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Mechanism of the Leakage Induced on Lipid Model Membranes by Rz1 Lipoprotein, the Bacteriophage λ *Rz1* Gene Product: A Fluorescence Study

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The leakage of aqueous contents of neutral (dipalmitoylphosphatidylcholine/cholesterol) liposomes as induced by Rz1, a proline-rich lipoprotein, the bacteriophage λ *Rz1* gene product, was studied. Fluorescence enhancement assay with Tb^{3+}/dipicolinic acid and self-quenching assays with carboxyfluorescein and fluorescein isothiocyanaten-dextran were used to monitor the Rz1-induced leakage from neutral liposomes. The results demonstrated that Rz1 caused local membrane destabilization leading to the leakage of the liposome contents. The extent of the leakage was independent of the molecular mass of the liposome-entrapped solutes in the range of 376–4000 Da. The kinetics of Rz1-liposome leakage was very similar to that obtained with detergent Triton X-100 for all the solutes used. The results suggested that Rz1 can act as a detergent; i.e., by interacting with lipids on both sides of the liposome membranes (inducing perturbation in the lipid packing within the bilayer), it facilitates the transfer of encapsulated molecules into the external liposome environment. The importance of this result for Rz1 physiological function is discussed.

KEY WORDS: Rz1 lipoprotein; lipid liposomes; leakage of liposomes; fluorescence methods.

INTRODUCTION

Rz1, a 6.5-kDa prolipoprotein, is the *Rz1* gene product of bacteriophage λ [1–3]. In induced *Escherichia coli* λ lysogens, Rz1 was found almost-exclusively in the outer membrane (OM). In a strain overproducing Rz1 from the pSB54 plasmid, it was distributed in all the fractions, OM, fraction A, and inner membrane (IM) [2]. The mature product contains the lipid (fatty acid esterified glyceryl-cysteine) as the N-terminal part. The lipid corresponds to that of Braun's mureni lipoprotein (Lpp) of *Escherichia coli* [4], but the peptide part of Rz1, composed of 60 amino acids (aa), shows no homology with that of the Lpp [2,3]: lipid–$_1$CTSKQSVSQCVK-PPPPPAWIMQPPPDWQTPLNGIISPSERG$_{60}$. The peptide part of Rz1 contains five charged amino acids: three charged positively (K, K, and R) and two charged negatively (D and E). Proline residues amount to nearly 25% of the aa of Rz1 and form two stretches of five and three residues. Rz1 does not contain transmembrane sequences, hence its localization in OM might depend on its lipid part, while the peptide part might be exposed to the periplasmic side of OM and thus be able to interact with IM. Several proline residues (proline stretches) tend to be exposed to the surface of a protein molecule, contributing to its hydrophobicity and facilitating the protein–protein interactions [5].

The physiological role of the Rz1 lipoprotein remains to be discovered. However, it follows from our previous research that the lysis of bacterial cell by bacte-

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riophage λ is preceded by a considerable enlargement of membrane adhesion sites, seen as the A fraction sedimenting in sucrose density gradient between fraction OM and fraction IM [6]. The same effect could be evoked by overexpression of the cloned *Rz1* gene [2]. It was interpreted as the result of possible Rz1-lipoprotein fusogenic activity.

We found evidence that Rz1 indeed induces the fusion of artificial membranes [7]. Moreover, it was demonstrated that Rz1 induces the fusion of neutral or negatively charged liposome membranes devoid of any proteins [8]. Therefore we postulated that Rz1-induced liposome fusion is mediated primarily by generation of a local perturbation in the lipid bilayer. Rz1 could cause the local membrane perturbation, for example, by mimicking a detergent [8].

The detergent-like action of Rz1 could be supported by the idea that a small perturbation in the lipid packing within contacting bilayers was necessary and probably sufficient to promote membrane fusion [9–11].

The aim of the experiments described here was to verify this hypothesis by examining the intrinsic properties of the Rz1 protein as the possible factor that perturbs (destabilizes) the lipid membrane. Usually, the membrane local perturbation is inferred from the occurrence of extensive leakage of the liposome contents [12–16]. The leakage of the liposome contents is inferred from the change in fluorescence of the fluorescent dyes due to their transfer from the internal into the external liposome environment [17–20]. The experiments demonstrated that the interaction of Rz1 liposomes with target liposomes loaded with Tb/DPA, CF, or FD-4 caused membrane perturbation to an extent allowing leakage of the solutes entrapped in the liposomes. The extent of leakage was independent of the molecular mass of the solutes entrapped in the liposomes. Similar experiments performed for the detergent Triton X-100 demonstrated that, at certain detergent concentrations, the extent of leakage and the leakage kinetics were very similar to those obtained for Rz1, for all the solutes used. It is proposed that Rz1 can act as a detergent interacting with lipids of both sides of liposome membranes.

