Asp-863 is a key residue for calcium-dependent activity of *Escherichia coli* RTX toxin α-haemolysin

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Abstract **α-Haemolysin is a protein toxin secreted by pathogenic strains of *Escherichia coli* and requires sub-millimolar Ca^{2+} for optimum lytic activity. As a member of the so-called RTX toxin family it contains a Gly-rich, Asp-rich Ca^{2+}-binding domain, consisting of a series of nonapeptides repeated in tandem. Asp-863 is located immediately after the last-but-one nonapeptide. A mutant in which Asp-863 has been substituted by Gly displays a requirement for Ca^{2+} that is 100-fold higher than the wild-type. Membrane lytic activity, as well as a conformational change revealed through an increase in intrinsic fluorescence, and the appearance of Ca^{2+}-bound protein monomers resolvable by fast protein liquid chromatography, are all three dependent on Ca^{2+} concentrations in the 2–20 mM range. Most RTX toxins have an Asp or Glu residue located at a position homologous to Asp-863, thus the key role of this residue for Ca^{2+} requirements of α-haemolysin may be a general feature of this family of toxins.**

Key words: α-Haemolysin; RTX toxin; Calcium-binding domain; Membrane lysis; Fluorescence spectroscopy; Phospholipid vesicle

1. Introduction

Escherichia coli α-haemolysin (HlyA) is a member of a family of Gram-negative bacterial toxins, the so-called RTX family, characterised by a number of nonapeptides repeated in tandem near the carboxy-terminus of the protein. RTX toxins are in turn members of the type I exoprotein secretion system present in a wide variety of Gram-negative bacteria (see [1–5] for reviews).

The nonapeptide consensus sequence is GGXGXDXUX, with U representing a large hydrophobic residue. The nonapeptide repeat domain is responsible for Ca^{2+}-binding, and Ca^{2+} is required for the lytic activity of HlyA [6–9]. The three-dimensional structure of the repeats domain of HlyA is still unknown, but a homologous domain of a non-toxin RTX protein, alkaline protease from *Pseudomonas aeruginosa*, has been solved to high resolution [10], and it can be reasonably assumed that the structure of the Ca^{2+}-binding C-terminal domain of HlyA follows a similar pattern. The core of the Ca^{2+}-binding structure consists of a parallel β-roll, built of a succession of nonapeptide motifs, each of them providing two half-sites for Ca^{2+}-binding, so that in the protease seven sequence motifs are found, contributing to the binding sites of five Ca^{2+}, two or three additional Ca^{2+} sites being found in other regions of this domain. As noted by Welch [5], a critical observation is that the repeats with exact matches to the consensus sequence form the highly regular, ordered central portion of the β-roll, whereas the repeats with approximate matches to the consensus give rise to frayed edges of the β-roll. Baumann et al. [10] observed that, at the C-terminal edge, two Ca^{2+} ions have one or two H_2O ligands in their octahedral structures, the two being easily exchangeable by Sr^{2+}.

HlyA contains 15 nonapeptide repeats in the Ca^{2+}-binding domain, thus a large number of Ca^{2+}-binding sites is to be expected. In fact in the presence of 3 M urea, when the protein is presumably in a partially unfolded conformational state, the binding of up to 12 Ca^{2+}/protein molecule has been measured [11]. However, in the absence of urea, only three high-affinity Ca^{2+}-binding sites were detected [9], corresponding probably to the easily exchangeable Ca^{2+} on α-haemolysin, i.e. the equivalents to the two exchangeable Ca^{2+} in the *P. aeruginosa* protease [10].

The C-end of the HlyA repeat domain has been the object of a recent study in this laboratory [12]. In the last-but-one nonapeptide a His (His-859) occupies the position occupied in all the other nonapeptides by either Asp or (occasionally) Asn. Substituting the anomalous His-859 by Asp had virtually no effect on the lytic activity of the protein, while a His → Asn substitution completely abolished the activity, probably because it shifted the protein monomer ↔ aggregate equilibrium towards the inactive, aggregated form [12]. In the present study our attention was drawn by the three consecutive Asp residues in positions 862–864. On the basis of the neutral H859D mutant obtained previously [12], a double mutant (DM HlyA) was obtained in which a D863G substitution occurred on top of the H859D replacement. This construction has the advantage of containing a mutation in the Asp-862–864 triplet while maintaining the same number of Asp residues, and the same net charge at neutral pH, than the wild-type (WT). The consequence of these mutations is that the modified protein has a much higher (two orders of magnitude) requirement for Ca^{2+}, i.e. that DM HlyA is fully active only at Ca^{2+} concentrations above 20 mM. The properties of DM HlyA at 50 mM Ca^{2+} parallel precisely those of the WT HlyA at 200 μM Ca^{2+}. Asp (or Glu) residues homologous to Asp-

