Interaction of the bacterial protein toxin α-haemolysin with model membranes: protein binding does not always lead to lytic activity

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Abstract **α-Haemolysin interaction with model membranes has been investigated by a 2-fold procedure. First, protein binding has been measured, by a direct method as well as through changes in the intrinsic fluorescence of the protein when incubated with liposomes and divalent cations. Then, the above results have been correlated with the protein lytic activity. The extent of protein binding is not significantly modified by the presence or absence of Ca^{2+}, or by changes in lipid composition, although these factors influence greatly the membrane lytic activity of the protein. Moreover, Ca^{2+} binding to the toxin must occur prior to protein binding to the bilayer, for a lytic effect to take place.**

Key words: Bacterial toxin; Ca^{2+}-binding protein; Lipid-protein interaction; Membrane protein insertion; Membrane protein intrinsic fluorescence; α-Haemolysin

1. Introduction

α-Haemolysin is an extracellular cytolytic protein toxin (M ≈ 107 kDa) produced by some strains of *E. coli* [1]. The molecular mechanism of its membrane lytic effects has been explored using model membranes [2–4]. In particular, previous studies from this laboratory have examined the leakage of large unilamellar vesicles of defined lipid composition by α-haemolysin [5]. Some of the data in the latter publication suggested that, under most conditions, a substantial proportion of the toxin remained in solution in equilibrium with the membrane-bound form. A quantitation of this equilibrium seemed thus desirable for a proper understanding of the lytic event.

Moreover, we have recently published the requirement by α-haemolysin of certain divalent cations (e.g. Ca^{2+}) for its lytic activity, and the competition for protein binding between Ca^{2+} and the non-activating cation Zn^{2+} [6]. The relationship between cation- and membrane-binding abilities of α-haemolysin had not been explored yet in pure phospholipid membranes. It is not known either whether membrane binding by the toxin leads necessarily to membrane lysis or not. In the present paper, we describe a direct method for measuring the proportions of free and bound α-haemolysin in the presence of large unilamellar liposomes and compare the results under conditions of varying lipid composition and presence or absence of cations. The studies of protein binding are complemented with data on the binding-related changes in intrinsic tryptophanyl fluorescence of α-haemolysin. The results reveal that divalent cations do not modify significantly toxin binding, although membrane lysis occurs only when Ca^{2+}-containing α-haemolysin binds the membrane.

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Abbreviations: ANTS, 8-aminonaphtalene-1,2,3-trisulphonic acid; DPX, *p*-xylenebispyridinium bromide; CHOL, cholesterol; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine

2. Materials and methods

2.1 Materials

Egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) were grade I from Lipid Products (South Nutfield, UK); cholesterol (CHOL) was from Sigma (St. Louis, MO) 8-Aminonaphtalene-1,2,3-trisulphonic acid (ANTS) and *p*-xylenebispyridinium bromide (DPX) were obtained from Molecular Probes (Eugene, OR) Plasmid-encoded α-haemolysin was purified from the culture filtrates of an overproducing strain of *E coli*, according to [7]; prior to its use, the protein was dialyzed against TCU buffer [7] containing 1 mM EGTA. Large unilamellar vesicles (LUV) of different compositions were prepared by extrusion and sized using 0.1-μm pore size Nuclepore membranes as described in [8]; the buffer was 20 mM Tris-HCl, 150 mM NaCl, pH 7 0, ± EGTA, Ca^{2+} or Zn^{2+}, in D_2O or H_2O as required.

2 2 Assessment of vesicle leakage

Release of vesicle contents was measured by the ANTS/DPX method, according to [9]. Additional details have been given elsewhere [5].

