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A synthetic peptide corresponding to the C-terminal 25 residues of phage MS2 coded lysis protein dissipates the protonmotive force in *Escherichia coli* membrane vesicles by generating hydrophilic pores

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The RNA phage MS2 encodes a protein, 75 amino acids long, that is necessary and sufficient for lysis of the host cell. DNA deletion analysis has shown that the lytic activity is confined to the C-terminal half of the protein. We have examined the effects of a synthetic peptide, covering the C-terminal 25 amino acids of the lysis protein, on the electrochemical potential, generated in *Escherichia coli* membrane vesicles and in liposomes reconstituted with cytochrome *c* oxidase. In all cases the peptide dissipates the electrochemical potential. The peptide also induces the release of carboxyfluorescein (376 daltons), but not of inuline (5500 daltons), from protein-free liposomes. The phenomena are observed at a lipid to peptide molar ratio of ~100:1. The possible connection between the dissipation of the proton-motive force and bacteriolysis is discussed.

Key words: cell lysis/hydrophilic pore/membrane potential/RNA phage/synthetic peptide

Introduction

Many phages conclude their infection cycle by lysis of the host cell. In the single-stranded RNA and DNA phage, such as MS2 and ϕX174, this process depends on the expression of a small overlapping gene (Atkins *et al.*, 1979; Beremand and Blumental, 1979; Model *et al.*, 1979; Henrich *et al.*, 1982; Kastelein *et al.*, 1982). In the RNA phage MS2 the lysis (L) gene encodes a protein of 75 amino acids, containing a strong lipophilic C-terminal half (Figure 1). Using cloned MS2 cDNA we have shown recently that the lipophilic C-terminal part of the protein comprising some 30 amino acids carries its lytic properties (Berkhout *et al.*, 1985). In that study we demonstrated that expression of the 3′ terminal 100 bases of the L gene resulted in lysis of the *Escherichia coli* culture.

Fractionation of cells infected with MS2 or carrying plasmids encoding the L function showed the protein associated with the membrane fraction (Beremand and Blumenthal, 1979) from which it could be extracted with sarcosyl (T.den Blaauwen and J.van Duin, unpublished results). It is inferred that the lysis protein is bound to the inner membrane and this location has been confirmed by sucrose gradient separation, although a substantial part was recovered from the outer membrane fraction and from a fraction of the gradient with intermittent density (J.V.Höltje, unpublished data).

We do not know how the protein causes cells to lyse. It does not possess a murein hydrolytic activity, which is not surprising in view of its small size. Also, the protein does not interfere with cell wall synthesis *in vivo*. Höltje and van Duin (1984) found that after induction of the lysis protein, the incorporation of diamino pimelic acid into SDS-insoluble material is not inhibited until the cells actually start lysing. On the other hand, some striking parallels with β-lactam-induced bacteriolysis have been noticed. Under certain growth conditions penicillin does not trigger cell lysis. Such phenotypic tolerance is established, for instance, at the stationary phase, growth at pH 5 or when the stringent response in *relA*$^+$ strains is activated (Goodell *et al.*, 1976; Kusser and Ishiguro, 1985). The observation is that physiological conditions that render bacteria tolerant to penicillin also curb lysis induced by the L protein (Höltje and van Duin, 1984; J.V.Höltje, unpublished data). In addition, Harkness and Ishiguro (1984) isolated an *E.coli* mutant that was tolerant to the murein synthesis inhibitor cycloserine. This mutant also failed to lyse when challenged *in vivo* with the lysis protein of ϕX174. These observations are an indication for the participation of murein hydrolases in phage-induced lysis and the suggestion is that the L protein interferes with host control over these so-called autolysins [for a review on autolysins see Tomasz and Waks (1975)].

Electron microscopy studies have not revealed significant differences between sacculi from intact and phage-lysed bacteria (T.den Blaauwen and J.van Duin, unpublished results). Presumably, limited hydrolysis of the peptidoglycan layer is sufficient to cause the collapse of the membrane by the prevailing osmotic pressure.

The question is how such a small protein can exert such dramatic effects on the integrity of the cell wall. Here we begin to analyse this problem by examining the effects of the MS2 lysis protein on the properties of the *E.coli* inner membrane. As it is not feasible to prepare the protein in sufficient amounts and of sufficient purity, we have used a chemically synthesized peptide corresponding to the 25 C-terminal amino acids. We refer to it as the lysis or L25 peptide. As our earlier experiments have shown this part to be active in *in vivo* bacteriolysis, we assume that the peptide can serve as a model substance to study changes in membrane properties.

```
1                                    10                                      20
Met-Glu-Thr-Arg-Phe-Pro-Gln-Gln-Ser-Gln-Gln-Thr-Pro-Ala-Ser-Thr-Asn-Arg-Arg-Arg-Pro-Phe-Lys-His-Glu-
                    30                                      40                                      50
Asp-Tyr-Pro-Cys-Arg-Arg-Gln-Gln-Arg-Ser-Ser-Thr-Leu-Tyr-Val-Leu-Ile-Phe-Leu-Ala-Ile-Phe-Leu-Ser-Lys-
                                     60                                      70
Phe-Thr-Asn-Gln-Leu-Leu-Leu-Ser-Leu-Leu-Glu-Ala-Val-Ile-Arg-Thr-Val-Thr-Thr-Leu-Gln-Gln-Leu-Leu-Thr.
