Segments Crucial for Membrane Translocation and Pore-forming Activity of Bordetella Adenylate Cyclase Toxin*

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Bordetella adenylate cyclase toxin-hemolysin (CyaA, AC-Hly, or ACT) permeabilizes cell membranes by forming small cation-selective (hemolytic) pores and subverts cellular signaling by delivering into host cells an adenylate cyclase (AC) enzyme that converts ATP to cAMP. Both AC delivery and pore formation were previously shown to involve a predicted amphipathic α-helix502–522 containing a pair of negatively charged Glu509 and Glu516 residues. Another predicted transmembrane α-helix565–591 comprises a Glu570 and Glu581 pair. We examined the roles of these glutamates in the activity of CyaA. Substitutions of Glu516 increased specific hemolytic activity of CyaA by two different molecular mechanisms. Replacement of Glu516 by positively charged lysine residue (E516K) increased the propensity of CyaA to form pores, whereas proline (E516P) or glutamine (E516Q) substitutions extended the lifetime of open single pore units. All three substitutions also caused a drop in pore selectivity for cations. Substitutions of Glu570 and Glu581 by helix-breaking proline or positively charged lysine residue reduced (E570K, E581P) or ablated (E570P, E581K) AC membrane translocation. Moreover, E570P, E570K, and E581P substitutions down-modulated also the specific hemolytic activity of CyaA. In contrast, the E581K substitution enhanced the hemolytic activity of CyaA 4 times, increasing both the frequency of formation and lifetime of toxin pores. Negative charge at position 570, but not at position 581, was found to be essential for cation selectivity of the pore, suggesting a role of Glu570 in ion filtering inside or close to pore mouth. The pairs of glutamate residues in the predicted transmembrane segments of CyaA thus appear to play a key functional role in membrane translocation and pore-forming activities of CyaA.

Bordetella pertussis, the etiological agent of whooping cough, secretes an adenylate cyclase toxin (CyaA, adenylate cyclase-hemolysin (AC-Hly), or ACT) that is a key virulence factor of the bacteria during early phases of respiratory tract colonization (1–4). The toxin paralyzes bactericidal activities of host phagocytes and induces macrophage apoptosis, possibly enabling the pathogen to escape host immune surveillance (5–8).

CyaA is synthesized as a single polypeptide of 1706 residues and consists of an amino-terminal AC domain of about 400 residues and a pore-forming RTX (repeat in toxin) Hly moiety of about 1306 residues (9). The Hly bears a C-terminal secretion signal (10, 11) and consists of a hydrophobic pore-forming domain (residues 500–700) (12), of a calcium-binding glycine- and aspartate-rich nonapeptide repeat domain (last 700 residues), and of a fatty acyl activation domain (residues 800–1000). In the last, the essential covalent post-translational modification is taking place (13) in the presence of the accessory acyltransferase, CyaC (14). The AC domain is enzymatically active by itself (15). The entire toxin is, however, needed for AC delivery into target cells (16, 17), where AC is activated by cytosolic calmodulin and catalyzes unregulated conversion of ATP to the key signal molecule, cAMP (18, 19). In turn, membrane insertion and pore-forming (hemolytic) activities of CyaA do not require the AC domain and are located to the Hly portion (20). This exhibits hemolytic activity on erythrocytes (16, 21) and can form small cation-selective membrane pores (12, 22).

CyaA binds target cells primarily via the αMβ2 integrin receptor known as CD11b/CD18, complement receptor 3 (CR3), or Mac-1 (23). This is expressed by granulocytes/neutrophils, macrophages, myeloid dendritic cells, NK cells, and certain subsets of CD8+ T and of B cells. It is noteworthy, however, that the toxin can penetrate and intoxicate to detectable levels also a variety of other cell types lacking the CD11b/CD18 receptor and/or endocytic mechanisms, such as mammalian erythrocytes (5, 6, 16, 24). Because of a lack of structural data, the mechanisms of membrane insertion of CyaA, its capacity to cross directly the cytoplasmic membrane, and its ability to form cation-selective membrane pores are poorly understood. Indirect evidence suggests that translocation of the AC domain...
across membrane and formation of CyaA pores are two independent and separable membrane activities, resulting from membrane insertion of CyaA (22, 25–33). Recently, we demonstrated that both of these activities involve a transmembrane α-helix predicted between residues 502 and 522 of the pore-forming domain of CyaA. This harbors a pair of negatively charged glutamate residues, Glu509 and Glu516. A helix-breaking proline substitution E509P within this segment abolished the capacity of CyaA to deliver the AC domain into erythrocytes. Moreover, the charge-reversing lysine substitutions E509K and E516K strongly enhanced the specific hemolytic activity of CyaA, by up-modulating its capacity to form pores while ablating AC domain translocation into cells. In addition, combination of the substitutions in CyaA-E509K/E516K strongly decreased the cation selectivity of formed pores, indicating that Glu509 and Glu516 are located within or close to the membrane pore (34). On this basis, we suggested that prior to interaction with target membrane, two conformational isomers of CyaA might form, one being a translocation precursor allowing AC domain delivery into cell cytosol and the other being a pore precursor, whose insertion into membranes yields formation of oligomeric membrane pores (34).

