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Functional significance of the highly conserved Glu^{570} in the putative pore-forming helix 3 of the *Bordetella pertussis* haemolysin toxin

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ABSTRACT

Adenylate cyclase-haemolysin toxin (CyaA) is a virulence factor secreted from the etiologic agent of whooping cough, *Bordetella pertussis*. Previously, the haemolysin or pore-forming domain (CyaA-PF) has been shown to cause cell lysis of sheep erythrocytes independently, and the predicted helix $3_{(570-593)}$ within the PF-hydrophobic stretch could be a pore-lining constituent. Here, a plausible involvement in haemolytic activity of polar or charged residues (Glu^{570}, Gln^{574}, Glu^{581}, Ser^{584} and Ser^{585}) lining the hydrophilic side of CyaA-PF helix 3 was investigated *via* single-alanine substitutions. All the 126-kDa mutant proteins over-expressed in *Escherichia coli* were verified for toxin acylation as the results are corresponding to the wild-type toxin. When haemolytic activity of *E. coli* lysates containing soluble mutant proteins was tested against sheep erythrocytes, the importance of Glu^{570}, which is highly conserved among the pore-forming RTX cytotoxin family, was revealed for pore formation, conceivably for a general pore-lining residue involved in ion conduction.

1. Introduction

Bordetella pertussis, a Gram-negative bacterium causing whooping cough in human, secretes a variety of toxins including the adenylate cyclase-haemolysin toxin (CyaA) which is important for initiating respiratory tract infection (Carbonetti et al., 2005). CyaA (~177 kDa) is a typical member of the pore-forming RTX cytotoxins (a subgroup of the **R**epeats-in-**T**o**X**in (RTX) protein family) that contains an adenylate cyclase (AC) domain attached to the *N*-terminus of the relatively conserved haemolysin or pore-forming (PF) domain (Fig. 1A). The CyaA-PF domain (~126 kDa) shares some common features with other RTX cytotoxins in that it contains an *N*-terminal hydrophobic region, a Gly–Asp-rich nonapeptide-repeat region and an unprocessed *C*-terminal signal peptide sequence (Welch, 1991; Linhartova et al., 2010). Moreover, these RTX toxins require post-translational acylation at an internal lysine, *e.g.* palmitoylation at Lys^{983} for CyaA by CyaC acyltransferase, to turn into an active form (Hackett et al., 1994) and be secreted subsequently by the type I secretion system (Welch, 2001; Linhartova et al., 2010). Following secretion, the CyaA toxin is stabilised by extracellular calcium ions which might act as a structural stabilising bridge in a β-roll motif of the Gly–Asp-rich repeats (Rose et al., 1995; Knapp et al., 2003; Chenal et al., 2009; Pojanapotha et al., 2011).

