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Calcium-dependent conformation of *E. coli* α-haemolysin. Implications for the mechanism of membrane insertion and lysis

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Abstract

Previous studies from this laboratory had shown that calcium ions were essential for the membrane lytic activity of *E. coli* α-haemolysin (HlyA), while zinc ions did not sustain such a lytic activity. The present data indicate that calcium-binding does not lead to major changes in the secondary structure, judging from circular dichroism spectra. However binding to Ca^{2+} exposes new hydrophobic residues at the protein surface, as indicated by the increased binding of the fluorescent probe aniline naphtholsulphonate (ANS), and by the increased tendency of the Ca^{2+}-bound protein to self-aggregate. In addition zinc ions are seen to decrease the thermal stability of HlyA which, according to intrinsic fluorescence and differential scanning calorimetry data, is stable below 95°C when bound to calcium, while it undergoes irreversible denaturation above 60°C in the zinc-bound form. Binding to phosphatidylcholine bilayers is quantitatively similar in the presence of both cations, but about one-third of the zinc-bound HlyA is released in the presence of 2 M NaCl. Differential scanning calorimetry of dimyristoylglycerophosphocholine large unilamellar vesicles reveals that Zn^{2+}–HlyA interaction with the lipid bilayer has a strong polar component, while Ca^{2+}–HlyA appears to interact mainly through hydrophobic forces. Experiments in which HlyA transfer is measured from phospholipid vesicles to red blood cells demonstrate that Ca^{2+} ions promote the irreversible binding of the toxin to bilayers. All these data can be interpreted in terms of a specific Ca^{2+} effect that increases the surface hydrophobicity of the protein, thus facilitating its irreversible bilayer insertion in the fashion of intrinsic membrane proteins. © 1998 Elsevier Science B.V.

Keywords: *E. coli* α-haemolysin; Calcium-binding protein; Membrane protein insertion; Protein toxin

1. Introduction

α-Haemolysin (HlyA), a 107 kDa extracellular protein toxin secreted by some pathogenic strains of *E. coli*, is able to bind eukaryotic plasma membranes and cause impairment of cell function and, eventually, cell lysis [1–3]. HlyA belongs to the so-called RTX protein family, characterized by a glycine-rich nonapeptide repeat region near the C-terminal end

Abbreviations: ANS, aniline naphtholsulphonate; ΔH, enthalpy change of the main gel–fluid transition; HlyA, *E. coli* α-haemolysin; T_m, gel–fluid transition temperature; MLV, multilamellar vesicles

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[4–6]. In addition to this relatively polar domain, that contains a large number of Ca^{2+}-binding sites, the protein has a hydrophobic region near the N-end [5] and two fatty acyl residues linked to internal lysines [7], so that HlyA contains the structural elements to make up an amphiphilic molecule.

In spite of contradictory results in the literature, previous studies from this laboratory were able to establish that Ca^{2+} (or, at higher concentrations, Sr^{2+} or Ba^{2+}) was essential for HlyA to display its lytic activity, while other divalent cations (e.g. Mg^{2+}, Mn^{2+}, Zn^{2+}) appeared to be inactive in this respect [8]. These results have received independent confirmation recently [9]. Our studies also showed that Ca^{2+} binding produces characteristic changes in the HlyA fluorescence emission spectrum [8], and that Ca^{2+} binding to the toxin must occur prior to protein binding to the bilayer for a lytic effect to take place [10]. It has also been found that fatty acyl binding to the protein during the so-called 'activation step' [7] may bring about an important conformational change in the protein, involving the Ca^{2+}-binding domain [11].

In summary, binding of calcium ions to HlyA has important functional consequences. What remains to be known, and is the object of the present paper, is the nature of the putative conformational changes brought about by Ca^{2+}, and their relationship to the functional ability (i.e. cell lysis) of HlyA. A variety of biophysical techniques (circular dichroism, quasi-elastic light scattering, fluorescence spectroscopy) are applied to this aim in our study. Also related to the present data is the recently published experimental methodology that enables us to distinguish between reversible adsorption and irreversible insertion of HlyA into lipid bilayers [12]. The main conclusion from the present study is that Ca^{2+} induces a change in HlyA conformation, that includes an increased surface hydrophobicity, in turn leading to the irreversible insertion of the toxin in the membrane.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine was grade I from Lipid Products (South Nutfield, England). Metallic salts ($CaCl_2$ and $ZnCl_2$) were of analytical grade. Plasmid encoded haemolysin was purified in the absence of added calcium from the culture filtrates of an over-producing strain of *E. coli* according to [13]. The protein was stored in 150 mM NaCl, 6 M urea, 20 mM Tris–HCl pH 7.0 at -20°C; under these conditions it was stable for months.