EXPERIMENTAL

Chemicals

Terbium(III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$) and hexane were purchased from Fluka Chemie AG (Buchs, Germany). Dipicolinic acid (pyridine-z, b-dicarboxylic acid) (DPA), fluorescein isothiocyanate–dextran (FD-4), cholesterol (Chol), chloroform, sodium citrate, Triton X-100, and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO). Carboxyfluorescein (CF) was obtained from Molecular Probes (Leiden, The Netherlands). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Birmingham, AL). Water was purified by means of a Millipore Milli-Q system. Buffer A (10 m*M* Tris–HCl and 150 m*M* NaCl, pH 7.4), buffer B (10 m*M* Hepes, 100 m*M* NaCl, pH 7.4, with or without 1 m*M* EDTA).

Rz1

The source of the Rz1 lipoprotein was *Escherichia coli* BL21(DE3)[pSB54] induced by IPTG to overproduce Rz1. pSB54 was a pT7-7 derivative [21]. Details of the Rz1 purification are given in Ref. 2. Briefly, it consisted in gentle lysis of the bacteria by sonication and preparative protein separation by SDS-PAGE. The position of the Rz1 protein on the gel was established by immunodetection using anti-Rz1 serum followed by goat anti-rabbit IgG-HRP with 4-chloro-1-naphthol and H_2O_2 as substrates. The Rz1-containing band was cut out of the gel and electroeluted as described in Ref. 22, then dialyzed against 20 m*M* Tris–HCl, 0.02% SDS, pH 7.0. The protein was precipitated with 5 vol of cold acetone and left overnight at −20°C. The precipitate was sedimented at 30,000*g* and stored at −20°C. The preparation was electrophoretically homogeneous.

Preparation of Liposomes

Liposomes were prepared by sonication followed by repeated extrusion through membranes [23]. Briefly, lipids in chloroform solutions were mixed, dried under nitrogen, resuspended in buffer A, and sonicated for 15 min with a 25% active cycle on an Ikasonic U 200 S Ultrasonic Disintegrator (IKA Labortechnik, Staufen, Germany) at 4°C. They were then extruded 10 times through a polycarbonate membrane with a pore size of 0.1 μm (Nucleopore Corp.). The composition of the lipid used was DPPC:Chol at a 6:1 mol ratio (neutral target liposomes).

Liposomes coupled with Rz1 [0.5% (w/w); Rz1-liposomes] were prepared in the same way. The dry lipid-Rz1 films were hydrated in buffer A by repeated sonication (100 10-s pulses with 30-s intervals). The coupling efficiency was about 90% as evaluated by measuring Rz1 (Trp) fluorescence at 345 nm before and after liposome purification by gel filtration through Sephadex G25, PD-10 columns (Pharmacia).

Target liposomes with encapsulated Tb/DPA complex, CF, or FD-4 were obtained in essentially the same way. For hydration of the dry lipid films the following solutions were used: (a) 50 m*M* FD-4 in buffer A, (b) 2.5 m*M* $TbCl_3$ and 50 m*M* DPA in buffer B without EDTA, and (c) 150 m*M* CF in buffer B without EDTA. Unencapsulated materials were separated from the liposomes by gel filtration on Sephadex G25, PD-10 or Sephacryl S-300 HR columns.

The mean diameters were approximately 150 nm for both types of liposomes, as determined by dynamic light scattering in a Coulter (N4S) submicron particle analyzer.

Measurements of Rz1-Induced Leakage

The Rz1-induced leakage was determined by fluorescence enhancement assay with Tb^{3+}/DPA and fluorescence self-quenching assay with CF or FD-4.

Enhancement Assay with Tb^{3+}/DPA. Terbium (Tb^{3+}) ions display weak fluorescence. However, the interaction of Tb ion with DPA leads to the formation of a $Tb(DPA)_3^{3-}$ complex (Tb/DPA complex) that produces a 10^4-fold enhancement of the Tb fluorescence [13]. The Tb/DPA complex is excited at 276 nm and the fluorescence emission is detected at 545 nm. The leakage is revealed as a decrease in Tb/DPA fluorescence due to immediate dissociation of the complex upon EDTA [13].