2.3 Direct measurements of α-haemolysin binding to liposomes

A modified form of a previously published method [10] has been used, based on the fact that free, but not lipid-bound protein will sediment when centrifuged in D_2O buffer. LUV, corresponding to 1 μmol of lipid were diluted to 1 ml, together with the appropriate amount of protein, to give lipid:protein molar ratios in the range 2000–20,000. LUV and protein were allowed to equilibrate for 30 min at room temperature, then centrifuged in a 120.2 Beckman rotor (500,000 × *g*, 20°C, 2 h). 100-μl fractions were recovered, starting from the top of the tube, and assayed for lipid P [11] and protein [12], the latter after addition of octylglucoside up to 1%

2 4 Measurements of intrinsic fluorescence of α-haemolysin

Bilayer-toxin interaction was also monitored through changes in the intrinsic fluorescence emission spectra of α-haemolysin. Small aliquots of a concentrated LUV suspension were added to a protein solution (0.15 -0.30 μM) in a cuvette with continuous stirring After equilibrating for 5 min, emission spectra were recorded with an excitation light of 295 nm (slit 5 nm). Fluorescence intensity measurements were corrected for light scattering [13]. From fluorescence titration curves, a dissociation constant K_d for the vesicle-toxin complex may be estimated, according to [14]:

$$\varepsilon - 1 = (\varepsilon_b - 1)\frac{(\varepsilon-1)n \cdot K_d}{m}$$

were ε corresponds to the relative change in fluorescence intensity F/F_0, ε_b is the limit value of ε, when all the protein in the system is membrane-bound, *m* is the lipid concentration, and *n* is the number of lipid molecules that make up a binding site for the protein. Although *n* is

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difficult to evaluate, the product $n \cdot K_d$ is easily estimated from the slope of $(\varepsilon-1)$ vs. $(\varepsilon-1)/m$. Thus, $n \cdot K_d$ is used as a valid parameter to compare the affinity of a protein for the various kinds of lipid bilayers, even when the precise value of n is not known.

3. Results

The basis of the centrifugation method for measuring α-haemolysin binding to LUV is shown in Fig. 1A. When either vesicles or protein are independently centrifuged in D_2O buffer, the lipid remains on top of the tube, while all of the protein sediments. When LUV and protein are incubated together and the mixture is then centrifuged, a substantial part of the protein remains in the top part of the tube, i.e. vesicle-bound (Fig. 1B). Note that the lipid and protein profiles in the top fractions are almost coincident, suggesting that they exist in the form of a lipid-protein complex. From experiments as shown in Fig. 1B, the proportion of bound protein under a variety of conditions may be estimated for a given total lipid:protein ratio (see, e.g. the data in Table 1). As expected, the fraction of bound protein varies with the total lipid:protein ratio (Fig. 2).

In a different series of experiments, changes in intrinsic fluorescence were used to monitor changes in protein structure occurring as a result of bilayer-protein interaction. In particular, an increase in Trp fluorescence is expected upon interaction with the hydrophobic lipid matrix [13–16]. This is indeed the case, as seen in Fig. 3. The binding may be quantified, by titrating the α-haemolysin intrinsic fluorescence with lipid vesicles (Fig. 3A); from the titration curve, a binding parameter $n \cdot K_d$ may be obtained (Fig. 3B) (see Section 2). Values of the binding parameter $(n \cdot K_d)$ are also collected in Table 1.

A collection of data of α-haemolysin binding to LUV, measured by the above techniques, as well as of toxin-induced vesicle leakage, assayed by the ANTS/DPX procedure (see Section 2), is shown in Table 1. The effects of lipid composition and divalent cations can also be conveniently examined. Membrane binding, as measured by any of the techniques, is not significantly influenced by the presence or absence of cations, or by changes in lipid composition (zwitterionic or neutral lipids were used in all cases). Even if small differences in binding are found, they cannot explain the widely differing membrane lytic activities, and are rather attributable to variations between protein batches. It can be concluded from the results in Table 1 that α-haemolysin binds zwitterionic lipid bilayers to an extent that is virtually unrelated to lipid composition or to the presence or absence of divalent cations. More significant, membrane binding, as detected by the above techniques, does not necessarily