2.2. Circular dichroism

Far-UV (195–250 nm) CD spectra were measured with a Jasco-720 spectropolarimeter. Spectra were recorded from each protein and buffer sample. The data were recorded every 0.5 nm and averaged over five runs, using a 0.5 mm quartz cell with HlyA at 80 μg/ml. The normalized spectra were independent of the protein concentration. Quantitation of α-helix contents in the samples was carried out from the normalized spectra according to the method of Deléage and Geourjon [14].

2.3. Differential scanning calorimetry

The effect of HlyA on the main gel–fluid phase transition of dimyristoylglycerophosphocholine was measured using lipid–protein samples prepared as follows. Dimyristoylglycerophosphocholine large unilamellar vesicles were prepared by extrusion and sized using 0.1 μm pore size Nuclepore membranes as described by Mayer et al. [15]; the buffer was 20 mM Tris–HCl, 150 mM NaCl, pH 7.0, ±EGTA, Ca^{2+}, or Zn^{2+} as required. Dimyristoylglycerophosphocholine vesicles (final concentration 0.6 mM) were incubated with HlyA (120 μg/ml) at 37°C for at least 1 h.

HlyA samples to measure protein thermal denaturation were prepared at a final concentration of 75 μg/ml in the presence of EGTA, Ca^{2+}, or Zn^{2+}. Higher concentrations provoked protein aggregation upon heating.

Samples or buffer were loaded into the sample or reference cell, respectively, of an MC-2 high sensitivity scanning calorimeter (Microcal, Northampton, MA). All samples were upscanned at least twice at a scan rate of 1°C per minute. Transition temperature (T_m), enthalpy changes (ΔH) and width at half-height were determined using the software ORIGIN (Microcal, Northampton, MA) provided with the calorimeter.

2.4. Measurements of protein binding to liposomes

The amount of liposome-bound toxin was measured by the centrifugation method [12]. Multilamellar liposomes (1 mM) were incubated with 25 μg of HlyA for 30 min at room temperature in 1 ml of 20 mM Tris–HCl, pH 7.0 buffer containing 10 mM Ca^{2+} or 10 mM Zn^{2+}, or 1 mM EGTA, as required. The mixture was then centrifuged in an Eppendorf centrifuge (14 000 rpm, 10 min). The pellet was resuspended in 200 μl of 20 mM Tris–HCl pH 7.0 or 2 M NaCl and assayed for lipid P and protein, the latter after addition of octylglucoside up to 1%. Control experiments demonstrated that, under the above centrifugation conditions, liposome-free protein did not sediment.

2.5. Measurements of membrane bound toxin transfer to erythrocytes

The method is essentially the one described by Tomita et al. [16], as applied by us to HlyA [12]. Horse red blood cells were used as indicators in assessing the haemolytic activity of HlyA bound to multilamellar vesicles (MLV). Briefly, toxin and MLV were incubated for 30 min at 37°C unless otherwise stated, and the liposome–protein complexes were recovered and washed by centrifugation (Eppendorf, 14 000 rpm, 10 min). Aliquots of the resuspended pellet were tested for haemolytic activity. Only HlyA that was reversibly bound to the lipid bilayer can produce haemolysis under these conditions.

2.6. Haemolysis assays

Haemolysis was tested quantitatively on microtitration plates containing horse red blood cells and successive two-fold dilutions of the free or liposome-bound toxin [12,13]. After 30 min at 37°C the absorbance value at 412 nm was measured in the supernatant.

2.7. Measurements of particle size distribution

The average size of protein aggregates was measured by quasi-elastic light scattering using a Malvern Zeta-sizer instrument. The protein concentration in these measurements was 50 μg/ml.