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Fig. 1. Deduced amino acid sequence of the lysis protein of RNA phage MS2. The corresponding nucleotide sequence is taken from Fiers *et al.* (1976).

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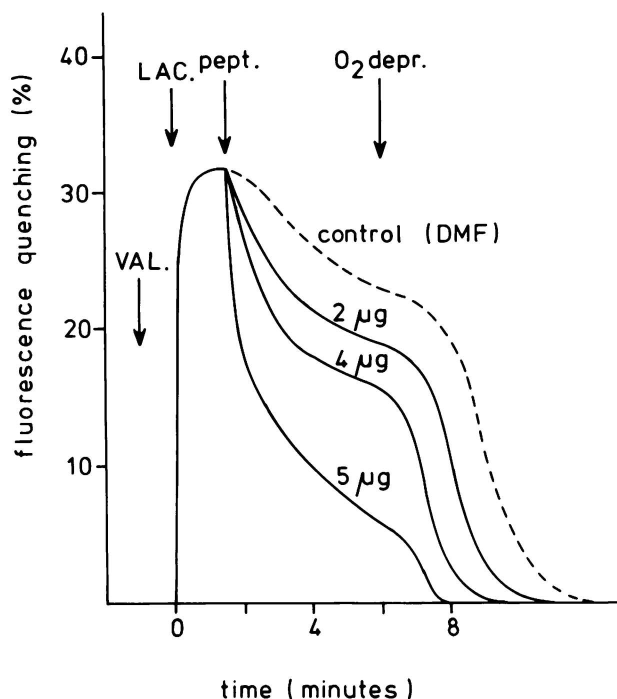

Fig. 2. Dissipation of the pH gradient in inverted *E.coli* vesicles by the L25 peptide. Reaction mixtures contained 50 mM potassium phosphate (pH 7.0), 2.5 mM $MgSO_4$ and 5 µM 9-aminoacridine in a total volume of 1 ml. Vesicles (246 µg protein) were added, followed by the addition of 5 nM valinomycin (VAL) and 5 mM D-lactate (LAC). Peptide was added in a volume of 2.5 µl dimethylformamide (DMF). The control contained only 2.5 µl DMF.

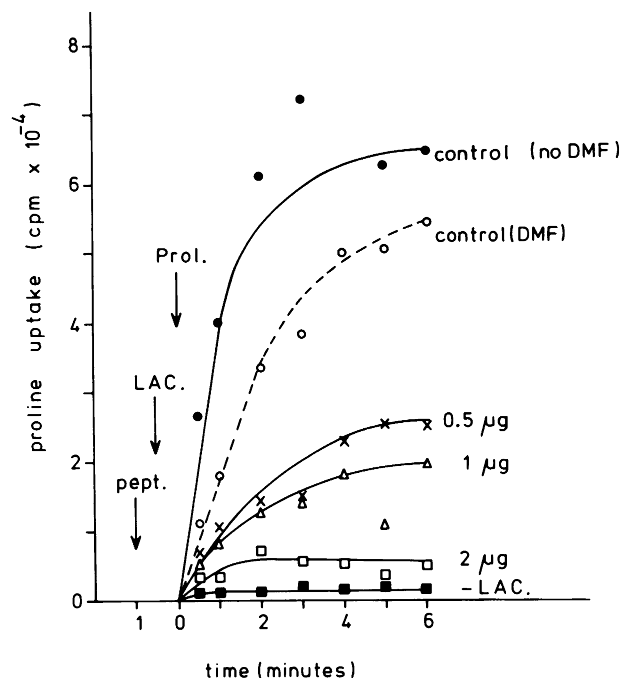

Fig. 3. Inhibition of proline uptake by right-side- out *E.coli* vesicles upon addition of the L25 peptide. Reaction mixtures contained 50 mM potassium phosphate (pH 7.0), 10 mM $MgSO_4$ in a total volume of 100 µl. Membranes (52 µg protein) were added followed by the addition of the indicated amounts of L25 peptide. Lactate (10 mM) and proline (0.1 µCi) were added as indicated. The L25 peptide was added in 1 µl DMF and the effect of the organic solvent on proline transport is given by the two control curves where no peptide is added.

We have measured the influence of this peptide on the proton-motive force generated *in vitro* in *E.coli* membrane vesicles and in proteoliposomes containing cytochrome *c* oxidase. Both components of the proton motive force, the electrical potential ($\Delta\Psi$) and the pH gradient (ΔpH), are dissipated by the peptide. In addition, peptide-induced changes in the permeability of protein-free liposomes were found. We suggest that the peptide elicits the formation of discrete hydrophilic pores in its target membrane. The possible relation between the induction of such pores and cell lysis is discussed.

Results

Dissipation of proton-motive force in E.coli membrane vesicles

Inverted *E.coli* vesicles were prepared by French press treatment of logarithmic phase cells. A pH gradient across the membrane was established by the addition of the electron donor D-lactate. Valinomycin was present to eliminate the electrical component ($\Delta\Psi$) of the proton-motive force. The accumulation of 9-aminoacridine inside the vesicles, evident from its fluorescence quenching, shows that the pH gradient (inside acid) has indeed formed (Figure 2). Addition of increasing amounts of L25-lysis peptide to the vesicle suspension progressively decreases the quenching, indicating that the peptide is able to dissipate the existing proton gradient. After ~8 min the oxygen supply is exhausted leading to the complete collapse of the pH gradient.