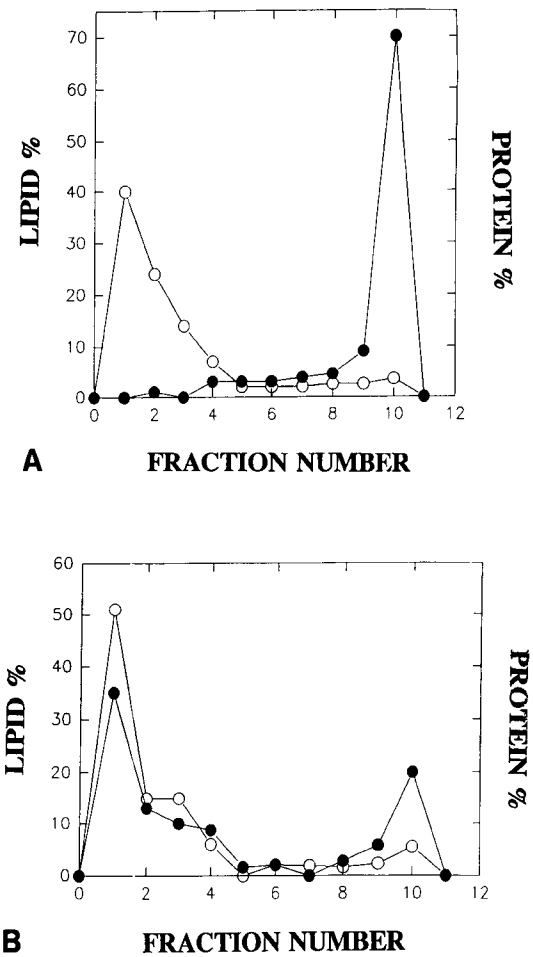

Fig. 1. Direct measurements of α-haemolysin binding to egg PC phospholipid vesicles (LUV). Protein and vesicles are incubated together, then centrifuged in D_2O buffer (as indicated in Section 2). Fractions (100 μl) are obtained starting from the top of the tube, and assayed for lipid (○) or protein (●). In (A) two control experiments are shown, in which only vesicles or only protein were run. (B) depicts a representative result, starting with 1 μmol of lipid and 50 μg protein (2173:1 molar ratio), in the presence of 1 mM Ca^{2+}.

Table 1
α-Haemolysin binding to liposomal membranes and α-haemolysin-induced leakage of large unilamellar liposomes. Effects of lipid composition and of divalent cations[a]

Lipid composition	Divalent cations	% Bound protein[b]	$n \cdot K_d$[c]	% Leakage[d]
PC	None[e]	35.2 ± 8.13	2.5×10^{-5}	0
PC	10 mM Ca^{2+}	31 2 ± 1 06	2.0×10^{-5}	32.0 ± 7.5
PC	10 mM Zn^{2+}	40 7 ± 9.25	1.5×10^{-5}	0
PC/PE/CHOL (2:1:1)[f]	10 mM Ca^{2+}	25.3 ± 5.65	1.8×10^{-5}	75.5 ± 10.5
PC/PE/CHOL (2:1:1)[f]	10 mM Zn^{2+}	34.0 ± 4.24	n.m.[g]	0
PC/CHOL (3:1)[f]	10 mM Ca^{2+}	25.0 ± 4.2	n.m.[g]	64 3 ± 7.85
PC/PE (2:1)[f]	10 mM Ca^{2+}	27.0 ± 1.41	n.m.[g]	66.2 ± 5.75

[a]Average values of three measurements ± S.E M
[b]Data from experiments as shown in Fig. 1B. Initial lipid:protein molar ratio: 428.
[c]Data from experiments as shown in Fig. 3. Initial lipid:protein molar ratio: 660
[d]Percent leakage after 10 min. Initial lipid:protein molar ratio: 537. See [5].
[e]Measurements in the presence of 1 mM EGTA.
[f]Molar ratios.
[g]Not measured.