2.8. Measurements of intrinsic fluorescence of haemolysin

Protein solutions (0.15–0.30 μM) were placed in a thermostatted cuvette under continuous stirring. After equilibrating for 5 min at each temperature, fluorescence intensity was read. Fluorescence intensity was measured at 295 nm excitation (slit 5 nm) and at the maximum emission wavelength (slit 5 nm). Fluorescence intensity measurements were corrected for light scattering.

2.9. Sensitivity of HlyA to trypsin

25 μg HlyA were incubated in 250 μl 20 mM Tris–HCl, pH 7.0 containing either 10 mM $CaCl_2$ or 10 mM $ZnCl_2$ or 1 mM EGTA, as required, after which 5 μl of 1 mg/ml trypsin (in 20 mM Tris–HCl, pH 7.0) was added to the solution. The mixtures were incubated at room temperature and phenylmethanesulphonyl fluoride was added to stop proteolysis. Preliminary experiments failed to reveal any significant dependence of trypsin activity on the divalent cations.

2.10. Gel electrophoresis

Sample diluter (125 mM Tris–HCl pH 6.8, 4% 2-mercaptoethanol, 20% glycerol, 0.005% bromphenol blue) was added to the protein solutions. The samples were electrophoresed on 12% acrylamide gels in the presence of SDS according to Laemmli [17]. Proteins were detected by Coomassie staining.

3. Results

3.1. Studies on HlyA in solution

α-Haemolysin has the property of self-aggregation, perhaps because of its amphipathic nature, and occurs indeed in the form of large and polydisperse multimolecular aggregates [13]. When the average size of these aggregates is measured by quasi-elastic light scattering, it is seen to be largely influenced by the presence of metal ions in the solution, the average diameter of the particles in the presence of Ca^{2+} being larger by about three-fold than in the presence

Table 1
The average size of α-haemolysin aggregates, measured by quasi-elastic light scattering, as a function of the presence of divalent cations in the solution. All samples in 20 mM Tris–HCl, pH 7.0. Protein concentration, 50 μg/ml

Addition	Aggregate size (Z average, nm)	S.D. (nm)	Polydispersity[a]
1 mM EGTA	107	48	0.507
10 mM $CaCl_2$	550	86	0.709
10 mM $ZnCl_2$	190	25	0.445

[a]Polydispersity gives an idea of the size heterogeneity. It may vary from 0 (monodisperse system) to 1 (fully heterogeneous).

of Zn^{2+}, or in the absence of divalent cations (Table 1). Since the main force driving the aggregation of proteins in aqueous media is the tendency of hydrophobic surface patches to come together to avoid unfavourable hydrocarbon–water contacts, the increase in average aggregate size may be indicative of a Ca^{2+}-induced conformational change leading to an increased exposure of hydrophobic patches at the protein surface. In order to test this hypothesis, HlyA in the presence and absence of Ca^{2+} was examined by circular dichroism and fluorescence spectroscopy (ANS binding).

The circular dichroism spectra of HlyA (80 μg/ml) in the presence and absence of Ca^{2+} are shown in Fig. 1. They are both very similar, with percentages of α-helix of 36% for both the Ca^{2+} and EGTA samples. This suggests that the conformational change brought up by Ca^{2+} involves mainly changes in the protein tertiary structure.

ANS binding to proteins has often been used as a semi-quantitative method for detecting hydrophobic binding sites in proteins [18]. When the protein (in EGTA) is incubated with ANS, addition of Ca^{2+} leads to an immediate increase in ANS fluorescent emission, suggesting an increase in the number of ANS binding sites (Fig. 2). When the order of reagent addition is inverted, i.e. Ca^{2+} is first added and then ANS, no such an increase in ANS fluorescence is observed, probably because the newly exposed hydrophobic patches that have surfaced in the presence of Ca^{2+} have been immediately made inaccessible by protein aggregation (see Table 1).