In our experiments, the Rz1-coupled liposomes and target liposomes with encapsulated Tb/DPA (as described under Materials and Methods) were mixed at a lipid molar ratio of 1:10 in buffer B containing 1 m*M* EDTA. The level of 100% fluorescence was considered as the fluorescence of the complex in the target liposomes in buffer B with EDTA. Zero percent fluorescence was determined after the addition of Triton X-100 (0.2%, v/v) to the sample at the end of the measurements. As controls, the fluorescence of Tb/DPA in target liposomes and a mixture of Tb and DPA in buffer B with EDTA was used.

Self-Quenching Assay with CF. Fluorescence of CF is >95% self-quenched at concentrations >100 m*M*. A concentrated solution of this water-soluble dye is encapsulated in the target liposomes. Dye release is accompanied by an increase in fluorescence (excitation/emission maxima, 490/520 nm). Complete lysis of the liposomes with detergent serves for the determination of the assay end point [20,24].

In our experiments CF (150 m*M* solution in buffer B without EDTA) was encapsulated in the target liposomes, which were then separated from the remaining dye by gel filtration. The Rz1-liposomes were added to the target liposomes at a lipid molar ratio of 1:10. The data are expressed as a percentage of F_{max}, determined after adding 0.2% (v/v) Triton X-100 to the sample at the end of the experiment. Mixed target liposomes were control samples for measuring spontaneous leakage of the liposomes.

Self-Quenching Assay with FD-4. The fluorescence of FD-4 is self-quenched at concentrations >40 m*M*. Dye release from liposomes is accompanied by an increase in fluorescence (excitation/emission maxima, 465/535 nm) [25].

The target liposomes were prepared in buffer A containing 50 m*M* FD-4. The Rz1-liposomes were added to the target liposomes at a lipid molar ratio of 1:10. The data are expressed as a percentage of F_{max}, determined after disrupting the liposomes in 0.2% (v/v) Triton X-100. Control samples for measuring the spontaneous leakage of the liposomes were prepared by mixing target liposomes.

RESULTS AND DISCUSSION

Rz1 Insertion into a Model Lipid Membrane (Liposome)

The peptide part of the mature Rz1 lipoprotein does not contain transmembrane sequences, hence the notion that the Rz1 lipoprotein is inserted into OM only through its lipid part seems to be justified [3]. If so, the peptide part should be exposed to the periplasm and thus be able to interact with IM. Similarly we imagine that in the liposome system, some Rz1 molecules are anchored by the lipid part, with their peptide part being exposed to the outer surface of the liposome, thus facilitating interactions with other liposomes and leading to their aggregation and fusion. The peptide part of Rz1 might also be exposed to the inner surface of the liposome, facing the aqueous contents entrapped in liposomes.

It has been well established that the intrinsic fluorescence of Trp increases when the amino acid senses a more hydrophobic environment. Concomitantly, a blue shift in the emission maximum is seen [26]. Hence, the Trp fluorescence can be used as an indicator of the protein position in a lipid blayer.

Rz1 contains neither Tyr nor Phe, the fluorescence spectra of which would partly overlap the Trp spectrum. Hence, Rz1 could be regarded as a one-component (Trp) system for spectrophotometric analysis. Moreover, it results from our earlier study [7,8] that mixing the Rz1-liposomes with target liposmes could lead to intermixing of the lipids, and as a result, Rz1 could be transferred from the Rz1-liposomes into the target liposomes. Figure

1 shows the Trp emission spectra for Rz1 in buffer (Fig. 1a) and in liposomes before (Fig. 1b) and after (Fig. 1c) mixing with the target liposomes. No significant shifts in wavelength were observed for Rz1 in either kind of liposomes. λ_{max} corresponded to 346 nm for Rz1 in liposomes and to 345 nm for Rz1 in liposomes after the addition of target liposomes. These λ_{max} values are close to the 348 nm characteristic for Rz1 in buffer (Fig. 1a) and Trp in aqueous solution [26]. The incorporation of Rz1 into the liposomes and Rz1-induced liposome fusion did not influence the Trp fluorescence spectra (Fig. 1). The shape and position of the maxima indicated that the peptide part of Rz1 was indeed exposed to the hydrophilic environment. This also indicated that Trp did not come into contact with the hydrophobic inside of the liposomal membrane due to liposome fusion. It might mean that Rz1 could cause (induce) the changes in the lipid structure that led to liposome fusion, without changing the peptide position within the membrane. These results suggest that if Rz1 introduced the perturbation into the lipid bilayers, this perturbation did not involve penetration of the peptide into the membrane.