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863 occur at the C-terminus of the repeat domain in several other RTX toxins, and may also regulate the Ca^{2+} requirement of those proteins.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) were obtained from Lipid Products (South Nutfield, UK). Horse erythrocytes were purchased from Microlab (Madrid, Spain). ANTS (8-aminonaphthalene-1,2,3-trisulphonic acid) and DPX (*p*-xylene bis(pyridinium bromine)) were from Molecular Probes (Eugene, OR, USA). Taq polymerase was supplied by Bioline (London, UK). The deoxyribonucleotides were from Boehringer Mannheim GmbH (Mannheim, Germany). The restriction enzymes *Nco*I and *Pac*I as well as the T4 DNA ligase were from New England Biolabs (Hertfordshire, UK). Oligonucleotides were synthesised by Amersham Pharmacia Biotech (Uppsala, Sweden).

2.2. Bacterial strains, growth conditions and plasmids

The *E. coli* D1210 strain was used throughout this work for DNA manipulation and HlyA expression. The recombinant plasmid pSU124, containing the *hly* genes [13], was used to introduce the mutations into the *hlyA* gene. *E. coli* (pSU124) was grown at 37°C in LB medium supplemented with 100 μg/ml of ampicillin in a shaking bath.

2.3. Preparation, expression and purification of WT and mutant HlyA

Site-directed mutants of HlyA at His-859 and Asp-863 were obtained by sequential PCR steps [14,15]. Mutations were confirmed by DNA sequencing. WT and H859DD863G were purified as described previously for the WT [16]. The purified proteins were stored at −20°C in 150 mM NaCl, 6 M urea, 20 mM Tris–HCl, pH 7.0.

2.4. Large unilamellar vesicles (LUV)

LUV made of PC:PE:Chol (2:1:1) were prepared by extrusion through polycarbonate filters (Nuclepore, Pleasanton, CA, USA), pore size 0.1 μm. Buffer was 150 mM NaCl, 20 mM Tris–HCl, pH 7.0. The diameter of the resulting vesicles was ca. 100 nm, according to quasi-elastic light scattering measurements. More details on the preparation of these vesicles can be found in Mayer et al. [17].

2.5. Release of liposomal contents

Leakage of vesicular aqueous contents was assayed according to Ellens et al. [18] with ANTS and DPX entrapped in the liposomes. LUV were prepared in 70 mM NaCl, 12.5 mM ANTS, 45 mM DPX, 20 mM Tris–HCl, pH 7.0. Non-entrapped probes were removed passing the LUV through a Sephadex G-75 column, eluted with 150 mM NaCl, 20 mM Tris–HCl, pH 7.0. Assays were performed at 100 μM lipid in a total volume of 1 ml, with continuous stirring, at 25°C. Buffer was as above, with the addition of $CaCl_2$ at the desired concentration. The assay was started by adding 100 nM calcium-free dialysed HlyA. ANTS fluorescence was recorded continuously ($\lambda_{ex} = 355$ nm; $\lambda_{em} = 520$ nm). When leakage reached equilibrium, Triton X-100 was added (final concentration 0.1% w/v) to induce 100% release. Percent release was computed as follows:

$$\%\ \text{release} = \frac{(F_f - F_0)}{(F_{100} - F_0)} \times 100$$

where F_f, F_{100}, and F_0 were respectively the fluorescence intensity values observed after addition of HlyA, after addition of Triton X-100, and before any addition.

2.6. Gel filtration by fast protein liquid chromatography (FPLC)

Gel filtration was performed according to Cortajarena et al. [12] on a Superdex HR-200 column using an FPLC system (Amersham Pharmacia, Uppsala, Sweden). The protein was diluted to 1.5 μM, left to equilibrate for 10 min, then filtered through a 0.45 μm pore size filter and injected into the column, that was equilibrated with 150 mM NaCl, 20 mM Tris–HCl (pH 7.0). Calcium was added, when required, to the protein and elution buffer, at the same concentration. In each case, 80 μg protein was injected in 0.5 ml. The column was eluted at 0.5 ml/min, and 1 ml fractions were collected.