Another way to measure the presence of a proton-motive force in *E.coli* vesicles is the uptake of proline. The dependence of proline uptake by right-side-out membrane vesicles on lactate is shown in Figure 3. In the absence of lactate proline uptake is negligible. It is clear that the lysis peptide inhibits the accumulation of proline suggesting the breakdown of the proton-motive force. It may be noted that in all the experiments shown the peptide is added in a fixed amount of dimethylformamide (DMF). The influence of this amount of DMF on the proton-motive force of the vesicles is indicated by the dashed line (control, DMF). The effects measured are thus not due to increasing amounts of organic solvent. Estimates of the peptide:lipid value in the assays are 1:100, which is about the same ratio that leads to cell lysis *in vivo* (B.Berkhout, G.Overbeek and J.van Duin, unpublished data). In the *in vitro* experiments this ratio is actually a maximal estimate since we cannot be sure that all peptide added is incorporated in the vesicles.

Dissipation of proton-motive force in liposomes reconstituted with cytochrome c oxidase

One can think of several ways in which the peptide may induce the breakdown of the proton-motive force. For instance, through direct inactivation of the lactate dehydrogenase or other components of the electron transport chain. To exclude this possibility we prepared liposomes composed of *E.coli* lipids wherein cytochrome *c* oxidase from beef heart was incorporated as described in Materials and methods. In this system a proton-motive force can be generated by the addition of ascorbate and cytochrome *c*, while *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine [$Ph(NMe_2)_2$] is included as an intermediary electron carrier. Generation of the membrane potential ($\Delta\Psi$) was monitored by a tetraphenyl-

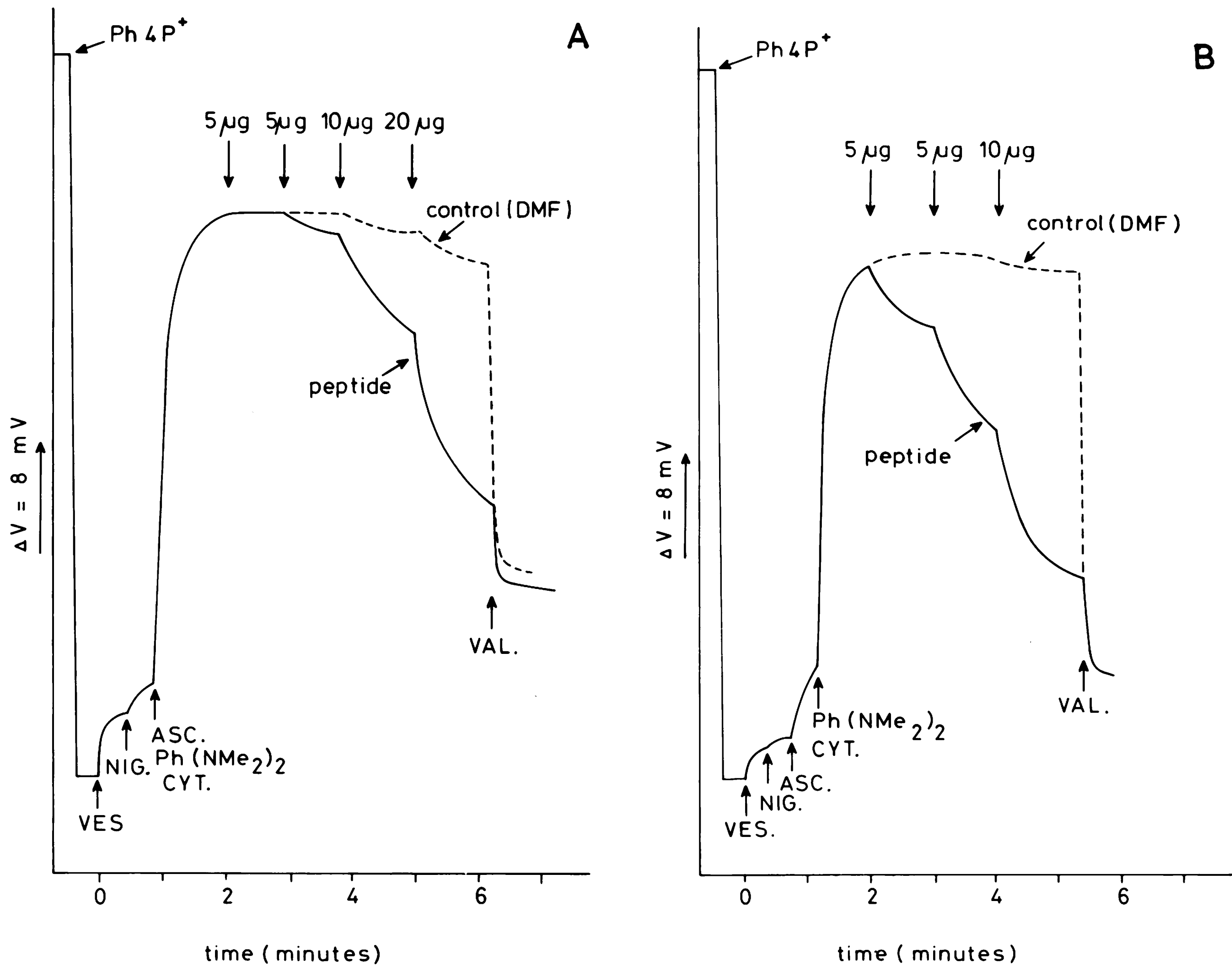

Fig. 4. Dissipation of $\Delta\Psi$ in liposomes reconstituted with cytochrome *c* oxidase upon addition of the L25 peptide. Reaction mixtures contained 50 mM potassium phosphate (pH 6.0), 5 mM $MgCl_2$ and 2 μM Ph_4P^+ in a total volume of 1 ml. Membrane vesicles (VES) (0.5 mg phospholipid/ml) were added as indicated followed by the addition of 20 μM cytochrome *c* (CYT), 10 mM ascorbate (ASC) and 0.4 mM Ph $(NMe_2)_2$. Nigericin (NIG) and valinomycin were used at final concentrations of 10 nM and 0.2 μM respectively. The L25 peptide is added as a 5 mg/ml DMF solution. (**A**) Phospholipids of the *E.coli* inner membrane composition (PE, PG and CL; molar ratio, 7:1.5:1.5). (**B**) Liposomes consisting of soybean phospholipid.