Membrane Perturbation Leading to Leakage of the Liposome Contents

Membrane perturbation was inferred from the occurrence of extensive leakage of three solutes of different molecular masses encapsulated in DPPC/Chol liposomes: Tb/DPA complex, CF, and FD-4.

Rz1-Induced Liposome Leakage. Fluorescence enhancement assay with Tb^{3+}/DPA is widely used for studying the fusion of artificial membranes [27] and as a diagnostic tool for distinguishing between leaky and nonleaky processes [9,28]. Tb^{3+} ions displays very weak fluorescence. The formation of Tb/DPA complex produces strong enhancement of the Tb fluorescence. An additional advantage of this assay is that the formation of the complex is very fast. On the other hand, the introduction of 0.1 m*M* EDTA into the solution containing Tb/DPA complex leads to immediate complex dissociation and produces a 90% decrease in the fluorescence intensity [13]. Since the complex dissociation can be induced by compounds present only in the outside medium, elegant experiments can be performed so as to demonstrate mixing of the contents of liposomes, separated from the outside medium, or real efflux of the liposome contents devoid of any interference from a dilution process [13].

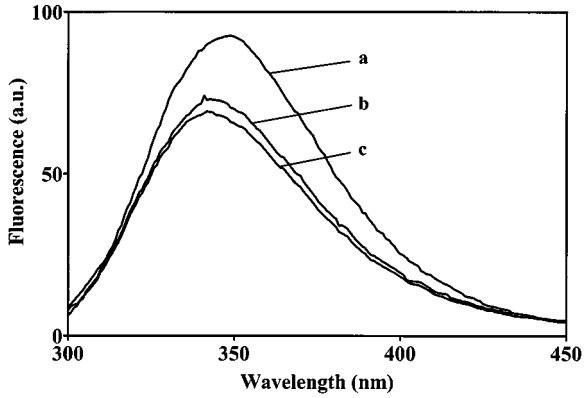

Fig. 1. Trp fluorescence spectra of Rz1. Rz1 in buffer (a) and liposomes before (b) and after (c) addition of the target liposomes. λ_{exc} = 280 nm. The spectra were corrected for light scattering and for the dilution caused by liposome addition.

In our experiments, the Tb/DPA complex in buffer B without EDTA was encapsulated in the target liposomes. The introduction of EDTA into the suspension of target liposomes did not influence the Tb/DPA strong fluorescence (Fig. 2; triangle line). This indicated that Tb/DPA did not enter the external liposome environment and leakage of liposome was not observed. The fluorescence intensity of the Tb/DPA complex in this system was regarded as 100%. For a control (reference) the fluorescence of the Tb^{3+} and DPA mixture (the same concentration as for complex formation), prepared in buffer B with EDTA, is presented as a zero line (Fig. 2; diamond line). The addition of Rz1-liposomes to the suspension of target liposomes induced the leakage of Tb/DPA (Fig. 2; solid line). After 10 min of experiment, the leakage reached an almost-constant level that represented a 34% decrease in fluorescence. The addition of Triton X-100 induced a further decrease in fluorecence The fluorescence intensity corresponding to the zero level indicated the complete lysis of liposomes and corresponded to the value obtained in control experiments for the dissociated Tb/DPA complex.

Leakage of Solutes of Different Molecular Masses. The enhancement assay with Tb^{3+}/DPA clearly demonstrated that Rz1 could induce the leakage of liposomes, causing the efflux of molecules as large as 657 Da, i.e., as for complex $Tb(DPA)_3^{3-}$.