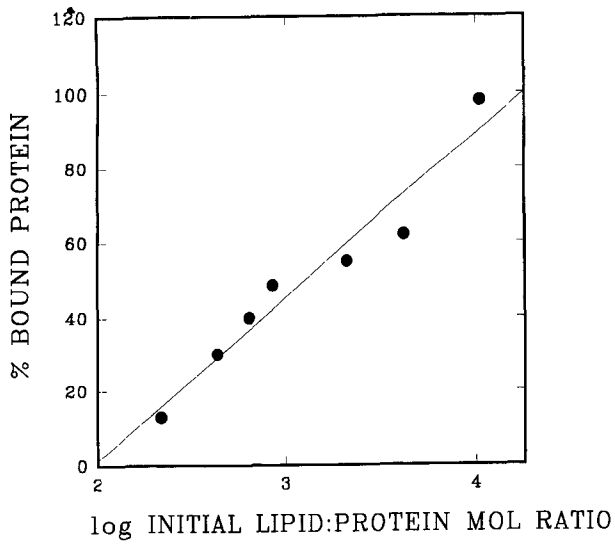

Fig. 2. The effect of varying initial lipid:protein ratios in the binding of α-haemolysin to egg PC LUV.

lead to membrane lysis, e.g. the toxin binds equally well the lipid bilayer in the presence or absence of Ca^{2+} (Table 1) although it is known that only in the former case will binding be followed by membrane destabilization.

Boehm et al. [17] described that, incubating red blood cells with α-haemolysin in the absence of Ca^{2+}, no lysis was observed; in order to test whether the protein had bound the cell membrane in the absence of Ca^{2+}, the cells were washed and resuspended in a Ca^{2+}-containing buffer. No lysis was observed either, and it was concluded that Ca^{2+} was required for the toxin to bind the membrane (17). However, an alternative hypothesis would be that Ca^{2+} binding to α-haemolysin must occur *prior to* toxin interaction with membranes for the lytic effect to occur. This was tested as follows: α-haemolysin, in a Ca^{2+}-free buffer, was preincubated (15 min) with LUV loaded with ANTS/DPX, then an excess (10-fold) of 'empty' (i.e. probe-fee) liposomes was added, and the mixture was allowed to equilibrate for a further 15 min. 'Empty' vesicles are added in order to remove any unbound protein from the aqueous medium [5]. Finally, the suspension is made 10 mM in $CaCl_2$. Virtually no release of aqueous contents is observed under these conditions (Fig. 4). As a positive control, the same amount of protein, now preincubated with 10 mM $CaCl_2$, is added to the above mixture (AH*). Release of vesicles loaded with ANTS/DPX starts inmmediately.

4. Discussion

The data on α-haemolysin interaction with LUV (Figs. 1–3, Table 1) demonstrate that the protein binds lipid bilayers irrespective of the presence or absence of divalent cations. This may explain some of the data in the literature. Oropeza-Wekerle et al. [18] observed, using gold immunolabeling, that intracellular Hly C-activated α-haemolysin was found preferentially bound to the bacterial inner membrane; considering that intracellular free Ca^{2+} concentration may be as low as 1 μM [19], it appears that the protein in this case binds the membrane without Ca^{2+} ions. Also relevant is the observation that leukotoxin, which requires Ca^{2+} for cell lysis, binds BL-3 cells in the presence or EGTA [20]. The data by Eberspacher et al. [21] on the binding of *E. coli* chromosomal α-haemolysin to erythrocytes or PC vesicles in the absence of added calcium are also in agreement with our measurements.

The experimental evidence in this and the previous [6] paper shows that Ca^{2+} ions are required for α-haemolysin-mediated cell lysis, but nor for α-haemolysin binding to membranes. In addition, the experiment in Fig. 4 indicates that Ca^{2+} must be added to the protein before it binds the lipid bilayer, in order to obtain lysis. This is in agreement with early data of Rennie et al. [22], who showed that, when α-haemolysin is incubated with Ca^{2+} for 2 min, and then mixed with erythrocytes, further addition of EDTA does not inhibit haemolysis.