Here, we show that AC membrane translocation and pore-forming (hemolytic) activity of CyaA further depends on an additional predicted transmembrane amphipathic α-helix, localized between residues 565 and 591 of the pore-forming domain and comprising yet another pair of glutamate residues, Glu570 and Glu581. Moreover, we show for the first time that hemolytic activity of CyaA further depends on an interaction with target membrane, two conformational isomers of CyaA might form, one being a translocation precursor allowing AC domain delivery into cell cytosol and the other being a pore precursor, whose insertion into membranes yields formation of oligomeric membrane pores (34).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Plasmids—Escherichia coli** XL1-Blue (Stratagene) was used throughout this work for DNA manipulation and CyaA expression. Bacteria were grown in Luria-Bertani medium supplemented with 150 μg/ml ampicillin. Wild-type CyaA and its mutant derivatives were expressed from the pCACT3 plasmid (29).

**Site-directed Mutagenesis—** Single amino acid substitutions were introduced into the cyaA gene by site-directed PCR mutagenesis using the TaqDNA polymerase and suitable pairs of mutagenic PCR primers (supplemental Table 1). PCR products with introduced substitutions were subcloned into the pCACT3, and the absence of other undesired mutations was verified by DNA sequencing. Complete sequences and detailed schemes of the plasmid constructs will be provided on request.

**Production and Purification of the CyaA-derived Proteins—** Intact CyaA and its mutant variants were produced in *E. coli* transformed with appropriate pCACT3-derived constructs. Exponential 500-ml cultures were grown at 37 °C and induced by isopropyl 1-thio-β-D-galactopyranoside (1 mM) for 4 h before the cells were washed in 50 mM Tris-HCl (pH 8), 150 mM NaCl, resuspended in 50 mM Tris-HCl (pH 8), 0.2 mM CaCl₂, and disrupted by sonication. The insoluble cell debris was resuspended in 8 M urea, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.2 mM CaCl₂. Upon centrifugation at 25,000 × g for 20 min, clarified urea extracts were loaded onto a DEAE-Sepharose column equilibrated with 8 M urea, 50 mM Tris-HCl (pH 8.0), 120 mM NaCl. After washing, the CyaA-derived proteins were eluted with 8 M urea, 50 mM Tris-HCl (pH 8.0), 2 mM NaCl, diluted four times with the 50 mM Tris-HCl (pH 8.0), 1 mM NaCl buffer, and further purified on a phenyl-Sepharose column equilibrated with the same buffer. Unbound proteins were washed out with 50 mM Tris-HCl (pH 8.0), and the CyaA-derived proteins were eluted with 8 M urea, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA and stored at −20 °C.

**Assay of AC, Cell Binding, and Cytotoxic and Hemolytic Activities—** Adenylate cyclase activities were measured in the presence of 1 μM calmodulin as previously described (35). One unit of AC activity corresponds to 1 μmol of cAMP formed per min at 30 °C, pH 8.0. Because toxin alterations outside the AC domain do not affect the specific AC activity, concentrations of mutant CyaAs in the extracts and in the purified preparations were equalized on the basis of their AC content prior to activity testing. Cell-invasive AC and hemolytic activities were measured as previously described (16) by determining the amount of AC enzyme activity protected against externally added trypsin upon internalization into erythrocytes and by hemoglobin release in time upon toxin incubations with washed sheep erythrocytes (5 × 10⁶/ml), respectively. Erythrocite binding of the toxins was determined as described in detail previously (17).