As a further test of the hypothesis of a calcium-driven conformational change of HlyA, the protein was subjected to the action of trypsin after having been incubated with either Ca^{2+}, Zn^{2+} or EGTA. The peptide patterns obtained by electrophoresis of partially digested proteins can often reveal changes in structure that make more or less accessible to the protease the protein target peptides. The tryptic patterns of HlyA in the presence of Ca^{2+} and Zn^{2+} are shown in Fig. 3. They are seen to differ greatly. The

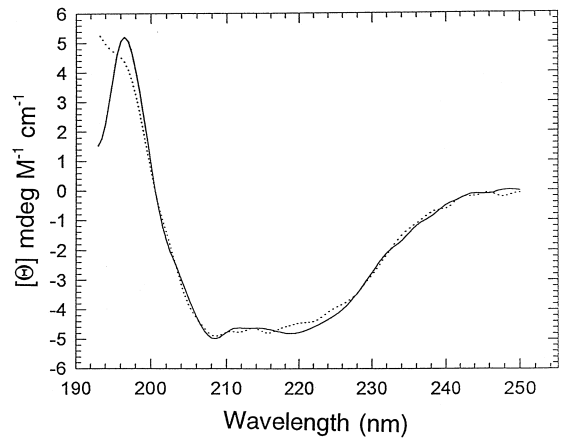

Fig. 1. Circular dichroism spectra of α-haemolysin in the presence (---) or absence (————) of 10 mM Ca^{2+}. Protein concentration: 80 μg/ml.

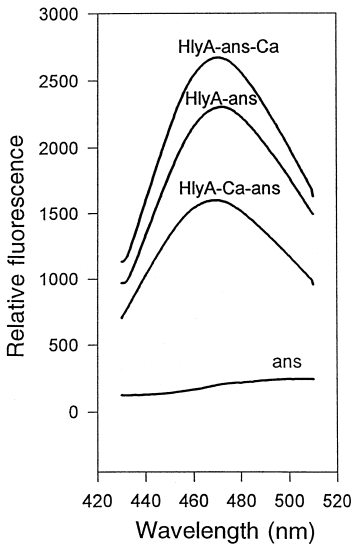

Fig. 2. ANS fluorescence emission in the absence and presence of HlyA. Protein concentration was 50 μg/ml. ANS concentration was 25 μM. $\lambda^{ex} = 295$ nm. The various reagents were mixed in the order given by each spectrum.

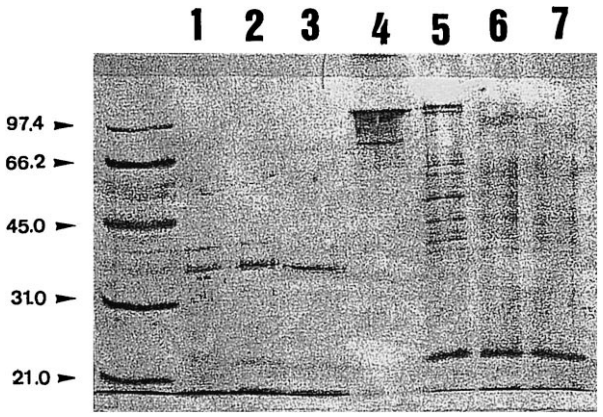

Fig. 3. Effect of divalent cations on HlyA proteolysis by trypsin. Far left, unlabeled lane, molecular weight markers. Lanes 1–3, HlyA preincubated with 10 mM Ca^{2+}, digested with trypsin for 15, 30 and 60 min respectively, Lane 4, control HlyA. Lanes 5–7, HlyA preincubated with 10 mM Zn^{2+}, digested with trypsin for 15, 30 and 60 min, respectively.

corresponding pattern for HlyA in EGTA (not shown) is very similar to the one obtained in the presence of Zn^{2+}. Once again the experimental data suggest that Ca^{2+} ions are inducing in HlyA a conformational state that is different from those obtained in the presence of Zn^{2+}, or in the absence of divalent cations.

Minor structural changes occurring in proteins at room temperature can sometimes be more fully appreciated by subjecting the protein to a thermal stress [19]. For that purpose the intrinsic fluorescence of HlyA, in the presence of either EGTA, $CaCl_2$ or $ZnCl_2$, was recorded in the 20–70°C interval. In general, fluorescence intensity of any fluorophore will decrease with temperature, because collisional relaxation is favoured. A decrease in intrinsic fluorescence is indeed observed for all three protein preparations (Fig. 4). Below 50°C the Ca^{2+}-containing sample shows a much steeper decrease in fluorescence intensity than the other two, which are, at this stage, undistinguishable. Above 50°C, the Zn^{2+}-treated protein presents a discontinuity in the ''fluorescence versus temperature'' plot, and above 60°C the signals of both divalent cation containing samples are similar, and clearly below the fluorescence level of the protein in the presence of EGTA. A discontinuity, or ''break'', as shown by the zinc-treated sample is most frequently interpreted in terms of a thermally-induced structural transition. This was confirmed by subjecting the same samples to a calorimetric study.

