Magainin oligomers reversibly dissipate DeltamuH in cytochrome oxidase liposomes.


DOI
10.1021/bi00181a017

Publication date
1994

Published in
Biochemistry

Citation for published version (APA):
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, and PGLa, to which we shall collectively refer as magainins, are cytotoxic for microbial and higher eukaryotic cells. Synthetic analogues of these antimicrobial assays than the parent compounds (Chen et al., 1987, 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 et al., 1991; 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% trifluoroethanol, 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.

At pH 7, magainins reversibly interfere with proton-mediated free-energy transduction. Consequently we confirmed the proposed (Westerhoff et al., 1989b) membrane permeabilization, cannot demonstrate 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 This study was supported by the Netherlands Organization for Scientific Research (NWO).

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1 Abbreviations: magainin 2, H2N-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Ala-Ser-Arg-Ser; PGLa, H2N-Gly-Met-Ala-Ser-Lys-Ala-Glu-Ala-Gly-Lys-Ile-Ala-Lys-Ala-Leu-Leu-Leu-Leu-COH; 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TMPD, N,N,N,N'-tetramethyl-p-phenylenediamine; TPP*, tetraphenylphosphonium 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 N₂ below –19 °C. The purified asolectin contained 0.88 mmol of phosphorus per gram.

Magainin 2, PGLa, (tyrosine)²magainin 2, “Z-peptide” (the stereoisomer of magainin 2 that has all the lysine and phenylalanine residues in the D configuration) and (Ala-Leu-Lys)₆, or “ALK peptide,” were 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 14,000) dialysis membrane (which had been soaked overnight and put into Spectrapor cutoff 12,000–14,000) dialysis membrane (which had been soaked overnight in water plus a pinch of Na₂EDTA). The suspension was dialyzed at 4 °C against 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 sonicating (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 Spectropor (M₄, cutoff 12,000–14,000) dialysis membrane (which had been soaked overnight in water plus a pinch of Na₂EDTA). 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 Sephacryl 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” (Oka 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 K₂SO₄, 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 Na₂EDTA, 6 mM ascorbic acid, 0.083 mM TMPD, and 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 (Jc) 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 electro 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 (Reynafarj 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 O₂ 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 O₂ (12 mL/min) and Ar (33 mL/min) were led over the suspension, the liposomes were allowed to develop a membrane potential. Subsequently, the O₂ 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 O₂ (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 O₂ 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 O₂/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.
Magainin Uncoupling of Cytochrome Oxidase Liposomes

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FIGURE 3: Inhibition of uncoupled respiration by large concentrations of magainin 2 (Δ), PGLa (O), and melittin (D). 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; (Δ) 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 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 (tyrosine5)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 (tyrosine5)magainin...
Table 1: Temperature Dependence of Half-Uncoupling Dose \( D_{1/2} \) and Cooperativity Parameter \( n \)

<table>
<thead>
<tr>
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<th>temp (°C)</th>
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<tr>
<td>magainin</td>
<td>( D_{1/2} ) (mg/g of PL)</td>
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<td>n</td>
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<tr>
<td>PGLa</td>
<td>( D_{1/2} ) (mg/g of PL)</td>
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<td>(ALSK)(_b)</td>
<td>( D_{1/2} ) (mg/g of PL)</td>
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*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.

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 \( (D_{1/2} / 2) \). 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 \( \text{Co}^{2+} \) (a quencher of calcein fluorescence \( \text{(Oku et al., 1982)\), we could monitor membrane permeability by fluorescence \( \text{[cf. Vaz Gomes et al. (1993)]. Figure 6 shows that magainin 2 allowed calcein and \( \text{Co}^{2+} \) to come in contact with one another. Part of this mixing occurred rapidly, while another part was appreciably slower, but more continuous. In contrast, Triton X-100 caused a single phase of increased contact \( \text{[cf. Grant et al. (1992)]\). The concentrations of the peptides necessary for this mixing were 20, 25, and 50 \( \mu \text{g/mL} \) for PGLa, melittin, and magainin 2, respectively. These are similar to the concentrations required in the case of liposomes without cytochrome \( c \) oxidase \( \text{(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 \( \text{TPP}^{+} \) (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
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 phenomenon we plotted the logarithm of respiratory rate 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 transformation 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 cytoxic 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 ($\Delta \mu _H^+$), 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 concentration. 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 concentration 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)
\[ K \cdot C = M^n = (T - nC)^n \] (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)
\[ \frac{J_e}{J_{e\text{max}}} = \frac{L_H}{L_{H\text{max}}} + (nH)^y \cdot yH \cdot L_e \] (2)
Here \( L_H \) 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 \( J_e/ (J_{e\text{max}} - J_e) \) 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:
\[ K^{\alpha y} = M^n = (T - n\alpha)^n \] (3)
Here \( y \) has been defined as the following function of the actual
respiratory rate:
\[ y = \frac{J_e - J_e(0)}{(J_{e\text{max}} - J_e)} \] (4)
\( \alpha \) is a measure for the proton (or \( \text{OH}^- \)) 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 \( \alpha \) is so
large that the \( y/\alpha \) 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
\( y/\alpha \) 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 \( I \) (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,
whereas 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 predomi-
nantly as tetramers and monomers with a dissociation constant
of some 1.6 \( \times 10^{-12} \) M\(^{-1}\) (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 Hermeter and Lako\'wie\'c (1986),
the majority of the membrane-bound melittin 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 phosphatidylethanolamine-
phosphatidylcholine-decanol lipid membranes, without lysing them.
Single-channel conductances could be discerned (Duclohier
et al., 1989; Cruciani et al., 1991, 1992). Interestingly,
ceruloplasmin (the antimicrobial agent produced by silk worms)
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(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 permeability change of the liposomal membranes rather than.

ACKNOWLEDGMENT

We thank Dr. G. Yewey for isolating the cytochrome oxidase, Dr. H.-C. Chen for discussions and peptides, Drs. P. Nicholls, J. Wrigglesworth, and C. Cooper for guidance in preparing the cytochrome oxidase liposomes, Drs. R. Zhou and P. B. Chock for help with setting up the SLM spectrofluorimeter, Dr. J. L. Rosner for discussions, and Dr. E. D. Korn for critical reading of the manuscript.

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