**Determination of the Intracellular cAMP Level—** Cytotoxic activity on sheep erythrocytes was determined as the capacity of various CyaAs to raise intracellular cAMP levels in 5 × 10⁸/ml of sheep red blood cells upon incubation with 5 μg/ml of the CyaA proteins for 30 min at 37 °C in HBSS (10 mM Na-HEPES, pH 7.4, 10 mM KCl, 140 mM NaCl, 3 mM MgCl₂, 2 mM CaCl₂, and 5 mM D-glucose) containing 100 μM 3-isobutyl-1-methylxanthine. The reaction was stopped by the addition of 100 mM HCl in 0.1% Tween 20, and the samples were boiled for 15 min at 100 °C to denature cellular proteins (cAMP is heat- and acid-resistant). The samples were neutralized by the addition of 150 mM unbuffered imidazol, passed through an Al₂O₃ column (aluminum oxide 90 active neutral, activity stage I; Merck), and cAMP concentration was determined by a competition immunoassay. Microtiter enzyme-linked immunosorbent assay plates (Nunc-Immuno; Maxisorp) were coated with a CAMP-BSA conjugate (gift of D. Ladant) diluted to 5 μg/ml in 0.1 M Na₂CO₃, pH 9.5. The plate wells were washed twice in 50 mM Tris-HCl, 0.15 mM NaCl, 0.1% Tween 20, pH 8.0 (TBS-Tween), saturated for 3 h with 2% BSA in TBS (TBS-BSA), and washed three times with TBS-Tween. 100 μl of sample or of the cAMP standard (Sigma) were directly added to the plate wells coated with CAMP-BSA and containing 50 μl of anti-cAMP rabbit antibodies (gift of A. Ullmann) diluted at 1:3000 in 2% TBS-BSA. Upon incubation at 4 °C overnight, the plates were washed four times with TBS-Tween, and anti-rabbit peroxidase conjugate (1:1000) was added in TBS-BSA. After incubation at 37 °C for 2 h, the wells were washed four times with TBS-Tween, and the peroxidase activity was determined using o-phenylenediamine (Sigma) as a substrate.

**Lipid Bilayer Experiments—** The methods used for black lipid bilayer experiments have been described previously (36). The experimental setup consisted of a Teflon cell with two water-filled compartments connected by a small circular hole. The
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FIGURE 1. Schematic representation of the two predicted transmembrane amphipathic α-helices in the hydrophobic pore-forming domain of CyaA. The pairs of negatively charged glutamate residues 509 plus 516 (α-helix<sub>502-522</sub>) (A) and 570 plus 581 (α-helix<sub>565-591</sub>) (B) are in both cases predicted to be localized on the same face of the given amphipathic α-helix and can be expected to form the ion filter of CyaA pores due to their negative net charge that may attract and/or relay cations through the pore. The incremental angle used to construct the helical wheel projections was 100° (performed by using Protean Lasergene software; DNASTar, Ltd.). For better readability, only 14 amino acid residues of each helix are displayed.

FIGURE 2. SDS-PAGE analysis of purified wild-type CyaA and of its mutant variants. The proteins were expressed in recombinant E. coli K12 strains transformed with the pCACT3-derived plasmids and purified from urea extracts of cell debris by a combination of ion exchange and hydrophobic chromatography as previously described (41). Proteins were separated on a 7.5% acrylamide gel and visualized by Coomassie staining.

hole had an area of about 0.4 mm<sup>2</sup>. Membranes were formed across the hole from a 1% solution of asolectin (lecithin type IIIs from soy beans from Sigma) in n-decane. The temperature was maintained at 20 °C during all experiments. All salts were obtained from Merck (analytical grade) and were buffered with 10 mM HEPES-KOH to a pH of 7. The electrical measurements were performed using Ag/AgCl electrodes (with salt bridges) connected in series to a voltage source and a homemade current-to-voltage converter. The amplified signal was recorded on a strip chart or tape recorder.

For the selectivity measurements, the membranes were formed in a 100 mM KCl solution. Toxin was added to both sides of the membrane, and the increase of the membrane conductance due to insertion of pores was observed with the electrometer (Keithley 617). After incorporation of 10–100 pores into a membrane, the instrumentation was switched to the measurement of the zero current potential, and a KCl gradient was established by adding 3 M KCl solution to one side of the membrane. Analysis of the zero current membrane potential was performed using the Goldman-Hodgkin-Katz equation (36).

For activity measurements, CyaA or CyaA mutants were added to both sides of the membrane, and the membrane conductance was taken 30 min after the addition, when further conductance increase in time was negligible (12). The aqueous phase contained 1 M KCl and was also buffered to pH 7. The applied membrane potential was 50 mV.

Statistical Analysis—Significance of differences in values was assessed by Student’s t test.