Abbreviations: 3D, three-dimensional; AC, adenylate cyclase; CyaA, adenylate cyclase-haemolysin toxin; CyaA-PF, CyaA pore-forming; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonylfluoride; RTX, Repeats-in-ToXin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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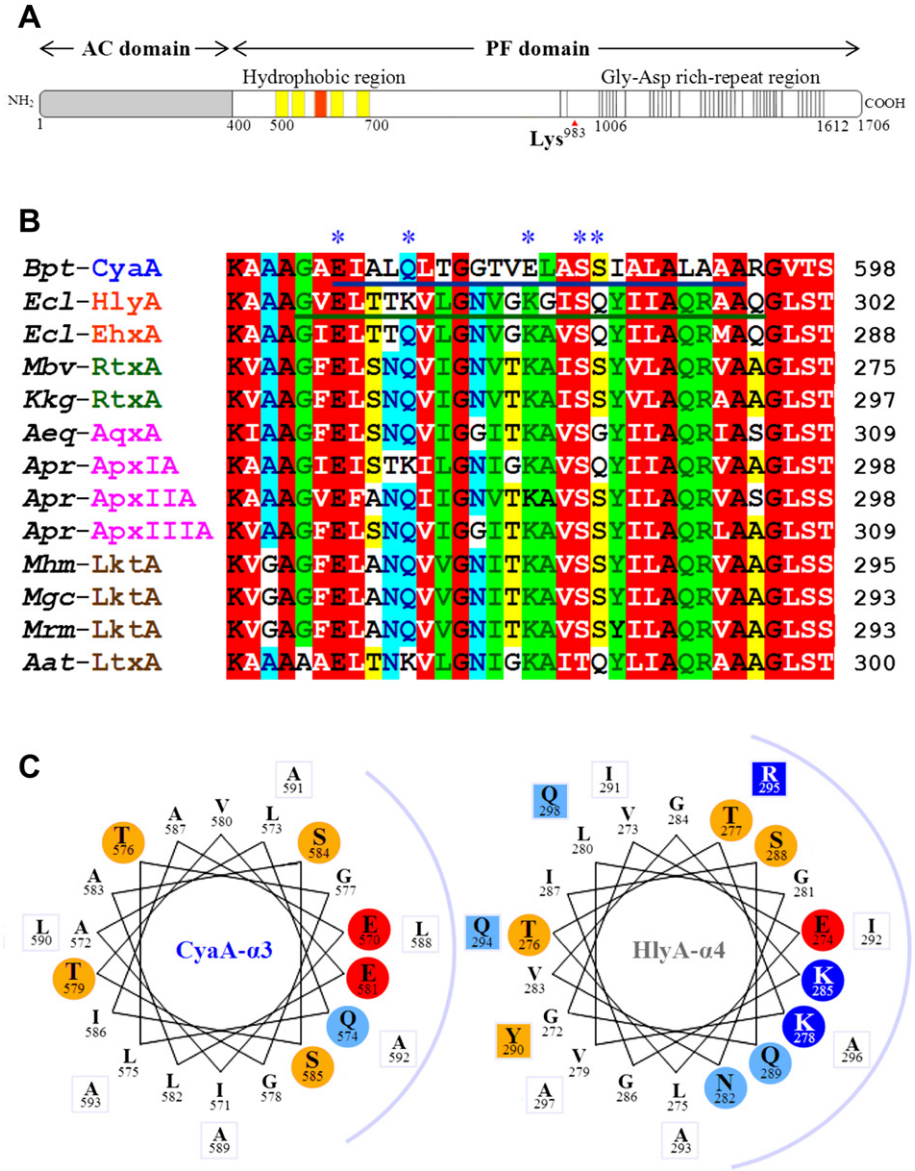

Fig. 1. A: Schematic diagram of CyaA showing adenylate cyclase (AC) and haemolysin or pore-forming (PF) domains. Five putative helices in the hydrophobic region (residues 500–700) are shown by yellow blocks, with red colour for α3. The calcium-binding region (residues 1006–1612) shows lines corresponding to each nonapeptide repeat (Gly–Gly–X–Gly–X–Asp–X–U–X). Lys^{983} indicates the palmitoylation site. B: Multiple sequence alignment of amino acid sequences covering the putative transmembrane $\alpha3_{(570–593)}$ of CyaA aligned with corresponding sequences of twelve pore-forming RTX cytotoxins. CyaA-$\alpha3_{(570–593)}$ and the putative membrane-spanning HlyA-$\alpha4_{(272–298)}$ are underlined. Degree of conservation among the sequences is highlighted by shading residues with red (black characters denote identical), blue, green and yellow for 100%, 80–90%, 65–75% and 50–60% homology, respectively. Asterisks indicate CyaA-PF residues selected for alanine substitutions. C: Helical wheel projections comparing CyaA-$\alpha3_{(570–593)}$ and HlyA-$\alpha4_{(272–298)}$. Amino acids were plotted every 100° consecutively around the helix axis (360° for 3.6 residues). Negatively and positively charged side-chains are shaded with red and blue, respectively. Orange and light-blue shadings represent oxygen- and nitrogen-containing uncharged side-chains, respectively. Relatively hydrophilic surface is indicated by arc. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CyaA toxin affects mainly human macrophages by binding to the $\alpha_M\beta_2$-integrin receptor through its non-apeptide-repeat region (El-Azami-El-Idrissi et al., 2003). Recently, the involvements of lipid rafts as well as the sugar moiety on the β_2 integrin in CyaA toxin binding were also reported (Morova et al., 2008; Bumba et al., 2010). Upon binding, the AC domain was internalised into the target-cell cytoplasm to catalyse the uncontrolled production of cAMP, disturbing the transcription of many inflammatory- and cell signalling-associated genes in apoptotic pathways that lead to cell death (Hewlett et al., 1989; Carbonetti et al., 2005; Cheung, 2008). However, the CyaA toxin can also

