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Escherichia coli hemolysin permeabilizes small unilamellar vesicles loaded with calcein by a single-hit mechanism

Gianfranco Menestrina

Dipartimento di Fisica, Universita' di Trento, I-38050 Povo (TN), Italy

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Escherichia coli hemolysin produces small unilamellar lipid vesicles permeable to the fluorescent dye calcein by forming pores through their membrane. The process of permeabilization proceeds as a pseudo first-order reaction, indicating that the toxin is active as a monomer; consistently no evidence for cooperativity has been found in a dose-response titration. The rate of interaction increases on lowering the pH of the solution and by introducing negatively charged lipids into the vesicles. The overall pore formation mechanism resembles that of other toxins of bacterial origin such as colicins, diphtheria, tetanus and botulinum toxin.

Hemolysin; Pore formation; Lipid vesicle; Single-hit mechanism; pH dependence; Surface potential; (*Escherichia coli*)

1. INTRODUCTION

Escherichia coli hemolysin is a 107 kDa polypeptide which is the primary substance responsible for the virulence of some pathogenic strains of these bacteria [1,2]. This toxin has been recently sequenced [3] and isolated to purity [4–6]. It appears to be the only example of a protein genuinely secreted by gram negative bacteria [7].

The nature of toxin attack on target cells is not yet understood. Hemolysis of red blood cells may proceed through a colloid osmotic shock due to the formation of hydrophilic pores on the cell membrane [8]. Consistently, hemolysin forms a voltage-dependent cation-selective ion channel of high conductance in planar lipid bilayer membranes (BLMs) [9].

I have now studied the interaction of *E. coli* hemolysin with small unilamellar lipid vesicles (SUVs) a system particularly suited to the determination of kinetic parameters for the reaction between toxin and target. Understanding the mode of action of this hemolysin could be relevant to the prophylaxis and prevention of deseases induced by virulent strains of *E. coli*.

Correspondence address: G. Menestrina, Dipartimento di Fisica, Universita' di Trento, I-38050 Povo (TN), Italy

2. MATERIALS AND METHODS

2.1. *Toxins*

E. coli hemolysin prepared as in [8] was kindly provided by Dr S. Bhakdi (University of Giessen, FRG) and was kept in lyophilized form at −20°C. A fresh sample was prepared each day and hemolytic activity tested as in [9]. The molar concentration of the toxin can be calculated from its hemolytic activity using the relation 1 HU = 10^{-10} M determined by an ELISA assay (Bhakdi, S. personal communication).

Lyophilized α-toxin and δ-lysin from *Staphylococcus aureus* were kind gifts from Dr Hungerer (Calbiochem-Behring, Marburg) and Dr J. Freer (University of Glasgow), respectively.

2.2. *Calcein release from lipid vesicles*

SUVs were prepared by sonication of multilamellar liposomes prepared as in [10] in a buffer containing 80 mM calcein (Sigma), 50 mM NaCl, pH 7.0. Lipids used were: egg phosphatidylcholine (PC) (PL Biochemicals), phosphatidylserine (PS) and phosphatidylinositol (PI) (Avanti Polar Lipids), and cholesterol (Fluka); lipid concentration was always 12.5 mg/ml but different compositions were used as specified; the molar ratio of binary mixtures was always 1:1.

To remove untrapped calcein SUVs were eluted through a Sephadex G-50 column using 200 mM NaCl, 10 mM Tris, pH 7.0 (buffer A), the final lipid concentration being approx. 2 mg/ml. Aliquots of these SUVs were introduced into a stirred

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and thermostatted quartz cuvette containing 3 ml buffer A at the pH indicated. Fluorescence was measured as in [10]; excitation was set at 494 nm and emission at 520 nm. Calcein release from the interior of the vesicles resulted in an increase of fluorescence as the dye became diluted into the external medium and self-quenching was relieved [11]. 100% release was determined by adding 0.7 mM Triton X-100. Comparison of the fluorescence produced by 100% release from fixed amounts of vesicles with a titration curve for pure calcein permitted calculation of their internal volume and thus estimation of the average diameter which was about 40 nm, in good agreement with published data [12,13]. Spontaneous release of calcein was slow and could be neglected in this study.

3. RESULTS AND DISCUSSION

3.1. *Permeabilization of lipid vesicles is a 1:1 reaction*

Addition of *E. coli* hemolysin to a solution containing SUVs loaded with calcein produces an increase of the fluorescence as the dye leaks out (fig.1). Assuming, on the basis of our previous results [9], that the toxin forms channels into the vesicles thus increasing their permeability to calcein, then the time course of fluorescence simply represents that of the formation of channels into the vesicles. Actually, the kinetics for dye release from a single vesicle are too fast to be resolved by our apparatus, lasting less than 1 s [14].

