respect to the magainins, this raised the question of whether they cause lysis in the same manner as melittin does and, if so, which of the two mechanisms may then be operative. A number of observations may shed light on this question. First, in an assay of erythrocyte lysis, magainin 2 did not exhibit any effect at concentrations 100-fold higher than the concentration at which melittin caused lysis (Zasloff, 1987). Second, the stimulatory effect of magainin 2 on liposomal respiration was reversed by the addition of pronase (Figure 7), suggesting that whatever magainin 2 causes is reversible, hence not consistent with the usual interpretation of lysis. Also the action of magainin 2 (and PGLa) on mitochondrial and bacterial membrane potentials was only transient and could be made more permanent by the addition of protease inhibitors (Westerhoff et al., 1989a,b). Third, magainin 2 caused a conductance increase in phosphatidylserine–phosphatidylcholine–decane lipid membranes, without lysing them. Single-channel conductances could be discerned (Duclohier et al., 1989; Cruciani et al., 1991, 1992). Interestingly, cecropins (the antimicrobial agent produced by silk worms)

(Christensen et al., 1988) and melittin (Tosteson et al., 1988) have been shown to exhibit similar properties. Finally, in *E. coli*, magainin 2 does not release β-galactosidase from the cells it kills (J. L. Rosner and H. V. Westerhoff, unpublished).

This supports the contention that the magainins, and perhaps also melittin, act primarily by causing an increase in membrane permeability. Only secondarily, at higher concentrations or dependent on additional forces (Williams et al., 1990), might they cause lysis. The mode of action depicted by Christensen et al. [1988; cf. Kempf et al. (1982)] for the action of cecropins could well hold true for magainins, notwithstanding the fact that the former are longer and contain a flexible and a hydrophobic domain in addition to a (smaller) amphipathic domain.

Although these data suggest that magainin 2 causes a limited permeability change of the liposomal membranes rather than complete lysis (rupture), we stress that this permeability is rather unspecific. Our assays suggest that the membranes become permeable for H^+ or OH^-, or phosphate and chloride anions, and Co^{2+} or calcein (Vaz Gomes et al., 1993). The minor change in the pH gradient in the experiment of Figure 1 suggests that PGLa does not act by solely increasing the H^+ or OH^- permeability. From mitochondrial experiments we know that magainin 2 can cause permeability to sucrose (Westerhoff et al., 1989a). Experiments in black lipid membranes (Duclohier et al., 1989; Cruciani et al., 1992) have suggested that magainin 2 causes ion permeability with limited discrimination between ions.

Recently, a working hypothesis concerning the permeabilizing action of magainins was made explicit (Vaz Gomes et al., 1993). Magainins in solution as monomers equilibrate with monomeric magainins in the interface between membrane and solution. The latter again equilibrate with an inactive multimeric form at a similar location and with a much lower concentration of a transmembrane oligomeric pore. The results obtained in this paper support such a scenario and specify that under these conditions (i) at room temperature most of the magainin 2 is in the membrane-bound monomeric form [cf. Matsuzaki et al. (1989, 1990)], (ii) at lower temperature some of the membrane-bound magainin might be dimeric, and (iii) at room temperature the membrane permeability complex may be a pentamer or hexamer, or at least a trimer. Experiments that (a) compare membrane affinity or permeabilizing activity between magainins and their covalently linked oligomers, (b) quantify fluorescence transfer between suitable magainin monomers [cf. Duclohier et al. (1989)] as a function of magainin concentration, or (c) examine heterocooperativity between different magainin monomers may further test the monomer–oligomer equilibrium model.

With respect to the mechanism by which the magainins kill bacteria, one now can formulate a concise working hypothesis: By permeabilizing the membrane to various substances, including anions, the magainins form a pore and cause the dissipation of the electric potential across the plasma membrane of the bacteria and hence greatly impair their ability to carry out anabolic processes (including the synthesis of ATP by the H^+-ATPase).

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