phosphonium (Ph_4P^+)-sensitive electrode and nigericin was included to avoid the generation of a ΔpH. In Figure 4A we demonstrate that in this system also the lysis peptide is capable of dissipating the membrane potential. The effects observed do not depend on the specific lipid composition of the membrane vesicles. As shown in Figure 4B similar results are obtained when the *E.coli* phospholipids are replaced by soybean phospholipids.

L25 peptide increases the permeability of protein-free liposomes

We have seen that the L25 peptide destroys the ΔpH and the $\Delta\Psi$ equally well. We have also shown that the breakdown of the proton-motive force is neither dependent on the components used to generate it, nor on the orientation, composition or origin of the vesicles employed. Such data suggest that the lysis peptide does not specifically interact with the protein components responsible for energizing the membrane. Rather, the results indicate that the peptide induces a general increase in permeability of the membrane to ions and other low molecular weight compounds. To investigate this possibility we examined the effect of lysis peptide on the permeability of protein-free liposomes. Carboxyfluorescein (CF) was encapsulated in liposomes at a concentration at which the fluorescence of the dye is almost completely self-quenched. Release of CF into the external medium results in relief of this self-quenching which can be monitored continuously as an increase of fluorescence (Wilschut *et al.*, 1980).

In Figure 5A, CF fluorescence is shown for large unilamellar vesicles consisting of dioleoylphosphatidylcholine (DOPC). Leakage of CF increases with rising peptide concentrations and is complete within 2 min at a peptide to lipid molar ratio of ~1:100. We have also surveyed the action of a peptide covering the C-terminal 36 amino acids of the lysis protein. Basically the same results were found, although contrary to