exert haemolytic activity against cells lacking the integrin receptor such as sheep erythrocytes (Osickova et al., 1999). In addition, the 126-kDa CyaA-PF fragment (haemolysin domain) was found sufficient to cause haemolysis of sheep erythrocytes (Ehrmann et al., 1992; Sakamoto et al., 1992; Powthongchin and Angsuthanasombat, 2008) but its structural basis of pore formation still remains to be investigated.

The hydrophobic region within the CyaA-PF domain or other related pore-forming RTX toxins has been suggested to be the membrane-inserting part for forming lytic pore in the target-cell membrane (Ludwig et al., 1991; Benz et al., 1994; Valeva et al., 2008; Powthongchin and Angsuthanasombat, 2009). By using various algorithms of membrane topology predictions, the PF-hydrophobic stretch covering residues 500–700 of CyaA was predicted to fold up into five potential helical transmembrane segments (Osickova et al., 1999; Powthongchin and Angsuthanasombat, 2009). In our previous work, single-proline substitutions as a means of disturbing each confined secondary structure revealed the importance of putative transmembrane α1, α2, α3 and α5 in pore formation, and we have proposed that the putative helical hairpin (α2-loop-α3) is part of the transmembrane pore in which α3 lines the lumen (Powthongchin and Angsuthanasombat, 2009). In this study, to characterise further the structural importance of the putative CyaA-PF pore-lining segment (α3), we have made single-alanine substitutions selectively at the polar and charged residues lining the hydrophilic side of this helix. The results revealed the involvement of charged residues for haemolytic activity against sheep erythrocytes of this haemolysin toxin, in particular the highly conserved Glu^{570} which conceivably serves as a general pore-lining residue involved in ion conduction.

2. Materials and methods

2.1. Amino acid sequence alignment and 3D modelling

Multiple sequence alignment of deduced amino acid sequences from related pore-forming RTX cytotoxins [CyaA from *B. pertussis* (*Bpt*-CyaA, gb:CAE41066); HlyA and EhxA from *Escherichia coli* (*Ecl*-HlyA, gb:ABE10329 and *Ecl*-EhxA, gb:BAA31774); RtxA from *Moraxella bovis* (*Mbv*-RtxA, gb:AKK84651) and *Kingella kingae* (*Kkg*-RtxA, gb:ABK58601); AqxA from *Actinobacillus equuli* (*Aeq*-AqxA; gb:AMM45569); ApxIA, ApxIIA and ApxIIIA from *Actinobacillus pleuropneumoniae* (*Apr*-ApxIA, gb:AAL55666; *Apr*-ApxIIA, gb:AAU84700 and *Apr*-ApxIIIA, gb:CAA48711); LktA from *Manheimia haemolytica* (*Mhm*-LktA, gb:AAL13281); *Manheimia glucosida* (*Mgc*-LktA, gb:AAG40306) and *Manheimia ruminalis* (*Mrm*-LktA, gb:AAR09165); LtxA from *Aggregatibacter actinomycetemcomitans* (*Aat*-LktA, gb:CAA34731)] was performed using ClustalW. Regions that are highly conserved in identity or similarity were again manually edited and the degree of sequence homology among the aligned sequences was defined by different coloured shadings.

For homology-based modelling, a template structure searched from the Protein Data Bank was selected based on the highest sequence homology to the CyaA-hydrophobic region which comprises five putative helical segments, $\alpha1_{(500–522)}$, $\alpha2_{(529–550)}$, $\alpha3_{(570–593)}$, $\alpha4_{(602–627)}$ and $\alpha5_{(678–698)}$ as assigned previously (Powthongchin and Angsuthanasombat, 2009). A plausible 3D model was consequently constructed based on the best-fit template structure of bovine rhodopsin (1gzm.pdb) using the SWISS-MODEL program. Structural refinement of the modelled five-helix bundle *via* energy minimisation was subsequently performed using GROMOS96 simulation software.