Differential scanning calorimetry is an ideal technique for detecting thermotropic transitions in biomolecules. The result of examining the three protein preparations by this technique is shown in Fig. 5. In this case the technique allows us to explore the temperature range up to about 100°C. Neither the Ca^{2+} nor the EGTA-treated samples gave rise to any thermotropic transitions, in agreement with the fluorescence data in Fig. 4. However, also as predicted from the fluorescence observations, the Zn^{2+}-containing sample does show a wide endotherm, indicative of a structural transition, between ≈65°C and 75°C, followed by large changes of opposite sign probably attributed to aggregation of the thermally denatured peptide. The transition starting at 65°C is no longer detected in the second heating scan (Fig. 5), confirming that it corresponds to a phenomenon of irreversible thermal denaturation. The difference in the onset temperatures of this transition (50°C by fluorescence, 65°C by calorimetry) is probably due to the different heating rates, slower in the case of fluorescence. This is an indication of a kinetically-controlled phenomenon, very common in protein denaturation

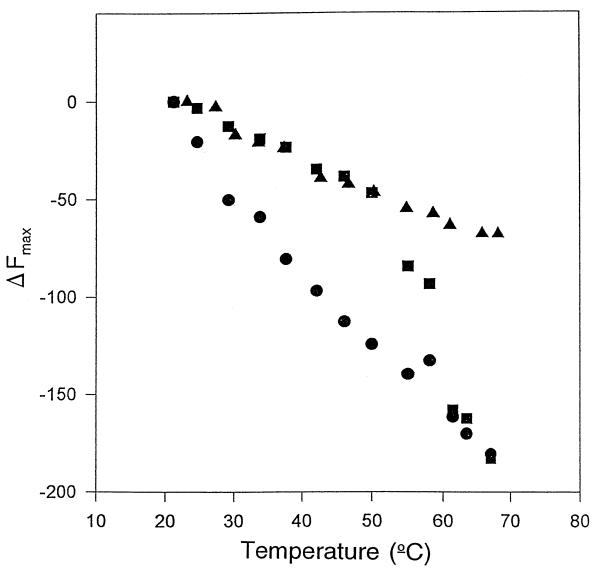

Fig. 4. Temperature dependence of the Trp intrinsic fluorescence of HlyA. Protein concentration: 10 μg/ml. Buffer was 20 mM Tris, pH 7.8 plus (▲) 1 mM EGTA, (■) 10 mM $ZnCl_2$, or (●) 10 mM $CaCl_2$.

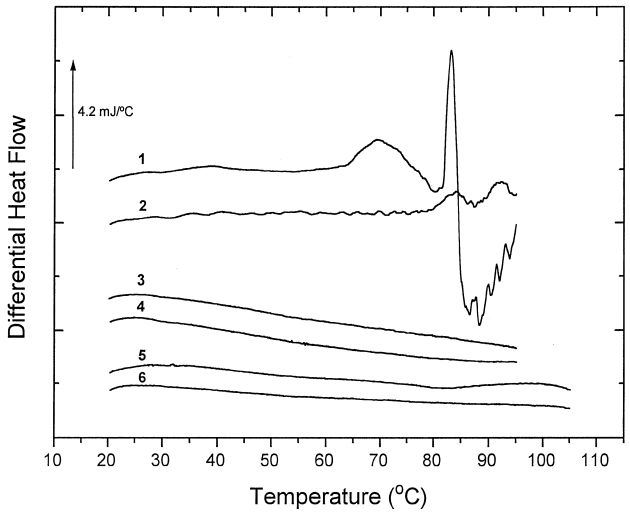

Fig. 5. Differential scanning calorimetry thermograms of HlyA in the presence or absence of divalent cations. Protein concentration was. 75 μg/ml. 1, HlyA in the presence of Zn^{2+}; 2, rescan; 3, HlyA in the presence of Ca^{2+}; 4, rescan; 5, HlyA in the presence of EGTA; 6, rescan.

processes [20]. Thus the calorimetric studies show a clear difference between the Zn^{2+}-containing protein preparation and the other two, more resistant to the thermal challenge. In turn, the fluorescence thermal data were able to discriminate between the Ca^{2+} and the EGTA samples, the fluorescence of the former decreasing more rapidly with temperature.