RESULTS

Substitutions of Glutamate 516 Increase Hemolytic Activity of CyaA by Extending Lifetime or by Enhancing Formation Frequency of CyaA Pores—In previous work, we showed that AC membrane translocation, as well as formation and cation selectivity of CyaA pores, depend on the structure and net charge of a potential amphipathic α-helical transmembrane segment (34). This is predicted to form between amino acid residues 502 and 522 (α-helix<sub>502-522</sub>) and comprises a pair of negatively charged glutamate residues, Glu<sup>509</sup> and Glu<sup>516</sup> (Fig. 1A). To analyze the function of this α-helix in membrane activities of CyaA in more detail, the glutamate 516 was replaced here by a helix-breaking proline residue and by a neutral glutamine residue. The mutant CyaA proteins were expressed in E. coli and purified by a combination of ion exchange and hydrophobic chromatography (Fig. 2), and their specific cell-binding, cell-invasive, and hemolytic activities were compared with those of intact CyaA, using sheep erythrocytes as target cells. The cell-invasive AC activity of CyaA constructs was first determined as the amount of the AC enzyme activity internalized into erythrocytes and protected against digestion by externally added trypsin (17).

As shown in Table 1, substitution of Glu<sup>516</sup> by the proline residue modestly reduced the capacity of CyaA-E516P to bind erythrocytes while causing ~4-fold reduction in the specific cell-invasive activity of the CyaA-E516P mutant, as compared with intact CyaA. In contrast, substitution of Glu<sup>516</sup> by the neutral glutamine residue, not expected to disrupt the α-helical structure of the mutagenized segment, had little effect on the cell association capacity of the CyaA-E516Q mutant and on the membrane translocation capacity of its AC domain (Table 1). These results are consistent with those obtained previously for proline and glutamine substitutions of the glutamate residue in position 509 in the same segment (34). The data, hence, further show that residues Glu<sup>509</sup> and Glu<sup>516</sup> are part of an α-helical structure that is crucial for AC domain translocation across target cell membrane.

On the other hand, although the specific hemolytic activity of the CyaA-E509P and CyaA-E509Q mutants was not altered, the substitution of Glu<sup>516</sup> by a glutamine residue raised this activity...
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Table 1

Activities of different CyaA mutants on erythrocytes and black lipid bilayer membranes

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Percentage of activity of CyaA on erythrocytesa</th>
<th>Pore properties of CyaA in lipid bilayers</th>
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<tr>
<td></td>
<td>Bindingb</td>
<td>Cell invasiveness</td>
</tr>
<tr>
<td>CyaA</td>
<td>100 ± 7</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>CyaA-E516P</td>
<td>76 ± 14*</td>
<td>26 ± 8*</td>
</tr>
<tr>
<td>CyaA-E516Q</td>
<td>97 ± 6</td>
<td>86 ± 9</td>
</tr>
<tr>
<td>CyaA-E516Kb</td>
<td>54 ± 6**</td>
<td>29 ± 4**</td>
</tr>
<tr>
<td>CyaA-E507P</td>
<td>68 ± 8**</td>
<td>1 ± 1**</td>
</tr>
<tr>
<td>CyaA-E507Q</td>
<td>103 ± 11</td>
<td>97 ± 6</td>
</tr>
<tr>
<td>CyaA-E507K</td>
<td>90 ± 9</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>CyaA-E581P</td>
<td>79 ± 13**</td>
<td>50 ± 11**</td>
</tr>
<tr>
<td>CyaA-E581Q</td>
<td>102 ± 10</td>
<td>85 ± 23</td>
</tr>
<tr>
<td>CyaA-E581K</td>
<td>74 ± 15*</td>
<td>2 ± 2**</td>
</tr>
</tbody>
</table>

a All activities are expressed as percentages of intact CyaA activity and represent average values ± S.D. at least three independent determinations performed in duplicate with three different toxin preparations. An asterisk indicates values significantly different from wild-type CyaA (*, p < 0.05; **, p < 0.001).

b Sheep erythrocytes (5 × 10^6/ml) were incubated at 37°C with 2 units/ml purified CyaA proteins, and after 30 min, aliquots were taken for determinations of the cell-associated AC activity and of the AC activity internalized into erythrocytes and protected against digestion by externally added trypsin (17).

c The hemolysis was measured photometrically at 541 nm as hemoglobin released in time upon incubation of sheep erythrocytes with the indicated toxins (17).

d The membranes were formed from asolectin dissolved in n-decane. The 1 M KCl solution was buffered by 10 mM HEPES and had a pH of 7.0. The applied voltage was 50 mV, and the temperature was 20°C. The most frequent single-pore conductance, G, was determined from at least 100 single pore opening events. The error corresponds to the half of the counting interval of the single pore conductance histogram.

e The number of pores with a given lifetime was plotted as a function of the mean lifetime. The graph was fitted with an exponential decay function, n = N_0 exp (−t/τ), and the lifetime was calculated as lifetime = nτ.

f Zero-current membrane potentials were determined for a 10-fold gradient of KCl. The aqueous salt solutions were buffered by 10 mM HEPES and had a pH of 7.0, T = 20°C. The permeability ratio Pcation/Pinert was calculated with the Goldman-Hodgkin-Katz equation (36) from the mean of at least three independent experiments. An asterisk indicates values significantly different from wild-type CyaA (*, p < 0.05; **, p < 0.001).