2.2. Construction of mutant plasmids by PCR-based directed mutagenesis

The pCyaAC-PF plasmid encoding both the 126-kDa CyaA-PF haemolysin fragment and the 21-kDa CyaC acyltransferase under control of the T7-promoter (Powthongchin and Angsuthanasombat, 2008) was used as a template. Complementary pairs of mutagenic oligonucleotide primers (see Table 1) were designed according to the sequence of the *cyaA-PF* gene to generate single-alanine-substituted mutant plasmids using a high fidelity Phusion™ DNA polymerase (Finnzymes, Finland), following the procedure of the QuickChange Mutagenesis Kit (Stratagene, USA). The *Dpn*I-treated PCR products were transformed into *E. coli* strain JM109. The selected clones were subjected to restriction endonuclease analysis and subsequently verified by DNA sequencing (Macrogen, Inc., Korea).

2.3. Toxin expression

The mutant plasmids were re-transformed into *E. coli* strain BL21(DE3)pLysS for protein expression. An overnight culture of each selected clone was grown at 30 °C in Luria–Bertani medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. When the cell culture at OD_{600} reached ~0.6, toxin expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.1 mM, and incubation was continued for another 6 h. *E. coli* cells were harvested and re-suspended in 50 mM Tris–HCl (pH 8.0) containing 5 mM $CaCl_2$ and 1 mM PMSF (phenylmethylsulfonylfluoride), and

Table 1
Oligonucleotide primers used in single-alanine substitutions.

Primers[a]	Sequences[b]	Restriction site
E570A-f	5′–CGCCG**C**GATCGCGCTG C–3′	*Pvu*I
E570A-r	3′–GGCCGCGGC**G**CTAGCG–5′	
Q574A-f	5′–GCGCTG**GC**GTT**A**ACAGGTGGAACGGT–3′	*Hpa*I
Q574A-r	3′–GCTCTAGCGCGAC**CG**CAATTGTCCAC–5′	
E581A-f	5′–CGGTCG**C**GCTGGCTTC–3′	*Alu*I[c]
E581A-r	3′–CTTGCCAGC**G**CGACCG–5′	
S584A-f	5′–CTGGCT**G**C**A**TC**G**ATCGCGTTGGCGG–3′	*Cla*I
S584A-r	3′–CCAGCTCGACCGA**C**G**T**AG**C**TAGCGC–5′	
S585A-f	5′–GGCTTCT**GCG**ATCGCGTTGG–3′	*Bst*XI[c]
S585A-r	3′–CGACCGAAGA**CGC**TAGCGC–5′	

[a] f and r represent forward and reversed primers, respectively.
[b] Underlined bases represent the recognition sites introduced for restriction enzyme analysis. Enlarge letters indicate the substituted nucleotide residues. The mutated residues in introduced restriction sites generate silent mutations.
[c] Deleted recognition sites.

subsequently disrupted in a French Pressure Cell at 10,000 psi. After centrifugation at 12,000*g* for 20 min at 4 °C, total proteins in soluble crude lysates were analysed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and concentrations were determined with the Bradford-based protein microassay (Bio-Rad, USA), with bovine serum albumin (Sigma–Aldrich, USA) as a standard.

Western blot analysis was performed to validate the identity of the CyaA-PF wild-type and its mutant proteins by probing with the 9D4 anti-RTX monoclonal antibody (Listlabs, USA, 1:2000 dilution). The immunocomplexes were subsequently detected with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Pierce, USA, 1:20,000 dilution) as described previously (Powthongchin and Angsuthanasombat, 2008).

2.4. Toxin acylation analysis

Palmitoylation of toxins at Lys^{983} was verified by mass spectrometry. The proteins were separated by SDS-PAGE (10% w/v gel), eluted from the excised gel and subsequently digested with trypsin according to the standard protocol. The peptide separation was performed in a nanobored C18 column prior to mass and LC-MS/MS analyses by using the ABI 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems) and LTQ-Orbitrap (Thermo Fisher Scientific, Inc., Germany), respectively.