2.7. Intrinsic fluorescence measurements

The fluorescence measurements were performed recording emission spectra in a SLM-Aminco 8.100 (Spectronic Instruments, Rochester, NY, USA) spectrofluorimeter fixing the excitation wavelength at 295 nm, to avoid the tyrosine contribution. The emission was recorded between 310 and 400 nm, with slits of 4 nm for both monochromators. The proteins used in the assay were previously dialysed for 12 h at 4°C against TCU (NaCl 150 mM, urea 6 M, Tris 20 mM, pH 7.0) buffer, with 0.1 mM EGTA (ethylene-bis(oxyethylenenitrilo)-tetraacetic acid) (Sigma, St. Louis, MO, USA) to remove cations in the samples, and then against TCU buffer alone for 6 h at 4°C in order to remove EGTA. Protein spectra were recorded in TC buffer (NaCl 150 mM, Tris 20 mM, pH 7.0) adding when necessary the corresponding $CaCl_2$ concentrations under continuous stirring. The spectra were recorded after binding equilibrium was reached. Dilution effect was corrected adding to another protein sample equal volumes of water and recording the corresponding spectra. The following equation was used to correct the values:

$$F_{pc} = F_p \times \frac{F_{d0}}{F_d}$$

where F_p and F_d are the fluorescence intensities of the protein sample and the protein used for dilution correction, respectively. F_{d0} is the initial signal of the sample used in the dilution correction. To follow the changes in fluorescence intensity produced by the addition of the cation, fluorescence intensity at the maximum wavelength was represented for each protein.

3. Results and discussion

3.1. Membrane lysis assays

The lytic activity of WT and DM HlyA was tested on LUV loaded with fluorescent probes at increasing Ca^{2+} concentrations [19,20]. The results are summarised in Fig. 1. DM HlyA requires ca. 100 times more Ca^{2+} than WT to induce a comparable solute efflux (Fig. 1B) so that, at 1 mM Ca^{2+} used in our HlyA assays, DM HlyA is totally inactive (Fig. 1A). Full activity of WT is found in the presence of about 200 μM Ca^{2+} [9], while DM HlyA is seen to require ca. 20 mM. When the haemolytic activities of WT and DM HlyA are assayed on horse erythrocytes, the same difference in Ca^{2+} requirement is found (data not shown). This underlines the importance of Asp-863 in Ca^{2+}-binding by HlyA and subsequent conformational changes leading to the breakdown of the bilayer permeability barrier. Note that DM HlyA contains the same number of Asp residues as WT in the C-terminus of the repeat domains, since it bears a His-859 Asp in addition to the Asp-863 Gly substitution. The His-859 Asp mutation alone has no effect on the affinity of HlyA for Ca^{2+} [12].

When WT, H859D and DM HlyA are assayed in the presence of 50 mM Ca^{2+}, a calcium concentration that permits full activity of DM HlyA (Fig. 1), the lytic activities of all three proteins are found to be superimposable. This is shown in Fig. 2 for the release of liposomal contents induced by WT and mutant HlyA in the 10–275 nM concentration range. Thus an increased requirement of Ca^{2+} appears to be the only effect of the double mutation with regard to the membrane lytic properties of HlyA.

3.2. Calcium-induced fluorescence changes in HlyA

Calcium-binding to HlyA is accompanied by an increase in intrinsic fluorescence at ca. 340 nm, i.e. fluorescence emission due to the protein Trp residues. For WT, the increase in fluorescence intensity reaches a maximum at ca. 0.25 mM Ca^{2+} [9]. When the Trp fluorescence intensities of WT and DM HlyA are recorded at increasing Ca^{2+} concentrations they