expectation, a four times higher concentration of this peptide was required to cause the same degree of liposome leakage. The induced permeability increase is dependent on the composition of the liposomes. For instance, vesicles mimicking the lipid composition of the *E.coli* inner membrane (Cronan and Vagelos, 1972) require more peptide

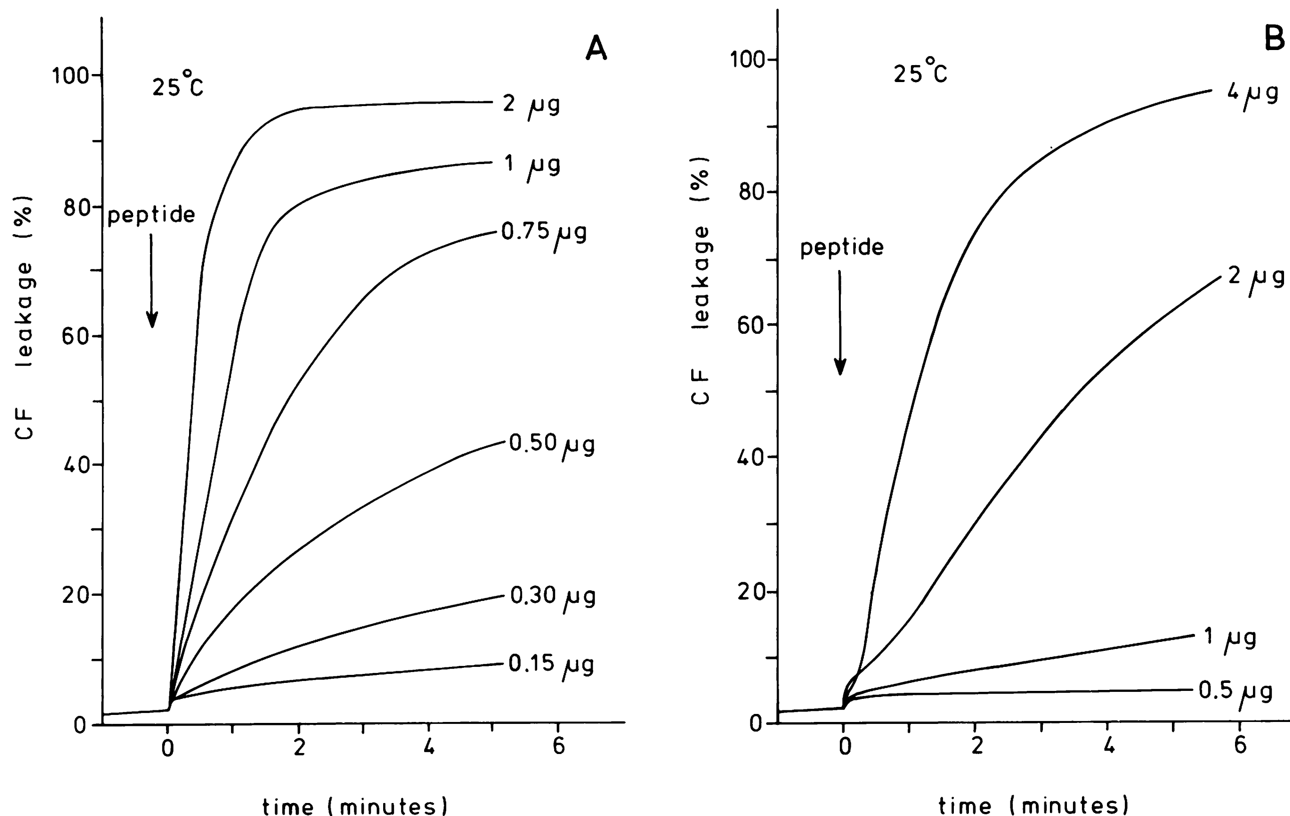

Fig. 5. Carboxyfluorescein efflux from large unilamellar vesicles as induced by the L25 peptide. Varying amounts of peptide were added to 1 ml solution containing liposomes at a concentration of 50 μM phospholipid. The peptide is added in 10 μl DMF. (**A**) DOPC. (**B**) Phospholipids of the *E.coli* inner membrane composition (PE, PG and CL; molar ratio, 7:1.5:1.5). 1 μg peptide equals 0.4 nmol.

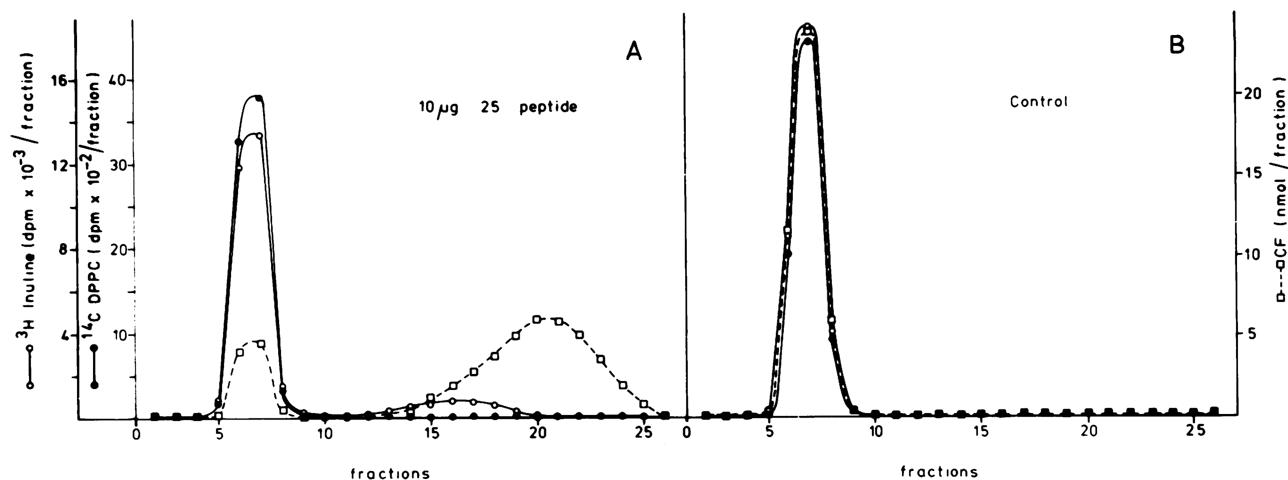

Fig. 6. Selective efflux of carboxyfluorescein from large unilamellar vesicles. (**A**) 10 μg of L25 peptide was added to 1 μmol of DOPC liposomes, labelled with a trace amount of [^{14}C]dipalmitoylphosphatidylcholine (DPPC) in which carboxyfluorescein and [^{3}H]inuline were encapsulated. The 1 ml reaction mixture was incubated for 20 min and subsequently separated on a Sephadex G100 column. (**B**) As (A) except for the addition of the peptide. Carboxyfluorescein was measured at 430 nm after adjusting the fractions to 0.1% (v/v) Triton X-100.

to give a comparable amount of carboxyfluorescein leakage than, for instance, DOPC liposomes (Figure 5B). This may be due to variations in lipid packing among liposomes of different composition. In a detailed study on the interaction between the lysis peptide and liposomes of varying composition, we have observed that the fluidity of the liposomal bilayer is a major factor determining the lytic capacity of the peptide (W.H.F.Goessens, J.van Duin and J.Wilschut, in preparation). Variations in lipid packing may also explain the quantitative differences between Figure 4A and 4B.