2.5. Haemolytic activity assay

The haemolytic activity test was performed in a 1-ml microcentrifuge tube by incubating 800 μl of sheep erythrocyte suspension (5×10^8 cells/ml) in buffer (150 mM NaCl, 2 mM $CaCl_2$, 20 mM Tris–HCl, pH 7.4) with 200 μl (~1 mg total proteins) of soluble *E. coli* lysate containing ~10 μg CyaA-PF (estimated from densitometer tracings of the band intensity compared to the standard protein markers in the stained gel, see Fig. 2). The mixtures were incubated at 37 °C for 5 h and thereafter the unlysed erythrocytes were pelleted down at 12,000*g* for 2 min. The supernatant containing the released haemoglobin was measured by spectrophotometer at OD_{540}. The same amount of total proteins in soluble *E. coli* lysate containing pET-17b was used as a negative control while the same amount of erythrocytes lysed with 0.1% Triton-X 100 was defined as 100% haemolysis. Percent haemolysis for each toxin sample was calculated by {[OD_{540} sample − OD_{540} negative control]/[OD_{540} of 100% haemolysis − OD_{540} negative control]} × 100. All samples were tested in triplicate for three independent experiments. Student's *t* test was performed to determine significance levels between the wild type and mutants.

3. Results and discussion

In our previous work, the predicted $\alpha 2_{529-550}$ and $\alpha 3_{570-593}$ within the PF-hydrophobic stretch were found to be crucial for haemolytic activity of CyaA-PF in line with the suggestion that the amphipathic α3 lines the lumen (Powthongchin and Angsuthanasombat, 2009). Therefore, molecular characterisation of this putative CyaA-PF pore-lining segment was further made in the present study for its functional importance.

3.1. Sequence conservation among the related pore-forming RTX cytotoxins

When the amino acid sequence corresponding to the putative $\alpha 3_{(570-593)}$ (E^{570}IALQLTGGTVELASSIALALAAA^{593}) of *B. pertussis* CyaA was aligned with that of twelve closely related pore-forming RTX cytotoxins, including both haemolysin (HlyA, EhxA and ApxIA) and leukotoxin subgroups (RtxA, AqxA, ApxIIA, ApxIIIA, LktA and LtxA), a high degree of sequence similarity (>90%) was found among these

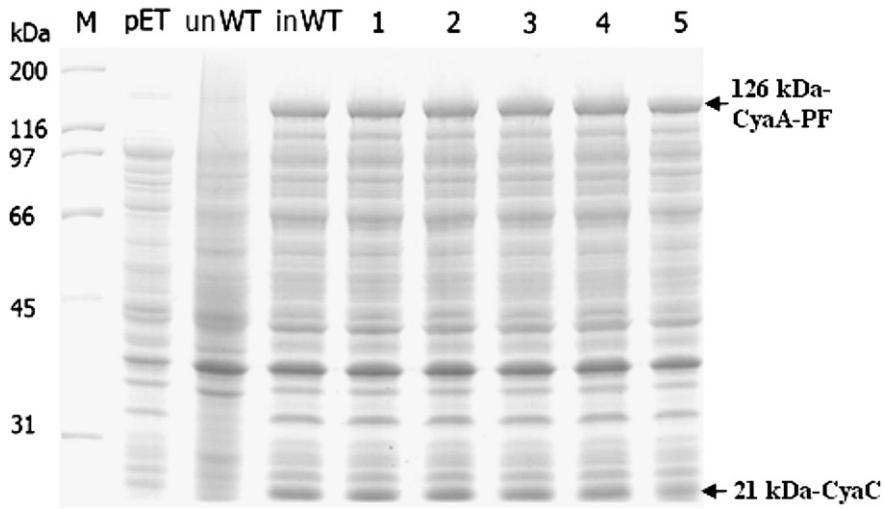

Fig. 2. SDS-PAGE (Coomassie blue-stained 10% w/v) of *E. coli* lysates (0.1 OD_{600}) expressing CyaA-PF or mutant toxins. M represents standard protein markers. pET represents the pET-17b empty vector. The CyaAC-PF wild-type was expressed in the absence (unWT) or presence of 0.1 mM IPTG (inWT). Lanes 1–5 represent the CyaA-PF mutants E570A, Q574A, E581A, S584A and A585A with 0.1 mM IPTG induction, respectively. The arrows indicate 126-kDa CyaA-PF and 21-kDa CyaC bands.