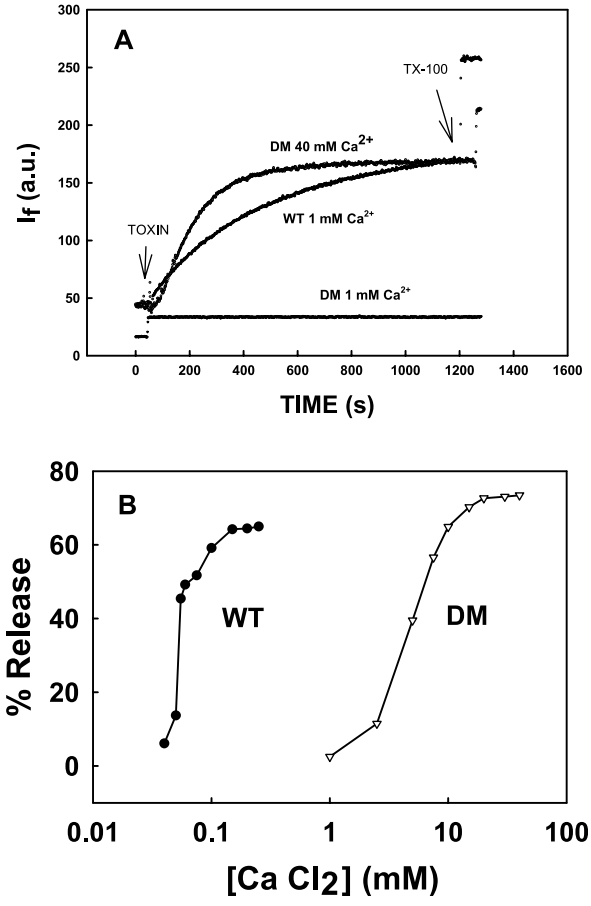

Fig. 1. The calcium requirements of WT and DM HlyA for toxin-induced release of liposomal aqueous contents. A: Time-course of release induced by WT and DM HlyA in the presence of 1 or 40 mM Ca^{2+}. B: Extent of release plotted as a function of Ca^{2+} concentration. % Release is measured from plots as shown in Fig. 1A, after 20 min. Liposomes were LUV composed of PC:PE:Chol (2:1:1). Total lipid concentration was 0.1 mM. Toxin concentration was 100 nM.

are seen to increase in parallel up to 1 mM Ca^{2+} (Fig. 3A). At higher Ca^{2+} concentrations, however, the fluorescence intensity of WT remains constant up to 50 mM Ca^{2+}, while the fluorescence of DM HlyA undergoes a further increase, reaching a plateau at ca. 10 mM Ca^{2+} (Fig. 3B). The 1–10 mM Ca^{2+} concentration range is precisely the one at which DM HlyA activity increases from virtually zero to near maximum (Fig. 1B). Thus the change in fluorescence observed in Fig. 3B is reflecting a conformational change that makes DM HlyA competent for membrane insertion and subsequent loss of impermeability.

For WT it had been established that Ca^{2+}-binding in the 0–250 μM region accompanied a conformational change, detected through changes in intrinsic fluorescence among other techniques, that in turn allowed the irreversible insertion of HlyA into the membrane bilayer, followed by membrane leakage [9,20–22]. The data in Fig. 3A indicate that DM HlyA fluorescence changes with Ca^{2+} concentrations up to 1 mM in parallel with WT. However, only after a further increase in fluorescence in the 1–10 mM Ca^{2+} range (Fig. 3B) is the mutant lytic activity elicited. The fluorescence data suggest that, for DM HlyA, a second Ca^{2+}-dependent conformational

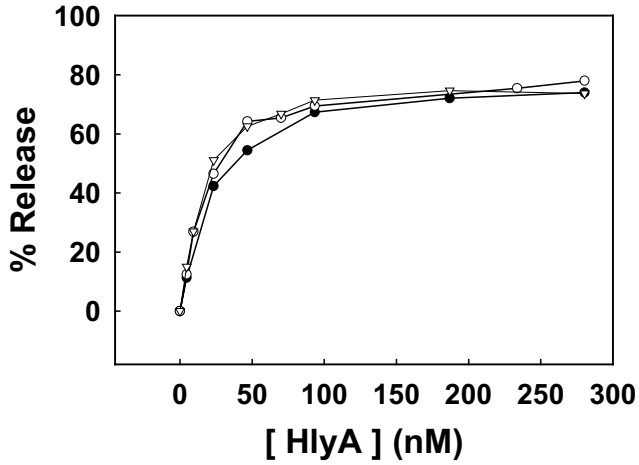

Fig. 2. Dose–response curves of toxin-induced release of LUV aqueous contents. (●) WT HlyA, assayed in 10 mM Ca^{2+}. (○) H859D, assayed in 10 nM Ca^{2+}. (∇) DM HlyA, assayed in 50 mM Ca^{2+}. % Release is measured from plots as shown in Fig. 1A, after 20 min. Other details as in Fig. 1.

change, in the millimolar concentration range, is required for the lytic activity to be observed.

3.3. Aggregate–monomer equilibrium

Like other RTX toxin proteins, HlyA exists in solution in equilibrium between the monomeric and aggregated forms [5,11,16]. As described in a previous paper [12], only the monomer containing bound Ca^{2+} appears to be competent to achieve target membrane insertion and subsequent lysis.