It is still conceivable that the L25-peptide behaves as some sort of detergent and that the phenomena observed can be attributed to the physical destruction of the liposomes. Though the peptide to lipid molar ratio at which leakage occurs argues against such an explanation it is desirable to have direct evidence that the liposomes stay intact during the course of the experiments. This can be achieved by showing that large molecules are not released from peptide-treated vesicles. Thus, we have prepared liposomes containing a mixture of carboxyfluorescein and [^{3}H]inuline. The mol. wt

of these compounds is 376 and 5500 daltons respectively. As a marker for the lipid a trace of [^{14}C]DPPC was incorporated. The vesicles were exposed to the lysis peptide and the reaction mixture fractionated over Sephadex G100. Figure 6A shows that the bulk (85%) of the inuline moves with the ^{14}C-labelled lipids in the void volume of the column. It must therefore still be enclosed in the vesicles. On the other hand, most of the carboxyfluorescein is retarded by the column, showing that it is released from the vesicles. In a control experiment, where no peptide was added to the liposomes, inuline and carboxyfluorescein elute together in the void volume (Figure 6B).

We conclude from this experiment that the peptide-induced release of carboxyfluorescein is not due to collapse of the vesicles. Rather the experiment suggests that the peptide causes the formation of discrete hydrophilic pores in the vesicle bilayer.

Table I. Amino acid composition of the L25 peptide

Amino acid	% measured	% theoret.
Asx	3.6	4.0
Thr	22.1	20.0
Ser	5.8	4.0
Glx	18.8	16.0
Pro	0	0
Gly	0.6	0
Ala	5.0	4.0
Val	8.5	8.0
Met	0	0
Ile	3.4	4.0
Leu	23.1	32.0
Tyr	0	0
Phe	2.4	4.0
Lys	1.7	0
His	0	0
Arg	5.0	4.0

Discussion

A peptide corresponding to the 25 C-terminal amino acids of the MS2 lysis protein has been used as a model substance to probe potential changes in the *E.coli* cytoplasmic membrane, that may be causally related to phage-induced lysis. We have observed the following. (i) The pH gradient as well as the electrical potential that can be built up *in vitro* across the membrane of *E.coli* vesicles is dissipated by the peptide. (ii) It makes no difference whether inside-out or right-side-out vesicles are used. (iii) Dissipation of $\Delta\Psi$ occurred in reconstituted liposomes that did not contain any *E.coli* proteins or lipids. In a second set of experiments we showed that the lysis peptide can release carboxyfluorescein from protein-free vesicles. Inuline, with a 15 times higher average mol. wt is not released under the same conditions.

The two sets of data can be coherently explained by a model in which the peptide short-circuits the membrane by the induction of size-restricted hydrophilic pores. Preliminary experiments on the electrical conductivity of black lipid films have confirmed this interpretation (R.Benz and J.van Duin, unpublished data). This presumed pore-forming ability of the L peptide is shared with a large variety of naturally occurring proteins, such as the microcins, some colicins, cecropins, mellitin and several other peptide antibiotics (Steiner *et al.*,1981; Dankert *et al.*, 1982; DeLorenzo and Aiguilar, 1984; Kayalar and Düzgünes, 1986; Slatin *et al.*, 1986; Baty *et al.*, 1987). Interestingly, also the S protein of phage λ, which has been implicated in cell lysis, has been reported to induce leaks in the *E.coli* cytoplasmic membrane (Garrett and Young, 1982; Wilson, 1982). We can only speculate about the structure of the induced pore. It is possible that the peptide elicits changes in lipid arrangement leading to transient formation of non-bilayer structures such as those induced by gramicidin (Killian *et al.*, 1985; Killian and de Kruijff, 1985). These structures have, however, only been observed at much higher peptide to lipid molar ratios than used in this study. Alternatively, and more likely, the peptide may form a hydrophilic channel by assuming an oligomeric structure that spans the bilayer. Length and high lipophilicity of the peptide meet these demands and a structure reminiscent of the one proposed for alamethicin is conceivable (Fox and Richards, 1982).

In view of the existing data it is not unreasonable to assume that cell lysis induced by the L protein *in vivo* is related to the *in vitro* observations that we describe in this paper. This assumption implies that cell lysis is the result of at least a two-step process; the first step being the association of the L protein with the cytoplasmic membrane, an interaction which will lead to its depolarization. In the second step, the loss of electrochemical potential must trigger the autolytic enzymes. This view suggests that other agents that de-energize the membrane will likewise induce bacteriolysis. The experiments of Jolliffe *et al.* (1981) showing that azide, cyanide or carbonylcyanide *m*-chlorophenylhydrazone (CCCP) cause lysis in *Bacillus subtilis* are consistent with this line of reasoning. We have found, however, that in *E.coli* this relationship is not so straightforward, possibly because the autolysins are much more strictly controlled in Gram-negative bacteria. Upon treatment of a logarithmic phase *E.coli* culture with azide or CCCP we observed onset of lysis, but this process stopped after a few minutes as if the cells were capable of a protective response. One could speculate that this protection is mediated by guanosine tetraphosphate (ppGpp), since de Boer *et al.* (1976) have found that the ionophor dinitrophenol increases the level of ppGpp in the cell by inhibiting the *spo*T-dependent breakdown pathway. Tetu *et al.* (1980) have confirmed that the half life of ppGpp is increased 10 times when the proton-motive force is dissipated. As mentioned in the Introduction, ppGpp protects cells from both penicillin and phage-induced lysis. Still, one is left to explain why *E.coli* cells can escape from uncouplers but not from the lysis protein.