g The membrane activity of the proteins was compared after 30 min of incubation with the membranes at a protein concentration of 13 ng/ml. The number of plus signs refers to the overall conductance of the membrane/cm² induced by the various CyaA proteins under these conditions in asolectin membranes and reflects the size (conductance), the lifetime, and the specific frequency of formation of pores by the various CyaA constructs.

h The CyaA-E516K mutant toxin was originally constructed and characterized in detail by Osičkova et al. (34), showing that despite reduced cell association capacity, the protein forms CyaA pores with importantly enhanced frequency as compared with intact CyaA. In this work, activities of CyaA-E516K were measured again under the same experimental conditions as those used for other mutants (pH 7).

i Not determined due to the extremely low membrane activity of the CyaA-E581P mutant.

About 2-fold, as compared with intact CyaA. Moreover, also for the CyaA-E516P mutant, the total hemolytic activity per unit of toxin added to red blood cells was significantly enhanced, despite a reduced cell association capacity of the mutant toxin (Table 1).

It was previously shown that CyaA can form small cation-selective pores that increase the conductance of lipid bilayer membranes (12, 22). The resulting conductance of the bilayer is the product of three values reflecting distinct pore parameters: (i) the unit pore size (conductance), (ii) the unit pore lifetime, and (iii) the specific frequency of formation of CyaA pores (the number of pores formed per given amount of membrane-inserted toxin per time interval). The resulting membrane conductance increase is, indeed, a highly cooperative, nonlinear function of the toxin concentration, most likely due to formation of conductive oligomers from nonconducting CyaA monomers in an association-dissociation reaction (Hill coefficient >3 for intact CyaA). Since the introduced mutations may have affected any or all of these three CyaA pore characteristics, the molecular basis of the enhanced specific hemolytic activity of CyaA-E516Q and CyaA-E516P constructs was analyzed by comparing the characteristics of pores formed by the mutant toxins in black lipid bilayer membranes made of 1% asolectin. As shown in Table 1, at pH 7.0 in a buffered 1 M KCl solution, the most frequent single-pore conductance for intact CyaA was 42 pS. A modest reduction in conductance was observed for the CyaA-E516Q mutant pores (30 pS), whereas the pores formed by the CyaA-E516P mutant exhibited a much reduced conductance of only 8 pS. This indicates that the glutamate to proline substitution at position 516 considerably altered the pore structure. Surprisingly, despite forming smaller pores, both mutants exhibited an enhanced hemolytic activity (Table 1).

A reduced single-pore conductance would be expected to yield a decreased rather than an increased hemolytic activity. Therefore, the lifetimes of single pores formed by both mutants were analyzed in lipid bilayer membranes. As also summarized in Table 1, both the CyaA-E516P and CyaA-E516Q mutant formed pores with lifetimes about 6 times longer than those of intact CyaA. A higher frequency of pore formation was, however, not observed for the CyaA-E516P and CyaA-E516Q mutants, when pore opening events per time unit were counted at a given protein concentration (Fig. 3). These results indicate that the increased specific hemolytic activity of the CyaA-E516P and CyaA-E516Q constructs was primarily due to the prolonged lifetimes of the pores formed by these proteins. This overpowered even the significantly reduced unit conductance (size) of the CyaA-E516P pores.

Previously, also a charge reversal by lysine substitution of the glutamate at position 516 was shown to significantly enhance the specific hemolytic activity of CyaA (Table 1) (34). However, in contrast to CyaA-E516P and CyaA-E516Q, the CyaA-E516K mutant exhibited the same single-pore conductance and mean pore lifetime as intact CyaA (Table 1). The increased hemolytic activity of CyaA-E516K, however, was due to its higher propensity to form conductive pores (its capacity to form higher numbers of pores per unit of added and membrane-inserted toxin), as already shown in detail earlier (34) and also documented here in Fig. 3. These data indicate that single amino acid substitutions at position 516 can increase the hemolytic activity of CyaA by two different molecular mechanisms, through extension of
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CyaA pore lifetime (E516P and E516Q) or by increasing the specific propensity of the E516K mutant to form membrane pores (Table 1 and Fig. 3). Both mechanisms, hence, conferred an increased capacity on the CyaA constructs to permeabilize cellular membrane and provoke colloid-osmotic lysis of erythrocytes (hemolysis).