The time course of channel formation can be described as a single-exponential function with time constant τ (fig.1, inset), which is inversely proportional to the concentration of toxin in the solution (fig.2). This is expected if hemolysin increases the vesicles' permeability by binding to them in a monomeric form; in fact, for this case, the following reaction scheme holds:

$$T + V_i \underset{K_{dis}}{\overset{K_{as}}{\rightleftharpoons}} V_p \qquad (1)$$

where T, V_i and V_p represent the concentrations of toxin monomers, intact vesicles and permeabilized vesicles, respectively, K_{as} and K_{dis} denoting the association and dissociation rates for the reaction. According to eqn 1 the rate of appearance of permeabilized vesicles is given by:

$$\frac{dV_p}{dt} = K_{as} V_i T - K_{dis} V_p \qquad (2)$$

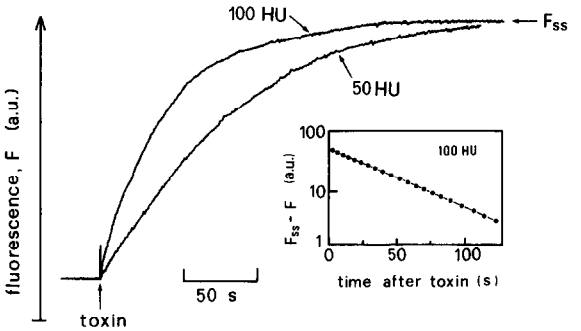

Fig.1. Time course of calcein release from lipid vesicles induced by *E. coli* hemolysin. Fluorescence, F, increases after the addition of toxin to a solution containing 10^{-9} M SUVs loaded with calcein to reach a steady value indicated as F_{ss}. (Inset) Semilogarithmic plot of the time course of relaxation, $F_{ss} - F$, with 100 HU *E. coli* hemolysin. The kinetics can be fitted by a single exponential with time constant $\tau = 41.2$ s and correlation coefficient $r = 0.997$. Other conditions: pH 5.0, $T = 23°C$, PC/PS vesicles.

which can be solved assuming that T does not appreciably decrease during the reaction and that K_{dis} is zero (in fact, the binding is irreversible, as indicated by experiments on BLMs [9] and red blood cells [8]) to give:

$$V_p = V_0(1 - \exp(-K_{as} T t)) \qquad (3)$$

in which V_0 indicates the total concentration of the vesicles, i.e. a single-exponential relaxation with the time constant:

$$\tau = 1/K_{as} T \qquad (4)$$

Provided that the total fluorescence and total number of permeabilized vesicles are proportional, the plots in figs 1 and 2 indicate that eqns 3 and 4 actually hold and allow evaluation of K_{as} as 2.5×10^6 $M^{-1} \cdot s^{-1}$.

In control experiments I have studied the effects of two other hemolytic toxins, δ-lysin and α-toxin produced by *S. aureus*, both of which are supposed to increase the permeability of cell membranes by forming lesions via an aggregating mechanism [15,16]. I have found that both toxins induce calcein release from lipid vesicles but that the kinetics of their interaction are characterized by an initial delay followed by a multiexponential relaxation (not shown but see [10,17] for similar results). This is a good indication that an aggregation

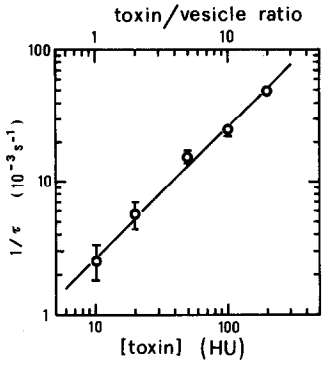

Fig.2. Dependence of permeabilization rate, τ^{-1}, on toxin concentration on a double logarithmic plot. The regression line has a slope of 0.98 and correlation coefficient $r=0.997$. Other conditions as in fig.1.

mechanism cannot result in such simple kinetics as those shown by *E. coli* hemolysin.