The experiments were extended on self-quenching assays with CF (molecular mass, 376 Da) and FD-4 (molecular mass, 4000 Da). The value obtained in measurements of the extent of the released aqueous contents was not dependent on the molecular mass of the entrapped solutes (Figs. 3 and 4; solid lines). The same can be observed for the kinetics of molecule efflux. After 10 min of the experiments, the leakage was 32% for CF and 31% for FD-4. The leakage was 34% for Tb/DPA. The lack of size dependence for the Rz1-induced leakage of

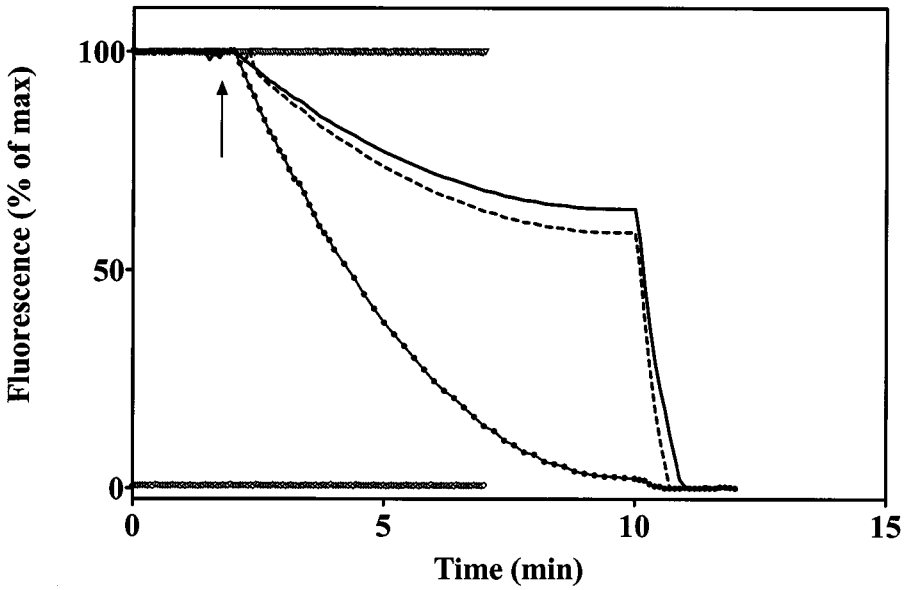

Fig. 2. Fluorescence enhancement assay with Tb^{3+}/DPA. Solid line: Rz1-liposomes and target liposomes were mixed at a 1:10 lipid molar ratio in buffer B containing 1 m*M* EDTA. Target liposomes were loaded with a 1:1 mixture of $TbCl_3$ (2.5 m*M*) and DPA (50 m*M*) in buffer B without EDTA. The decrease in fluorescence resulted from $Tb(DPA)_3^{3-}$ complex dissociation in an external liposome environment containing EDTA. The fluorescence intensity (λ_{exc} = 276 nm, λ_{em} = 545 nm) is reported as a percentage of F_{max}. F_{max} is defined as the fluorescence of the Tb/DPA encapsulated in the target liposome. The fluorescence intensity of Tb^{3+} after addition of 0.2% (v/v) Triton X-100 in buffer B containing 1 m*M* EDTA to the sample represented 0% fluorescence. Diamond line: Control samples for referring the total dissociation of the Tb/DPA complex were prepared by adding $TbCl_3$ (2.5 m*M*) and DPA (50 m*M*) to the suspension of lipids in buffer B with 1 m*M* EDTA and 0.2% Triton X-100. Triangle line: Control samples for measuring the spontaneous leakage of the liposomes were prepared by mixing the target liposomes (loaded with Tb/DPA in buffer B without EDTA) in buffer B with EDTA. Addition of detergent diluted 52 times (circle line) and 168 times (dashed line) to the suspension of the target liposomes. The arrow indicates the addition of Rz1-liposomes to the suspension of the target liposomes.

solutes of different molecular masses should be related to a mechanism different from that responsible for the formation of discrete channels or pores in the bilayer [18]. This might be valid at least for molecules sized up to 1.2 nm, i.e., the Stokes radii of FD-4 [29]. A plausible explanation for the observed phenomenon was that the Rz1 acted on the lipid membrane as a detergent would act. The specific structure and properties of detergents, however, limit the group of chemical compounds that can be considered detergent-like compounds. These specific properties are related to two clearly distinguished parts of the compound with different features: hydrophilic and hydrophobic. It seems that Rz1 fulfills this requirement. Rz1 contains a lipid (three fatty acid residues) as its N-terminal part and a peptide part composed of 60 aa. The peptide part of Rz1 contains five charged amino acids: three charged positively (K, K, and R) and two charged negatively (D and E). The hydrophobic and hydrophilic aa amount to nearly 48 and 52%, respectively. As already demonstrated with Trp fluorescence, the peptide part of Rz1 is located rather closely to the hydrophilic part of the liposome bilayer. It is reasonable to assume that the lipid part of Rz1 packs into the hydrocarbon chain region (and probably slightly fluidizes the chains).