Negative Charge of Glu516 Is Crucial for Cation Selectivity of CyaA Pores—CyaA pores were previously shown to be cation-selective (12). To assess the ion selectivity of the pores formed by mutant CyaAs, zero-current membrane potentials were measured for a 10-fold KCl gradient (36) on membranes containing inserted CyaA-E516P and CyaA-E516Q. The determined ratios of cation permeability, $P_{\text{cation}}$, divided by anion permeability, $P_{\text{anion}}$ (Table 1), revealed that cation selectivity of the pores formed by CyaA-E516P and CyaA-E516Q mutants was about 4 times lower (2.5 and 3.0, respectively) than that of intact CyaA pores (10.8). This shows that a negative charge at position 516 was as such crucial for the cation selectivity of the CyaA pore, since its replacement by an uncharged residue yielded a strong decrease of pore selectivity for cations. This is in agreement with the conclusion reached earlier (34) that the residue Glu516 is localized inside or close to the membrane pore and is involved in ion filtering, most likely by repelling anions. Intriguingly, a slightly lower cation selectivity was observed for the CyaA-E516P and CyaA-E516Q pores than for the CyaA-E516K pore bearing a charge-reversing substitution. In contrast to the E516K substitution, however, the $\alpha$-helix-breaking (E516P) and neutral (E516Q) substitutions affected pore structure, reducing its size and prolonging its lifetime, and may have, hence, affected also the charge distribution at the pore mouth that also contributes to cation selectiveness of the pores.

AC Membrane Translocation and Hemolytic Activities of CyaA Are Controlled by a Potentially $\alpha$-Helical Transmembrane Structure Comprising Glutamates 570 and 581—Analysis of CyaA by the algorithm of Eisenberg et al. (37) predicted an additional transmembrane amphipathic $\alpha$-helix to form between residues 571 and 591 of the pore-forming domain (34). This $\alpha$-helix also comprises a negatively charged glutamate residue, Glu581. Moreover, analysis of the same domain by the algorithm of Rao and Argos (38) showed that a transmembrane $\alpha$-helix can already be predicted to start from the residue 565 ($\alpha$-helix565–591, AAAGAEIALQTLGTVELAS-SIALALAGA) and is involved in ion filtering, most likely by repelling anions. Moreover, the negative charge at Glu570 does not seem to be abolished, the AC translocation capacity of the CyaA-E570P, CyaA-E581P constructs to bind erythrocytes but caused an important reduction of the hemolytic activity of the proteins. Although the capacity of the CyaA-E570P mutant to translocate AC across the membrane of erythrocytes was abolished, the AC translocation capacity of the CyaA-E581P toxin was moderately reduced, showing that an $\alpha$-helical structure at residue 581 was not essential for toxin penetration into cells.

At position 570, however, it clearly was the $\alpha$-helical structure that was crucial for AC translocation capacity of CyaA, since a glutamine substitution reduced specifically only the hemolytic activity of CyaA. The E570Q substitution thus represents the first point mutation that selectively reduces the hemolytic activity of CyaA without altering its cell association and AC translocation capabilities in membranes lacking the CD11b/CD18 receptor.

Moreover, the negative charge at Glu570 does not seem to be involved in AC domain translocation and appears to be controlling only the cation selectivity, size, and frequency of CyaA pore formation, as further documented in Table 1. Indeed, the decrease of hemolytic activity of the CyaA-E570P, CyaA-E570Q, and CyaA-E570K constructs appeared to be due to a drop of the conductance (size) of pores formed by these proteins and/or due to a decreased propensity of the mutant toxins to form membrane pores. Pores of all of these mutant toxins, however, exhibited the same or very similar lifetimes as the pores formed by intact CyaA (Table 1).

Intriguingly, the charge reversing glutamate to lysine substitution at position 581 had an effect on the hemolytic activity...
opposite to that of the same substitution at position 570. Despite partially reduced erythrocyte binding capacity, the specific hemolytic activity per unit of the CyaA-E570K toxin was enhanced 4 times (Table 1). Moreover, in contrast to CyaA-E570K, the cell-invasive activity of the CyaA-E581K mutant was almost nil (Table 1). The two glutamate residues and their negative charges appear, hence, to play a quite different role in toxin activity. Given the different impacts of the proline substitutions of Glu570 and Glu581, these residues appear to be part of distinct structures, rather than being part of a single α-helix.

Altogether, these data demonstrate that the segment of CyaA comprising the pair of glutamate residues 570 and 581 plays a critical role in translocation of the AC domain of CyaA across the cellular membrane, as well as controlling the propensity of formation of CyaA pores, in that various alterations of its charge and structure can modulate the hemolytic activity of CyaA in both directions, up and down.