Further experimental evidence for the monomeric action hypothesis is provided by the results shown in fig.3. Here, successive additions of small amounts of *E. coli* hemolysin were made to a cuvette containing a fixed amount of PC/PI vesicles and the extent of calcein release was measured after each addition. The linear increase in percentage release at very low *E. coli* hemolysin concentration (well below a protein/vesicle ratio of 1:1, where each vesicle is supposed to bear at most one toxin molecule) is a good indication that hemolysin molecules act in a 1:1 fashion on the vesicles. Actually, an S-shaped dependence is expected for an aggregation mechanism with a flat increase at low toxin concentrations, where most of the vesicles do not carry enough monomers to form a conducting aggregate, and a very steep increase when a critical toxin concentration is reached in the vesicles which allows for the formation of aggregates. *S. aureus* δ-lysin indeed behaves in this way, as shown in the inset to fig.3, the critical toxin concentration being reached at a protein/vesicle ratio of around 500:1; qualitatively similar results have also been obtained with α-toxin (not shown).

3.2. *pH and lipid dependence of the permeabilizing action*

The pH dependence of the time constant τ is depicted in fig.4 for the case of PC/PS vesicles. *E. coli* hemolysin is more active at low pH with a pK for activation around 6.

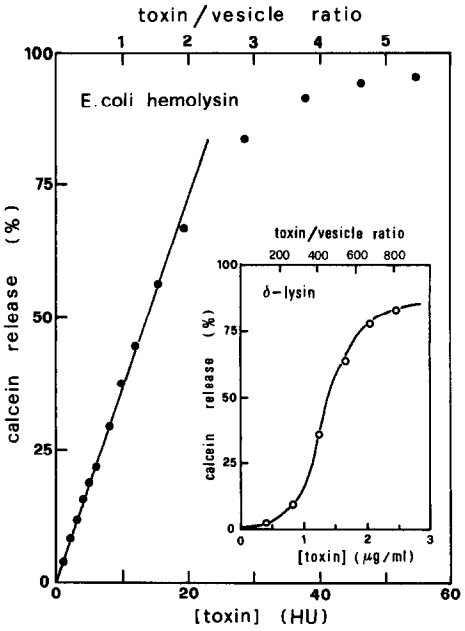

Fig.3. Lack of cooperativity in the action of *E. coli* hemolysin on lipid vesicles. Percentage release of calcein upon successive additions of toxin is shown. The release increases linearly at low toxin concentrations. (Inset) Similar experiment performed with *S. aureus* δ-lysin; in this case the release increases sigmoidally, i.e. cooperativity is shown. Other conditions: pH 6.0, $T=21.4$°C.

The lipid composition of the SUVs is also important: the toxin has a preference for vesicles containing negative lipids (PS and PI) rather than neutral lipids (e.g. PC and cholesterol). Effects due to possible size varations in vesicles of different compositions (which are kept within a factor of 2 [13]) are probably small and have been neglected to a first approximation. As shown in table 1, both the rate constant, τ^{-1}, and percentage

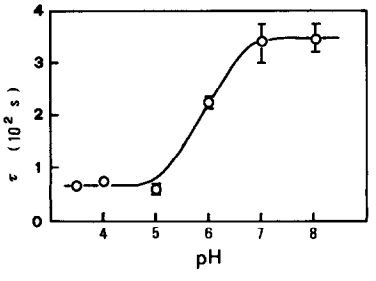

Fig.4. pH dependence of the time constant for permeabilization of PC/PS vesicles upon addition of 50 HU *E. coli* hemolysin. Other conditions as in fig.1.

Table 1

Lipid dependence of the action of 10 HU *E. coli* hemolysin on SUVs at pH 5.0

	SUV composition				
	PC:PI	PS	PC:PS	PC	PC: cholesterol
Calcein release[a] (%)	60	57	52	5	3
Rate constant[b] $(\times 10^{-3})(s^{-1})$	7.9	5.4	3.7	$\lesssim 1$	$\lesssim 1$
Surface potential[c] (mV)	−62	−66	−40	0	0

[a] 100% release obtained by addition of Triton X-100

[b] Determined from a semi-logarithmic plot as in fig.1

[c] Evaluated according to the Guy-Stern theory [25] using the experimental binding constants given in [26].

of lysis are roughly proportional to the surface potential of the SUVs, indicating that this is a driving force for the insertion process.

It is intriguing to note that the potentiating effects of low pH, negative surface potential and negative transmembrane voltage [9] that we have found with *E. coli* hemolysin are also shared by other membrane-seeking toxins of bacterial origin like colicins [18,19], diphtheria toxin [20,21], botulinum and tetanus toxin [22–24], suggesting the existence of a common mechanism which has been carefully conserved during evolution.

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