In conclusion, the sequence of events leading to membrane disruption by α-haemolysin appears to be as follows. First, the toxin binds Ca^{2+} [6]; this would induce a (hypothetical) conformational change. Then, the Ca^{2+}-containing protein binds the lipid bilayer. The protein may now induce membrane lysis, perhaps after oligomerisation [5]. The toxin, in the absence of the divalent cation, may also bind the lipid bilayer, but then the lytic event does not take place, and the bilayer remains stable.

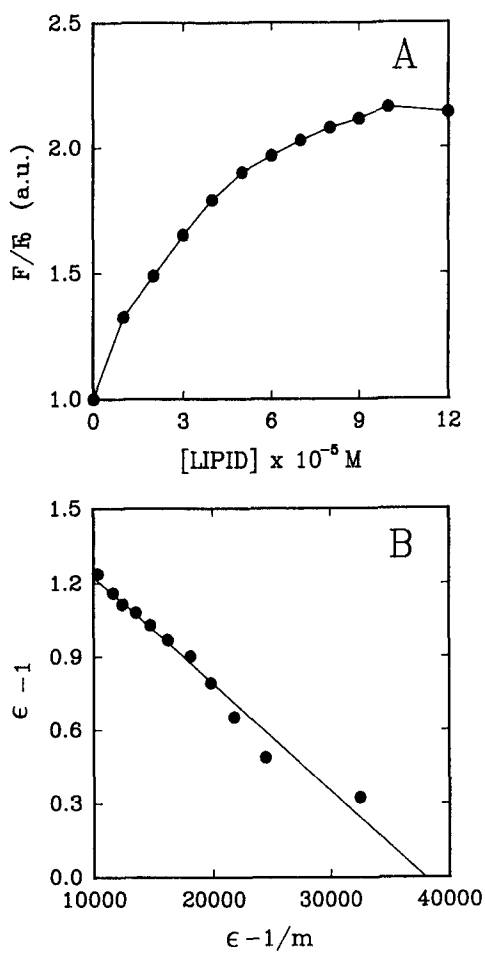

Fig. 3. α-Haemolysin binding to lipid bilayers, as seen through changes in protein intrinsic fluorescence. (A) Lipid (PC LUV) titration of α-haemolysin intrinsic fluorescence. Fluorescence intensity was measured at the maximum emission wavelength in each case [13]. (B) Plot of $(\varepsilon-1)$ vs. $(\varepsilon-1)/m$, derived from the titration curve in (A). $\varepsilon \equiv F/F_0$, and m is lipid concentration (see Section 2) This experiment was conducted in the presence of 1 mM EGTA.

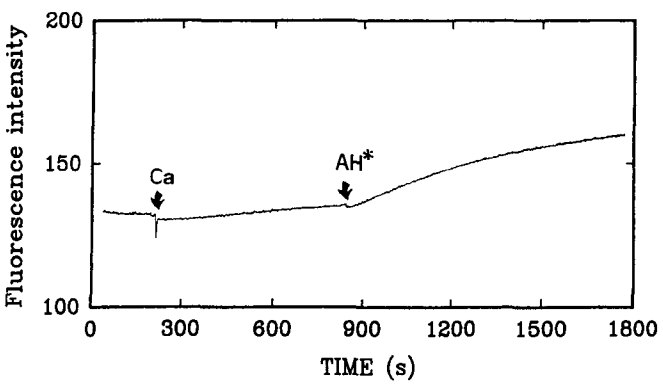

Fig. 4. α-Haemolysin requirements of calcium prior to protein binding to membranes. The protein was incubated for 15 min with PC LUV loaded with ANTS/DPX, in the absence of calcium, then 10 mM $CaCl_2$ was added (arrow, Ca). After sometime, the same amount of protein, this time pre-incubated with 10 mM $CaCl_2$, was added (arrow, AH*).

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