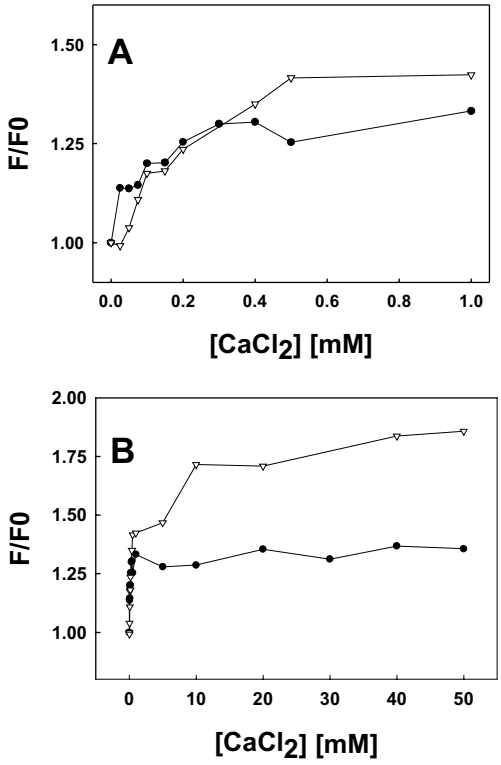

Fig. 3. Ca^{2+}-dependent changes in intrinsic fluorescence intensity of the toxin. A: Calcium concentrations 0–1 mM. B: Calcium concentrations 0–50 mM. (●) WT; (∇) DM HlyA.

Cortajarena et al. [12] optimised an FPLC method to separate Ca^{2+}-bound monomers from protein aggregates. Under their conditions, aggregates eluted at ca. 10 ml, and Ca^{2+}-bound monomers at ca. 18 ml. A peak at 18 ml could only be seen under conditions leading to HlyA lytic activity, i.e. 0.2 mM Ca^{2+} for WT. DM HlyA requires Ca^{2+} concentrations in the millimolar range to allow the separate elution of a (small) peak at 18 ml (Fig. 4). This confirms the idea that the behaviour of DM HlyA as related to membrane lysis parallels that of WT, except that the mutant requirements for calcium are greatly increased.