To verify the Rz1-detergent hypothesis, experiments with the detergent Triton X-100 were performed. The scenario of these experiments was similar to those performed with Rz1. The liposomes did not contain Rz1. The detergent at a proper concentration was added to the target liposomes with encapsulated Tb/DPA, CF, or FD-4. The concentrations of fluorescent species were identical to those used for Rz1 experiments. As Rz1 was expected to behave like detergent, the detergent Triton X-100 (typically used for experiments with liposomes) should induce changes in liposomes similar to the perturbations induced by Rz1, which was manifested by the leakage curves.

It was difficult to estimate the concentration of detergent at which the expected changes in the liposomes should appear. The starting Triton X-100 concentration

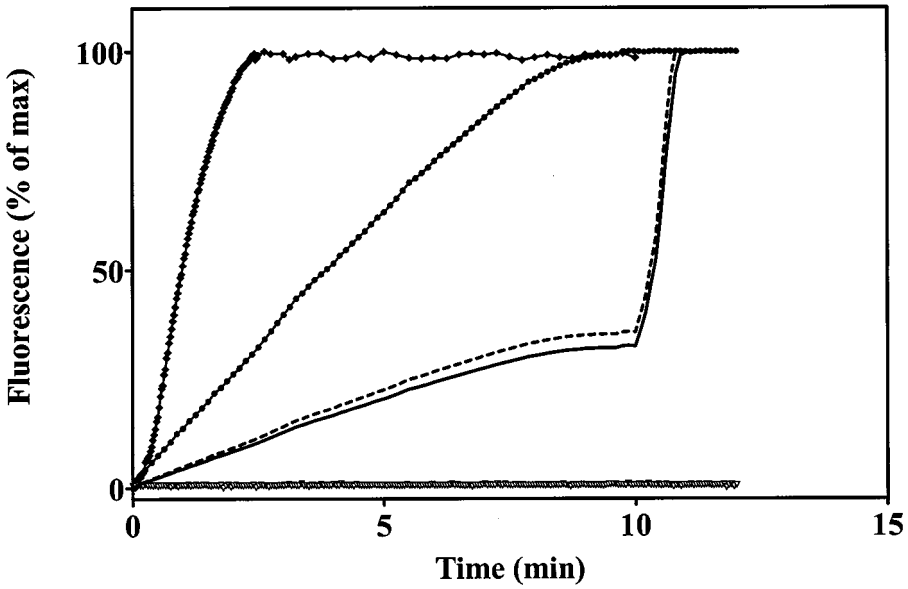

Fig. 3. Self-quenching assay with carboxyfluorescein. Solid line: Target liposomes were prepared in buffer B without EDTA, containing 150 m*M* carboxyfluorescein. Rz1-liposomes were added to the target liposomes at a 1:10 lipid molar ratio. The release of carboxyfluorescein from liposomes was followed by measuring its fluorescence (λ_{exc} = 490 nm, λ_{em} = 520 nm). The data are expressed as a percentage of F_{max}, determined after adding 0.2% (v/v) Triton X-100 to the sample at the end of the experiment. Diamond line: Control samples for measuring the detergent-induced leakage of the liposomes were prepared by adding 0.2% (v/v) Triton X-100 to the target liposomes. Triangle line: Control samples for measuring the spontaneous leakage of the liposomes were prepared by mixing the target liposomes. Addition of detergent diluted 54 times (circle line) and 170 times (dashed line) to the suspension of target liposomes.

was 0.2% (v/v). Triton X-100 at this concentration caused complete lysis of liposomes and was used for calibration of the fluorescence intensity. The detergent induced total lysis of liposomes filled with Tb/DPA, CF, or FD-4 (Figs. 2–4; diamond lines). In the next steps in the experiment, gradually diluted detergents were used. The circle curves in Figs. 2–4 represent typical leakage curves obtained for moderately diluted detergent: diluted 52 times for Tb/DPA, 54 times for CF, and 54 times for FD-4. Surprising results were obtained for much more diluted detergent. The addition of diluted detergent into the suspension of liposomes (Figs. 2–4; dashed lines) caused leakage characterized by leakage curves similar to those obtained for Rz1-induced leakage (Fig. 2–4; solid lines). The leakage was again not dependent on the molecular masses of the solutes encapsulated in the liposomes. These results support the hypothesis that the Rz1-induced perturbation in the lipid membrane that led to liposome leakage can be explained by a mechanism similar to that for the interaction of detergent with the liposome lipids.