In summary our studies with HlyA in solution, in the absence of membranes, may be interpreted as indicating that binding of Ca^{2+} ions induces a conformational change in the protein, affecting mainly the tertiary structure, and leading to the appearance of additional hydrophobic patches on the protein surface.

3.2. Studies in the presence of membranous systems

Binding is the earliest, and conceptually simpler, step of HlyA interaction with a membrane. We shall initially ignore the difference between binding and insertion [12], considering only total binding as measured by the ''centrifugation method'' [12]. Table 2 shows the binding of HlyA to multilamellar vesicles consisting of egg phosphatidylcholine. As expected from previous studies [10] there are no significant differences in the total binding of HlyA to liposomes, irrespective of the presence or absence of divalent cations. However, when the liposomes containing bound HlyA are washed with 2 M NaCl, only the protein that had been preincubated with Ca^{2+} ions stays virtually unaffected by the high ionic strength solution. Both hydrophobic and electrostatic forces are involved in the interaction of HlyA with bilayers [21], but in the presence of Ca^{2+} hydrophobic forces appear to be more important, in agreement with the results in the above section.

Bakás et al. [12] were able to show experimentally the difference between reversible adsorption and non-reversible insertion of HlyA in lipid bilayers: both concepts would be encompassed by the ''binding'' parameter, as measured by the centrifugation method (Table 2). The method of HlyA transfer from liposomes to red blood cells [12,16] is a useful one in order to assess the proportion of bound protein that is irreversibly inserted in the bilayer. The corresponding measurements have been made for liposomes containing bound α-haemolysin in the presence of either EGTA, Ca^{2+} or Zn^{2+}. In this experiment liposomes are incubated with HlyA in the presence of 1 mM EGTA, 1 mM Zn^{2+} or 1 mM Ca^{2+}; after washing, they are incubated with red blood cells in the presence of 10 mM Ca^{2+} in order to induce maximum haemolytic activity. The results are shown in Fig. 6. In the presence of Ca^{2+} the protein binds irreversibly the liposomal membrane, as shown by the fact that even the largest amounts of protein that could be tested failed to produce more than 30% haemolysis, while ten times less protein, bound to the liposomes in the absence of metal ions, produced 80% haemoly-

Table 2
Binding of α-haemolysin to multilamellar phospholipid vesicles, and its partial removal by high ionic strength solutions. All samples in 20 mM Tris–HCl, pH 7.0. Binding measured by the centrifugation method [12] (see Section 2). Average of three independent measurements

Addition	% Bound protein (± S.E.M.)	
	After washing with buffer	After washing with 2 M NaCl
1 mM EGTA	25.1 ± 1.28	10.6 ± 1.40
10 mM $CaCl_2$	28.7 ± 2.30	28.5 ± 2.07
10 mM $ZnCl_2$	30.9 ± 1.82	20.0 ± 1.07

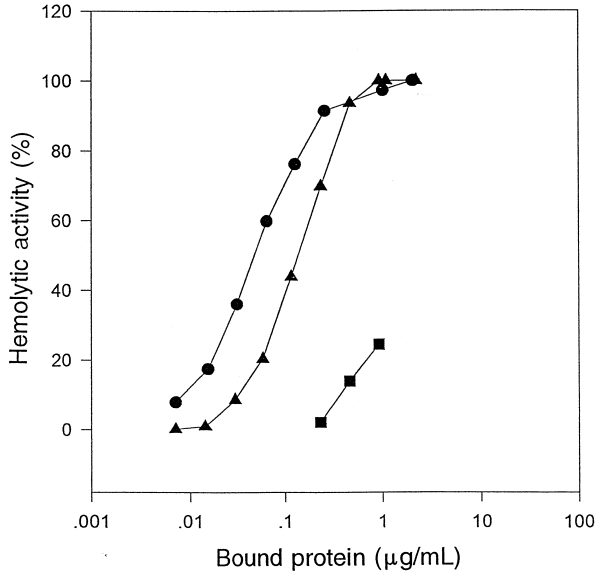

Fig. 6. Accessibility of liposome-bound HlyA to red blood cells. See Section 2 for details. (▲) HlyA/EGTA, (●) HlyA/Zn^{2+}, (■) HlyA/Ca^{2+}.