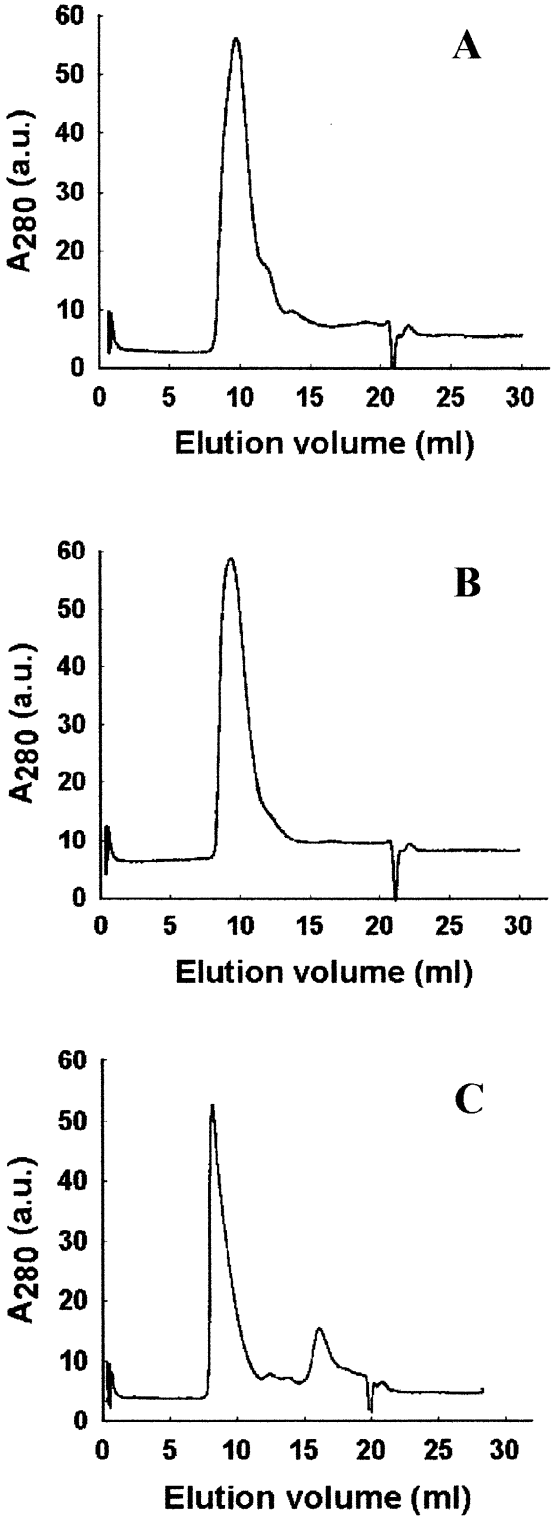

Fig. 4. Separation of DM HlyA-Ca monomers by FPLC. Urea- and calcium-free DM HlyA (1.5 μM) was incubated in the presence of 0 mM (A), 1 mM (B) and 40 mM (C) $CaCl_2$, and gel-filtrated through a Superdex HR-200 column.

TOXIN	ORGANISM	GGXGXD/HXUX		ACCESSION N°
HlyA	*Escherichia coli*	854 865	SGYGHHIIDDD GGK.DDKIS	PO8715
EhxA	*Enterohaemorragic Escherichia coli*	836 847	QNYGHHTIADE GGKG.DRLH	AAC24352
ApxAI	*Actinobacillus pleuropneumoniae*	850	KEYGRHIII**E**	I39643
ApxAII	*Actinobacillus pleuropneumoniae*	793	TGDGNDSIT**D**	P15377
LktA	*Pasteurella haemolytica*	790	QGDGNDIIT**D**SD	P55118
CyaA	*Bordetella pertussis*	1594 1604	VGYGHDTIY**E** SGGGHDTIR	P15318
AktA	*Actinobacillus actinomycetemcomitans*	850	KEYGHHTIT**E**	P16462
AshA	*Actinobacillus suis*	793	TGDGNDSIT**D**	Q00951

Fig. 5. Alignment of the last nonapeptide repeats of RTX toxins. The Asp/Glu residue at the end of the last (or last-but-one) nonapeptide is written in bold.

3.4. Sequence homologies

Comparing the sequences of the various toxins of the RTX family provides the interesting observation that in many cases an Asp (or Glu) residue occurs in a homologous position to Asp-863, i.e. immediately after the last, or last-but-one of the nonapeptide repeats (Fig. 5). This is observed in eight out of 10 RTX toxins whose sequences are known. When the Asp/Glu residue follows the last-but-one repeat, as in HlyA, or in *E. coli* EhxA, or in *Bordetella pertussis* CyaA, the last repeat does not follow immediately the previous one, but instead one or two amino acid residues occur between them (Fig. 5). The two RTX toxins with no Asp/Glu after the last/last-but-one nonapeptide, i.e. *A. pleuropneumoniae* ApxA II and *V. cholerae* RtxA, are non-haemolytic, but other non-haemolytic members of the family do have homologues to Asp-863. Considering the very scarce number of regularities found in the RTX family of toxins [5], the presence of Asp/Glu after the last (or last-but-one) nonapeptide repeats in eight of its members is a point that deserves consideration. The experimental data in this paper demonstrate that, in HlyA, this Asp residue is essential for keeping the Ca^{2+} requirements of the protein in the sub-millimolar range (in practice, Ca^{2+} requirements of 10 mM or higher are equivalent to having an inactive toxin under physiological conditions). In the absence of experimental data from the other toxins, the hypothesis can be put forward that the homologues of HlyA Asp-863 in the other toxins mentioned in Fig. 5 may also be important in establishing the toxin requirements for calcium ions.

4. Conclusions

Asp-863 is a very important residue for the calcium-dependent lytic activity of HlyA. A double mutant of this RTX toxin, in which Asp-863 has been mutated to a Gly, and His-859 has been substituted by Asp, so that the number of Asp residues and the net charge in that portion of the polypeptide remain constant, requires 100-fold higher concentrations of Ca^{2+} for its lytic activity than the WT. The H859D

mutation in itself has no effect on HlyA haemolytic activity or calcium-binding properties. The range of Ca^{2+} concentrations that elicit a lytic activity in the double mutant also induce a protein conformational change (according to changes in intrinsic fluorescence emission), and allow the existence of Ca^{2+}-bound toxin monomers, that are supposed to be the species that binds irreversibly the target membrane. The presence in most other toxins of the RTX family of Asp or Glu residues in positions homologous to Asp-863 with respect to the Ca^{2+}-binding nonapeptide repeats suggests that the observations in this paper may have a more general validity.

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