A puzzling aspect of the activity of the peptide is its relation to the amino acid sequence. Will any hydrophobic peptide have the same consequences or are specific residues at critical positions required? Comparison between the lysis proteins of several closely related RNA phages shows that six out of 25 C-terminal amino acids are conserved. However, the degree of conservation may be overestimated since the genetic information for this essential part of the L protein lies in overlap with the replicase gene and thus conservation may relate to the replicase function. More sequences are needed before meaningful suggestions on this point can be made.

Materials and methods

Growth of cells and isolation of membrane vesicles

Escherichia coli ML308-225 (i^-,z^-,y^+,a^-) were grown on minimal medium A (Davis and Mingioli, 1959) with 1% sodium succinate and 0.1% yeast extract. Right-side-out membrane vesicles were prepared as described by Kaback (1971). Inside-out membrane vesicles were prepared by French press treatment (Poolman *et al.*, 1983).

Preparation of liposomes

Dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), *E.coli* phosphatidylethanolamine (PE), *E.coli* cardiolipin (CL) and dipalmitoylphosphatidylglycerol (PG) were from Avanti Polar Lipids (Birmingham, AL) and soybean phospholipids were from Sigma Chemical Co. (St Louis, MO). Headgroup-labelled [^{14}C]DPPC (2.5 Ci/mol) was prepared by a demethylation–remethylation procedure using [^{14}C]CH_3I, as described by Stoffel (1975). Liposomes (large unilamellar vesicles with an average diameter of 100 nm) were prepared according to Mayer *et al.* (1986). Thoroughly dried phospholipid (usually 10 μmol) was suspended in 1.0 ml of aqueous medium by vortex mixing. The preparation was passed through five cycles of freezing (in liquid nitrogen) and thawing (in water at room temperature) and subsequently extruded 10 times through two stacked unipore polycarbonate filters with a pore diameter of 0.1 μm (Nuclepore Co., Pleasanton, CA).

Encapsulation of 5,6-carboxyfluorescein (CF) was achieved by preparing the liposomes in 50 mM CF, 5 mM Hepes (pH. 7.4). CF was obtained from Eastman Kodak Co. (Rochester, NY) and purified by chromatography on Sephadex LH-20 (Ralston *et al.*, 1981). The non-encapsulated dye was separated from the liposomes by gel filtration on Sephadex G-75 in 100 mM NaCl, 5 mM Hepes (pH. 7.4). In some experiments, in addition to CF, 0.5 mM [^{3}H]inuline (Amersham International, UK) diluted with unlabelled inuline was also incorporated in the liposomes. In this case gel filtration was done by Sephadex G-100 using the elution buffer mentioned above.

Measurement of carboxyfluorescein fluorescence

Release of CF from liposomes, resulting in relief of its fluorescence self-quenching, was determined in an SLM-800 spectrofluorometer (SLM/Aminco, Urbana, IL), equipped with a thermostatted cell holder. Measurements were carried out, under continuous stirring, at a liposome concentration corresponding to 50 μM phospholipid in a volume of 1.5 ml. Excitation was at 430 nm (at which wavelength the inner-filter effect is negligible), while fluorescence emission was recorded at 520 nm. Maximal fluorescence (100%) was set by lysing the vesicles with Triton X-100 (0.1%, by vol.). Residual fluorescence of the liposomes after gel filtration was 2–3%.

Preparation and characterization of the L25 peptide

A peptide corresponding to the C-terminal 25 amino acids of the MS2 lysis protein was synthesized on a Biosearch SAM2 peptide synthesizer using Boc-protected amino acids. Amino acid analysis of the peptide was performed on a Beckman amino acid analyser model M. The results, the average of two independent runs, are presented in Table I. The low recovery of leucine is not unusual in these determinations and generally results from incomplete acid hydrolysis. The four N-terminal amino acids were determined on a Beckman sequenator and the expected residues were found in a >95% yield. The water-insoluble peptide is dissolved in 100% dimethylformamide and added to the *in vitro* assay systems in this solvent.

Incorporation of cytochrome c oxidase in liposomes

Cytochrome *c* oxidase was isolated from beef heart as described by Yu *et al.* (1975) and reconstituted into liposomes composed of either *E.coli* or soybean phospholipids as described by Driessen *et al.* (1985) and de Vrij *et al.* (1986) with minor modifications.

Determination of $\Delta\Psi$ and ΔpH

The $\Delta\Psi$ (interior negative) was determined from the distribution of tetraphenylphosphonium (Ph_4P^+) across the membrane using a Ph_4P^+-sensitive electrode (Shinbo *et al.*, 1978). Uptake of Ph_4P^+ was determined by measuring the decrease of Ph_4P^+ concentration in the external medium.

ΔpH (interior acid) was estimated from the fluorescence quenching of 9-aminoacridine. The reaction was performed at 20°C in a Perkin Elmer MPF-4 spectrofluorometer using excitation and emission wavelengths of 405 and 455 nm respectively.

Proline transport assay

ΔpH-driven accumulation of proline was performed by incubating the *E.coli* membrane vesicles in 50 mM potassium phosphate (pH 7.0) and 10 mM $MgSO_4$. Energization was initiated by the addition of 10 mM D-lactate. After 0.5 min [^{14}C]proline (285 Ci/mol) was added at a final concentration of 12 μM. Reactions were carried out at 25°C and transport was terminated by the addition of 2 ml ice-cold 100 mM LiCl. The samples were filtered on 0.45-μm cellulose nitrate filters (Millipore) and washed once with 2 ml ice-cold 100 mM LiCl.