**Charge Reversal of Glutamate 581 Increases Pore Lifetime and Enhances Frequency of CyaA Pore Formation**—To explain the molecular basis of the highly increased specific hemolytic activity of the CyaA-E581K mutant, we determined the characteristics of pores formed by the protein in black lipid bilayer membranes. As shown in Table 1 and documented in Fig. 4A, contrary to expectation, the single-pore conductance of the pores formed by CyaA-E581K was about 2 times lower than that of intact CyaA. On the other hand, the pores formed by the CyaA-E581K toxin appeared to be importantly more stable, since their mean lifetime was approximately 4 times longer than that of pores formed by intact CyaA (10.5 s versus 2.8 s), as also shown in Table 1. Occasionally, even CyaA-E581K pores opened for times of up to several tens of seconds were observed (Fig. 4A). Furthermore, also the frequency of formation of CyaA-E581K pores was substantially enhanced over that of intact CyaA (Fig. 4B).

It should also be noted that at ~70 pm toxin concentration (13 ng/ml; Fig. 4A), single pore units could be resolved, and the membrane conductance was stable in time for both intact CyaA and the CyaA-E581K protein, whereas already at 2 times higher amounts of the proteins (~140 pm, 25 ng/ml), the CyaA-induced membrane conductance was rapidly rising, and its increase in time was particularly steep for the CyaA-E581K mutant (Fig. 4B). This is in agreement with the previously observed high concentration dependence of the pore-forming activity of CyaA, for which a Hill cooperativity coefficient of >3 was found for intact CyaA, indicating that CyaA pores consisted of three or more toxin molecules (22, 34). The recordings shown in Fig. 4B suggest that CyaA-E581K formed oligomers with an enhanced propensity, yielding smaller conductance units that assembled and opened more frequently. These data,
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TABLE 2
Activities of different CyaA mutants on erythrocytes and black lipid bilayer membranes
See the footnotes to Table 1.

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</tr>
<tr>
<td>CyaA-D557K</td>
<td>107 ± 10</td>
<td>101 ± 15</td>
</tr>
<tr>
<td>CyaA-D557N</td>
<td>80 ± 20</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>CyaA-D558K</td>
<td>91 ± 25</td>
<td>103 ± 4</td>
</tr>
<tr>
<td>CyaA-D558N</td>
<td>86 ± 32</td>
<td>97 ± 15</td>
</tr>
<tr>
<td>CyaA-D557K/D558K</td>
<td>83 ± 18</td>
<td>101 ± 15</td>
</tr>
<tr>
<td>CyaA-D557N/D558N</td>
<td>91 ± 19</td>
<td>109 ± 22</td>
</tr>
</tbody>
</table>

As shown in Table 2, the resulting toxin mutants exhibited the same cell association capacity, AC translocation, and hemolytic activities on erythrocytes as intact CyaA. Moreover, measurements of the pore characteristics of these constructs revealed similar characteristics of their single pores as those found for intact CyaA, with the exception of ion selectivity that was significantly decreased upon replacement of aspartate residue 557 or 558 by asparagine or lysine residues (Table 2). The $P_{cation}/P_{anion}$ ratios for all CyaAs bearing substitutions of Asp^{557} or Asp^{558} were, indeed, about one-third lower (6.1–7.4) than that of intact CyaA (10.8). Nevertheless, when the D557N and D558N or D557K and D558K substitutions were combined in a single molecule, the ion selectivity of their pores was not reduced any further and remained similar to that of the single mutants (Table 2). This would indicate that the residues Asp^{557} and Asp^{558} are not localized within or in close proximity of the pore mouth and may modulate the ion selectivity at a distance.

hence, show that enhanced frequency of formation and pro-
longed lifetime of the pores formed by CyaA-E581K both syn-
ergized in bringing about the highly increased specific hemol-
hytic activity of the protein.

Negative Charge of Glu{570} but Not That of Glu{581} Is Important for Cation Selectivity of CyaA Pores—Zero-current membrane potential measurements with mutant toxins having sub-
stitutions of glutamates at positions 570 and 581 revealed that the negative charge at position 570 was essential for cation selectivity of the toxin pore (Table 1). A change of charge from −1 to 0 or −1 to +1 at residue 570 led, indeed, to an important drop of the pore selectivity for cations. This indicates that the negatively charged carboxyl group of residue Glu{570} was localized within or close to the pore and influenced its ion selectivity, most likely being involved in ion filtering, similarly to residues Glu{509} and Glu{516} (see above) (34). In contrast, Glu{581} appears to be outside of the CyaA pore and part of another structure than Glu{570}, since pores formed by the mutant toxins having the negative charge eliminated at position 581 exhibited a high cation selectivity even when Glu^{581} was substituted by the charge-reversing lysine residue (Table 1).