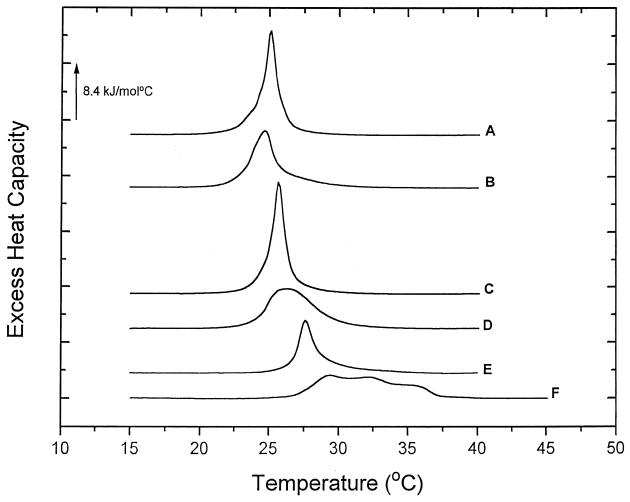

Fig. 7. Differential scanning calorimetry thermograms of dimyristoylphosphatidylcholine vesicles (A, C, E) and mixtures of this lipid with HlyA (B, D, F), in the presence or absence of divalent cations. (A, B), EGTA; (C, D), Ca^{2+}, (E, F), Zn^{2+}. The second or third heating scan is shown for each sample. Each sample contained about the same amount of phospholipid, and was ≈ 1200:1 lipid:protein ratio.

sis. Protein bound in the presence of Zn^{2+} acted very much like the sample with EGTA. The irreversible binding of HlyA in the presence of Ca^{2+} is in agreement with the hypothesis of hydrophobic patches exposed on the surface of the protein upon its binding to Ca^{2+}, and also with its resistance to washing by high-salt solutions.

Finally, the role of metal ions in the binding of HlyA to bilayers was examined through the effect of the bound protein on the gel-to-liquid crystalline transition of dimyristoylglycerophosphocholine. For that purpose, liposomes (large unilamellar vesicles obtained by extrusion) consisting of that phospholipid were prepared in the appropriate buffer, with or without divalent cations, and incubated with HlyA. The samples were prepared in such a way that, after washing, the lipid:protein ratio was ca. 1200:1 in all cases. The corresponding thermograms are shown in Fig. 7. In the presence of EGTA the protein has little effect on the phospholipid gel–fluid transition, suggesting a low degree of insertion. In the presence of Ca^{2+}, the main effect of the protein is to widen the transition endotherm, without changing the transition midpoint (these parameters are collected in Table 3). ΔH does not change very much at this relatively high lipid:protein ratio. This is the typical behaviour of intrinsic membrane proteins, i.e. those interacting with the hydrophobic matrix of the bilayer [22]. Studies carried out in the presence of Ca^{2+} for lipid:protein ratios ranging between ca. 2500:1 and 500:1 further confirm the insertion of HlyA in the way of intrinsic proteins (M.P.V., unpublished results). In the presence of Zn^{2+}, however, the phospholipid transition is shifted to higher temperatures, and the area under the curve, corresponding to the

Table 3
The effect of HlyA on the gel-to-liquid crystalline thermotropic transition of dimyristoylglycerophosphocholine bilayers, and the influence of divalent cations. Lipid:protein mole ratio was of ca. 1200:1 in all cases. Width is measured as full-width at half-height

Sample	T_m (°C)	Width (°C)	ΔH (kJ/mol)
Lipid/EGTA	25.1	1.1	26
Lipid:HlyA/EGTA	24.7	1.8	25
Lipid/Ca^{2+}	25.7	1.0	25
Lipid:HlyA/Ca^{2+}	26.0	3.9	24
Lipid/Zn^{2+}	27.7	1.6	18
Lipid:HlyA/Zn^{2+}	32.2	7.9	24

Average values of 2–3 measurements are given. Temperatures and widths are repetitive to ±0.1°C. The errors in ΔH are estimated at about 5% of the respective values.

transition enthalpy change, is increased with respect to that of the pure lipid (Table 3). All this is characteristic of extrinsic proteins, i.e. those interacting mainly with the phospholipid polar headgroups [22]. Thus the calorimetric data suggest that, in the presence of Ca^{2+}, the interaction of HlyA with membranes is hydrophobic in nature, while the zinc-treated protein interacts mainly through electrostatic and/or polar forces.