Our suggestion that Rz1 caused leakage of the artificial membrane may parallel the distortion of the bacterial OM into which the Rz1 lipoprotein has been inserted during λ bacteriophage infection becomes very probable. Therefore, it is also probable that when the mureni layer undergoes destruction by the λ gene R, the OM destabilization may be dependent (by simple detergent-like mechanism) on the Rz1 lipoprotein.

The suggested mechanism for Rz1 action, i.e., not the formation of specific structures (channels or pores) or specific protein–protein interaction (between proline stretches), may also facilitate understanding the action of sites responsible for adhesion. These sites were suggested to participate in the export of proteins and polysaccharides, the components of OM, and also in the adsorption of bacteriophages (supposedly they facilitate the transit of phage DNA through the membranes) [1–3,6]. The transition of these species through a membrane may be facilitated by the Rz1-perturbed local places (sites). There is an additional point regarding the proposed mechanism that should be emphasised. Rz1 alone can induce leakage (and the membrane fusion). It has been suggested that the resultant effect of Rz1 physiological activity might be connected with Rz1 cooperation with other proteins

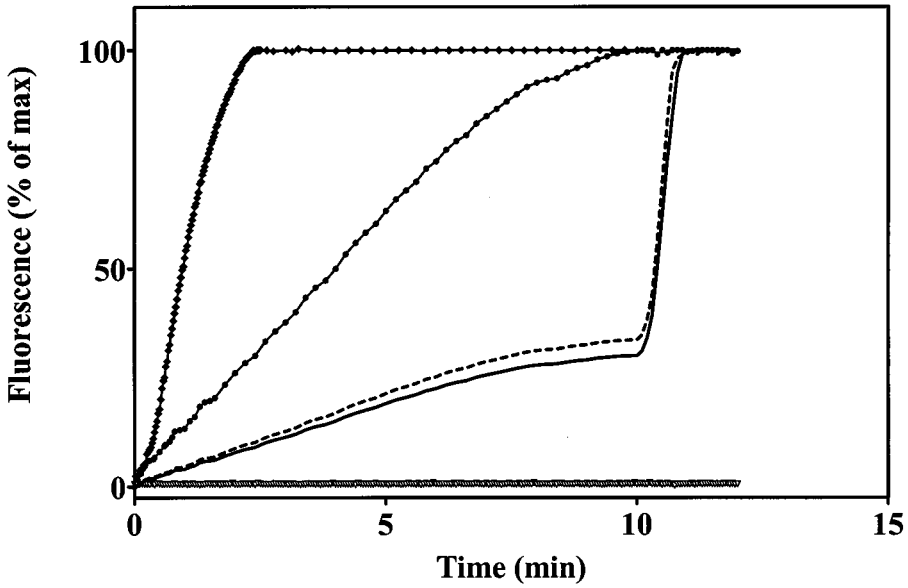

Fig. 4. Self-quenching assay with FD-4. Solid line: Target liposomes were prepared in buffer B without EDTA, containing 50 m*M* FD-4. Rz1-liposomes were added to the target liposomes at a 1:10 lipid molar ratio. The release of FD-4 from liposomes was followed by measuring its increasing fluorescence (λ_{exc} = 490 nm, λ_{em} = 520 nm). The data are expressed as a percentage of F_{max}, determined after adding 0.2% (v/v) Triton X-100 to the sample at the end of the experiment. Diamond line: Control samples for measuring the detergent-induced leakage of the liposomes were prepared by adding 0.2% (v/v) Triton X-100 to the target liposomes. Triangle line: Control samples for measuring the spontaneous leakage of the liposomes were prepared by mixing the target liposomes. Addition of detergent diluted 54 times (circle line) and 172 times (dashed line) to the suspension of target liposomes.

[2]. Our results indicate that the interaction of Rz1 with other proteins is not a prerequisite for membrane destabilization (and fusion), at least in the model system.

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