sequences (Fig. 1B). This could imply a common membrane-spanning segment of these related pore-forming RTX cytotoxins regardless of their target-cell specificity. Moreover, the region flanking CyaA-α3 (K^{564}AAAGA^{569} and G^{595}VTS^{598} being assigned as loop regions) is also conserved. It is noteworthy that Glu^{570} located at the *N*-terminal end of the CyaA-helix is entirely identical throughout the aligned toxins whilst Glu^{581} in the helix's centre is opposite in charge to the corresponding position (*i.e.* Lys) of all other toxins. It was also interesting to note that the three toxins, *E. coli* HlyA, *A. pleuropneumoniae* ApxIA and *A. actinomycetemcomitans* LtxA, which all have relatively high activities, have a positively charged lysine in place of the polar uncharged glutamine at position 574 of CyaA, suggesting the requirement of an additional positively charged moiety lining the pore lumen.

As illustrated in Fig. 1C, the helical wheel projection of the CyaA-α3 was also compared with that of *E. coli* HlyA (G^{272}VELTTKVLGNVGKGISQYIIAQRAAQ^{298}), which has been proposed to serve as a transmembrane helix (Ludwig et al., 1991; Valeva et al., 2008). It reveals that these two amphipathic transmembrane helices possess a very similar hydrophilic surface. Additionally, the projections of the other aligned toxins also show the amphipathic nature of these segments along with their hydrophilic sides resembling that of CyaA and HlyA (data not shown), supporting the functional importance of these conserved sequences in serving as a lumen-pore-lining segment.

3.2. Common biochemical characteristics of the expressed CyaA-PF mutant proteins

Further attempts were made to characterise the structural importance of this putative CyaA-PF pore-lining segment (E^{570}IALQLTGGTVELASSIALALAAA^{593}). Single-alanine substitutions were conducted selectively at the polar (Gln^{574}, Ser^{584} and Ser^{585}) and charged residues (Glu^{570} and Glu^{581}) lining the hydrophilic side of this helix (see Fig. 1C). All five alanine-substituted mutants (E570A, Q574A, E581A, S584A and S585A) of the 126-kDa CyaA-PF toxin, when overproduced in *E. coli* together with its accessory protein (the 21-kDa CyaC acyltransferase), still remained as a soluble form with yields comparable to the wild-type toxin (Fig. 2). This could indicate that these single-alanine substitutions did not affect the folding of the mutant molecules, as protein misfolding can lead to the formation of an insoluble aggregate (no 126-kDa CyaA-PF protein was detected in the pellet fraction of each mutant lysate, data not shown). It should be noted that the wild-type and mutant CyaA-PF toxins were all recognised by anti-RTX specific monoclonal antibody in Western blot analysis (data not shown), but not for the 21-kDa protein band which was previously verified to be CyaC acyltransferase by LC/MS/MS (Thamwiriyasati et al., 2010). In addition, some other smaller immuno-reactive bands were also observed for both the wild-type and mutant toxins (data not shown), indicating that all the expressed CyaA-PF fragments are somewhat sensitive to proteolytic degradation (Powthongchin and Angsuthanasombat, 2008).

As mentioned earlier, palmitoylation at Lys^{983} is needed for activating the haemolytic fragment precursor (CyaA-PF) (Powthongchin and Angsuthanasombat, 2008). Therefore, verification *via* MALDI-TOF/MS and LC-MS/MS analyses was conducted to prove such *in vivo* acylation of each mutant toxin. As was determined by MALDI-TOF/MS analysis, the molecular weight (MW) of trypsin-treated fragments derived from each mutant protein matched their primary sequences. Furthermore, LC-MS/MS analysis identified the peptide fragment of *m*/*z* 1619.63 to be E^{972}GVATQTTAYGK$_{C16:0}$R^{984}, containing the palmitoylated Lys^{983} (MW of the added palmitoyl group ~238). It was found that all the mutant toxins reveal the palmitoylated peptide mass as described. These results indicate that toxin palmitoylation at Lys^{983} was not affected by each alanine substitution, confirming that no severe conformational change had occurred as a consequence of these mutations.