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References

Atkins,J.F., Steitz,J.A., Anderson,C.W. and Model,P. (1979) *Cell*, **18**, 247–256.

Baty,D., Knibichler,M., Verhey,H., Pattus,F., Shire,D., Bernadac,A. and Lazdunski,C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1152–1156.

Beremand,M.W. and Blumenthal,T. (1979) *Cell*, **18**, 257–266.

Berkhout,B., de Smit,M.H., Spanjaard,R.A., Blom,T. and van Duin,J. (1985) *EMBO J.*, **4**, 3315–3320.

Cronan,J.E., Jr and Vagelos,P.R. (1972) *Biochim. Biophys. Acta*, **265**, 25–65.

Dankert,J.R., Uratani,Y, Grabau,C., Cramer,W. and Hermodson,M. (1982) *J. Biol. Chem.*, **257**, 3857–3863.

Davis,B.D. and Mingioli,E.S. (1959) *J. Bacteriol.*, **60**, 17–28.

de Boer,H.A., Bakker,A.J., Weyer,W.J. and Gruber,M. (1976) *Biochim. Biophys. Acta*, **432**, 361–368.

de Vrij,W., Driessen,A.J.M., Hellingwerf,K.J. and Konings,W.N. (1986) *Eur. J. Biochem.*, **156**, 431–440.

DeLorenzo,Y. and Aguilar,A. (1984) *Trends Biochem. Sci.*, **9**, 266–269.

Driessen,A.J.M., de Vrij,W. and Konings,W.N. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7555–7559.

Fiers,W., Contreras,R., Derinck,F., Haegeman,G., Iserentant,D., Merregaert,J., Min Jou,W., Molemans,F., Raymakers,A., van den Berghe,A., Volckaert,G. and Ysebaert,M. (1976) *Nature*, **260**, 500–507.

Fox,R.O. and Richards,F.M. (1982) *Nature*, **300**, 325–330.

Garrett,J.M. and Young,R. (1982) *J. Virol.*, **44**, 886–892.

Goodell,E.W., Lopez,R. and Tomasz,A. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 3293–3297.

Harkness,R.E. and Ishiguro,E.E. (1983) *J. Bacteriol.*, **155**, 15–21.

Henrich,B., Lubitz,W. and Plapp,R. (1982) *Mol. Gen. Genet.*, **185**, 493–497.

Höltje,J.V. and van Duin,J. (1984) In C.Nombela (ed.), *Microbial Cell Wall Synthesis and Autolysins*. Elsevier Science Publishers, Amsterdam, pp. 195–199.

Jolliffe,L.K., Doyle,R.J. and Streips,U.N. (1981) *Cell*, **25**, 753–763.

Kaback,H.R. (1971) *Methods Enzymol.*, **22**, 99–120.

Kagawa,Y. and Racker,E. (1971) *J. Biol. Chem.*, **246**, 5477–5487.

Kastelein,R.A., Remaut,E., Fiers,W. and van Duin,J. (1982) *Nature*, **295**, 35–41.

Kayalar,C. and Düzgünes,N. (1986) *Biochim. Biophys. Acta*, **860**, 51–56.

Killian,J.A. and de Kruijff,B. (1985) *Biochemistry*, **24**, 7881–7890.

Killian,J.A., Verkley,A.J., Leunissen-Bijvelt and de Kruijff,B. (1985) *Biochim. Biophys. Acta*, **812**, 21–26.

Kusser,W. and Ishiguro,E.E. (1985) *J. Bacteriol.*, **164**, 861–865.

Mayer,L.D., Hope,M.J. and Cullis,P.R. (1986) *Biochim. Biophys. Acta*, **858**, 161–168.

Model,P., Webster,R.E. and Zinder,N.D. (1979) *Cell*, **18**, 235–246.

Poolman,B., Konings,W.N. and Robillard,G.T. (1983) *Eur. J. Biochem.*, **135**, 41–46.

Ralston,E., Hjelmeland,L.M., Klausner,R.D., Weinstein,J.N. and Blumenthal,R. (1981) *Biochim. Biophys. Acta*, **649**, 133–137.

Shinbo,T., Kama,N., Kurihara,K. and Kobataka,Y. (1978) *Arch. Biochem. Biophys.*, **187**, 414–422.

Slatin,S.L., Raymond,L. and Finkelstein,A. (1986) *J. Membrane Biol.*, **92**, 247–254.

Steiner,H., Hultmark,D., Engström,A., Bennich,H. and Boman,H.G. (1981) *Nature*, **292**, 246–248.

Stoffel,W. (1975) *Methods Enzymol.*, **35**, 533−541.
Tétu,C., Dassa,E. and Boquet,P.L. (1980) *Eur. J. Biochem.*, **103**, 117−123.
Tomasz,A. and Waks,S. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4262−4166.
Wilschut,J., Düzgünes,N., Fraley,R. and Papaladjopoulos,D. (1980) *Biochemistry*, **19**, 6011−6021.
Wilson,D.B. (1982) *J. Bacteriol.*, **151**, 1403−1410.
Yu,C.A., Yu,L. and King,T.E. (1975) *J. Biol. Chem.*, **250**, 1383−1392.

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