4. Discussion

Calcium is an essential requirement for α-haemolysin activity [8–10]. The results in this paper shed some light on the basis for such a requirement. In summary, the circular dichroism data (Fig. 1) do not suggest any significant change in secondary structure after addition of Ca^{2+}, while the thermal effects on HlyA intrinsic fluorescence (Fig. 4) and the pattern of tryptic peptides (Fig. 3) speak of a change in structure. More specifically, changes in the size of aggregates (Table 1) and in ANS fluorescence (Fig. 2) are suggestive of an increased hydrophobic area on the protein surface. Calcium ions would then induce a conformational change, based mainly on changes in the tertiary structure, that would result in surfacing hydrophobic portions of the protein. The increased hydrophobic surface would undoubtedly favour either membrane binding (through hydrophobic forces) or self-aggregation.

α-Haemolysin is a member of the so-called RTX family, characterized by a domain in which a glycine-rich nonapeptide containing one aspartic acid is repeated several times (15 in the case of HlyA) [4–6,8,9]. This kind of structure was found to constitute a novel calcium-binding entity [6]. According to our measurements [8] about three exchangeable calcium ions per protein molecule must be bound for optimal activity. Very similar calcium requirements and calcium binding data have been found for another member of the RTX family, the adenylate cyclase toxin from *Bordetella pertussis* [23,24]. In this case, a calcium-dependent conformational change has been detected by a variety of techniques, that appears to imply modifications in the tertiary structure [23,24]. Similar changes may be required for the activation of HlyA by Ca^{2+}, and the case of the adenylate cyclase toxin shows that changes in the Ca^{2+}-binding domain may be transmitted to regions far away in the protein structure.

Once the superficial hydrophobic area is increased as a result of Ca^{2+}-binding, the next step in HlyA-dependent cell lysis is membrane insertion (in competition, or maybe in equilibrium, with self-aggregation). Our previous studies [10,12] have shown the difference between adsorption and insertion, and the requirement of a fluid (''liquid disordered'') bilayer for irreversible insertion. The data in this paper (Table 2, Fig. 6) demonstrate that Ca^{2+}-binding is an additional requirement for irreversible insertion. This kind of insertion appears to be essential for the lytic process to go on (through yet unknown steps). Thus the requirement of Ca^{2+} ions for HlyA-dependent haemolysis refers at least to the requirement of Ca^{2+} for a proper (irreversible) insertion into the membrane. Such an insertion, that converts the toxin into an intrinsic membrane protein (Fig. 7, Table 3), is undoubtedly favoured by the calcium-dependent change in tertiary structure leading to the exposure of hydrophobic patches. Various other proteins follow a similar pathway to membrane insertion. The pore-forming domain of colicin A suffers a ''collapse of the native tertiary structure'', although ''a large proportion of the helical secondary structure remains preserved'' [25,26]. In *Pseudomonas* exotoxin A, ''denaturation-like conformational changes appear to play an important role in membrane insertion'' [27]. Finally, in α-lactalbumin, that may be either absorbed or inserted in membranes [28,29], a flexible structural intermediate must form in solution for membrane insertion to occur. Note that in the last three examples, the conformational change is brought about by pH, or temperature, while in HlyA and in the adenylate cyclase toxin the process is triggered by Ca^{2+}. This might be a common pattern in other members of the RTX family.

The effects of the binding of Zn^{2+} are interesting. A rather large number of zinc ions, about 14, appear to bind [8]. As a consequence, a large conformational rearrangement may occur (though different from the one elicited by Ca^{2+}), so that HlyA binds the membranes as an extrinsic protein (Fig. 7) and, as such, it may be removed, at least partially, by high ionic strength solutions (Table 2). Finally, the zinc effects appear to be somehow reversible, since, in the pres-

ence of 10 mM Ca^{2+}, at least part of the protein that had been incubated with liposomes in the presence of 1 mM Zn^{2+} can move onto the red blood cell surface and produce cell lysis there (Fig. 6).

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