3.3. Haemolytic activity of the single-alanine-substituted CyaA-PF mutants

To determine an effect of these single-alanine substitutions (E570A, Q574A, E581A, S584A and S585A) on toxicity, we examined the soluble crude lysates from *E. coli* expressing each CyaA-PF mutant toxin for their relative haemolytic activities against sheep red blood cells as shown in Fig. 3. It was revealed that single-alanine substitutions at Glu^{570} or Glu^{581} caused a drastic decrease in haemolytic activity to 19 ± 1 and 39 ± 7%, respectively, compared to 66 ± 6% of the wild-type (~70% and ~40% decreased from the control wild-type value (66 ± 6%) for E570A and E581A, respectively). In contrast, these substitutions at Gln^{574}, Ser^{584} or Ser^{585} did not severely affect the toxin activity, as the Q574A, S584A and S585A mutant toxins still retained relatively high haemolytic activity (53 ± 4, 60 ± 8 and 70 ± 5%, respectively) at a level more than 80% of the wild-type activity against sheep erythrocytes, perhaps suggesting that these residues (Gln^{574}, Ser^{584} and Ser^{585}) are not importantly involved in toxin haemolysis. It should be noted that the detrimental effects on haemolytic activity seen for the E570A and E581A mutants are least likely to be caused by improper folding and non-acylation of the mutant proteins since both are still palmitoylated at Lys^{983} as mentioned earlier. These results thus suggest that Glu^{570} and Glu^{581} in the putative pore-lining helix (α3) play a role in haemolytic activity of the CyaA-PF toxin.

3.4. Structural model with implications for ion conduction

Thus far, no 3D crystal structure of the pore-forming RTX cytotoxins has yet been resolved. Most structure–function relationship studies were based on homology modelling and prediction algorithms, categorising this group of toxins to be alpha-pore-forming toxins (α-PFT) (Ludwig et al., 1991; Menestrina et al., 1994; Osickova et al., 1999). The α-PFTs utilise their amphipathic α-helices as a fundamental constituent to form pores or channels in the target-cell membrane (Gonzalez et al., 2008). Insecticidal crystal proteins (Cry δ-endotoxins) from *Bacillus thuringiensis* (*Bt*), one of the well-characterised α-PFTs, are notable for the crucial role of the α4-loop-α5 hairpin which provides a proper structure for inserting and spanning the target-cell membrane to form pores (Angsuthanasombat,

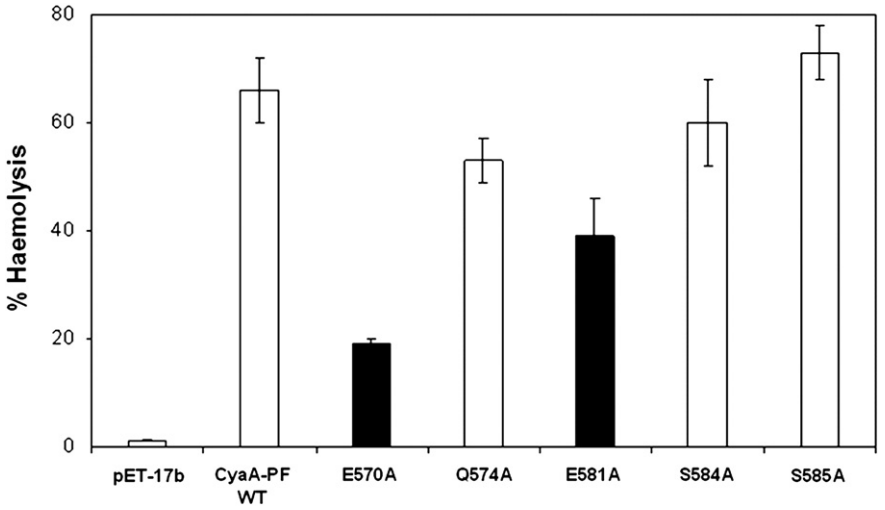

Fig. 3. Haemolytic activities of soluble crude extracts of *E. coli* cells expressing CyaA-PF wild-type (WT) or each mutant toxin (E570A, Q574A, E581A, S584A and S585A) were tested against sheep erythrocytes. The crude lysate containing the pET-17b empty vector alone was used as a negative control. CyaA-PF wild-type toxin exhibited 66 ± 6% haemolysis. The E570A, Q574A, E581A, S584A and S585A mutant toxins showed 19 ± 1, 53 ± 4, 39 ± 7, 60 ± 8 and 70 ± 5% haemolysis, respectively. Error bars indicated standard errors of the mean from three independent experiments with each performed in triplicate. The activities of mutants which show significant differences (P values $\leq$ 0.05) from that of the wild type are denoted by black shading.

2010). The relatively hydrophobic α5 of Cry toxins is likely to interact with the membrane and is involved in toxin-pore oligomerisation (Likitvivatanavong et al., 2006) whilst the amphipathic α4 suitably lines the lumen and explicit charged residues (*e.g.* Glu^{129} and Asp^{136} for Cry1Aa, see Fig. 4B) are shown to participate in ion permeation through the pore (Masson et al., 1999).

Here, we have built a plausible 3D model for the putative helical bundle of CyaA-PF (Fig. 4A). As can be inferred from the model, the lumen-lining α3 reveals the two critical negatively charged residues, *i.e.* Glu^{570} and Glu^{581}, with good spatial and geometrical relations similar to Glu^{129} and Asp^{136} of Cry1Aa-α4 (Fig. 4A and B). Interestingly, it has been reported that substitutions of Glu^{570} with Pro, Gln or Lys decreased CyaA haemolysis whereas this activity markedly increased for the replacement of Glu^{581} with polar uncharged- (Gln) or oppositely charged- (Lys) residues (Basler et al., 2007). As was noted earlier, Glu^{570} presented at the cap of this lumen-lining helix is completely conserved throughout the aligned cytotoxins but Glu^{581} in the core helix is reverse in charge to the equivalent position (*i.e.* Lys) of all other toxins (see Fig. 1B). Together with our mutagenesis data as presented above (Fig. 3), it is thus conceivable that the terminal negatively charged residue (i.e. Glu^{570}) which has a tendency to be at the pore entrance could contribute to the primary interaction with an incoming cation. For the interior Glu^{581}, it seems that oppositely charged requirement might also contribute in part to haemolysis since reversal of this charge (E581K) showed enhanced activity of CyaA as mentioned earlier (Basler et al., 2007) and all the cytotoxins, including the highly active haemolysin-HlyA from *E. coli*, possess the acquired positive charge at this position.

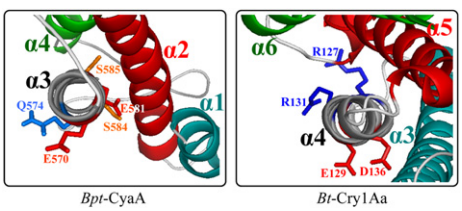

Fig. 4. Comparison of amino acid arrangement between two analogous lumen-lining helices, *Bpt*-CyaA α3 (*left*) and *Bt*-Cry1Aa α4 (*right*), together with the relative positions of other helices in the CyaA-PF helical model and the Cry1Aa crystal structure (top view generated by the PyMol program). The relatively hydrophobic helix (CyaA-α2 or Cry1Aa-α5) encircled by other amphipathic helices is shown in red. The two critical negatively charged side-chains (Glu^{570} and Glu^{581} for CyaA-PF, Glu^{129} and Asp^{136} for Cry1Aa) are denoted with red sticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In conclusion, this study provides further insights into a functional significance of two negatively charged residues in the putative lumen-lining α3 of *B. pertussis* CyaA-haemolysin. The highly conserved Glu^{570} which is relatively more critical for haemolytic activity against sheep erythrocytes could act as a gateway of the pore lumen, whilst Glu^{581} in the middle of the helical segment might recruit the hydrated ions through the toxin-induced pore. The inward ions along with water molecules would finally lead to colloid-osmotic lysis of target cells. However, it remains to be tested whether the substitutions at Glu^{570} and Glu^{581} are conducive to alterations in the passage of ions through the pore.

Conflict of interest

There are no conflicts of interest.

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