Negative Charge of Aspartate 557 and/or 558 Also Contributes to Cation Selectivity of CyaA Pores—Further analysis of the CyaA sequence by the algorithm of Hofmann et al. (39) suggested a model for transmembrane topology of the pore-
forming domain, with the two predicted transmembrane α-helices (residues 529–549 and 563–595) being linked together by a short extracellularly localized loop, comprising two aspartate residues at positions 557 and 558 (Fig. 5). To test whether the negative net charge of these aspartates might be located at the pore mouth, attracting cations and thus contributing to the cation selectivity of the toxin pore, the Asp^{557} and Asp^{558} residues were substituted by neutral asparagine or oppositely charged lysine residues.
Mutagenesis of Adenylate Cyclase Toxin Transmembrane Segments

A

Wild-type CyaA

Translocation

Insertion

Equilibrium

Cations

ATP cAMP

AC

Translocation precursor

Pore precursor

Oligomeric cation-selective pore

B

CyaA-E509K/E516K or CyaA-E581K

Equilibrium

Cations

No translocation precursor

Pore precursor

Oligomeric cation-selective pore

C

CyaA-E509P, CyaA-E516P or CyaA-E581P

Trypsin

Translocation intermediate

Equilibrium

Insertion

Cations

ATP cAMP

Translocation precursor

Pore precursor

Oligomeric cation-selective pore
Mutagenesis of Adenylate Cyclase Toxin Transmembrane Segments

DISCUSSION

We show here that two transmembrane amphipathic α-helices, predicted to be located between residues 502–522 and 565–591 and comprising two pairs of negatively charged glutamate residues, 
Glu509 plus Glu516 and Glu570 plus Glu581, respectively, play crucial roles in activities of the toxin on target cell membrane.

Previously (34), we have shown that replacement of Glu509 or Glu516 by oppositely charged lysine residues had a substantial influence on the cation selectivity of the formed mutant CyaA pores, where the $P_{cation}/P_{anion}$ permeability ratio ($P_{cation}/P_{anion}$) of CyaA-E509K and of CyaA-E516K pores was reduced to one-half ($P_{cation}/P_{anion} = 0.5$), or one-third ($P_{cation}/P_{anion} = 0.3$) of that determined for intact CyaA ($P_{cation}/P_{anion} = 10.8$), respectively. Moreover, when the two substitutions were combined in a single molecule and the charge of the potential α-helix502–522 was changed from −2 to +2, the cation selectivity of the formed membrane pores was further decreased ($P_{cation}/P_{anion} = 2.3$). The results reported here show that a quite similar reduction of the cation selectivity is obtained for the CyaA-E516P ($P_{cation}/P_{anion} = 2.5$) and CyaA-E516Q ($P_{cation}/P_{anion} = 3.0$) mutants, which have the glutamate Glu516 replaced with neutral proline or glutamine residues and thus harbor an α-helix502–522 with a total negative charge reduced from −2 to −1. This strongly suggests that the charged segment is an important part of the cation filter inside CyaA pores, since its selectivity is highly decreased even when only one of the two glutamate residues is substituted by an uncharged or an oppositely charged residue.

The Glu570 residue of the second predicted transmembrane α-helix565–591 could constitute the other part of this filter. Its substitution with an uncharged proline or glutamine residue substantially decreased the cation selectivity of the formed membrane pores ($P_{cation}/P_{anion} = 3.3$ or 4.4, respectively). The same decrease was, moreover, observed when Glu570 was replaced by an oppositely charged lysine residue ($P_{cation}/P_{anion} = 3.6$). In contrast, the cation selectivity of the pores formed by CyaA was affected only slightly ($P_{cation}/P_{anion} = 8.2$) for the mutant having the negative charge removed at position 581 (CyaA-E581Q). Moreover, when Glu581 was substituted by a charge-reversing lysine residue, the selectivity of the single pores formed by the CyaA-E581K mutant was not affected at all ($P_{cation}/P_{anion} = 11.4$). This strongly suggests that in contrast to Glu570, the Glu581 residue does not participate directly in the transport of cations across CyaA pore and may be part of a different structure than Glu570.

Aside from the altered cation selectivity, indeed, the constructs with substitutions of Glu570 exhibited rather different properties than constructs harboring the same amino acid substitutions of Glu581: (i) the CyaA-E570P mutant had the AC translocation capacity completely abolished, whereas that of CyaA-E581P was slightly reduced; (ii) CyaA-E570Q and CyaA-E570K exhibited about half of the specific hemolytic activity of intact CyaA, whereas the hemolytic activity of the CyaA-E581Q was slightly increased, and that of the CyaA-E581K was even 4 times enhanced; and finally, (iii) the mean lifetime of the CyaA-E581K pores was substantially prolonged, as compared with pores formed by CyaA-E570K. This suggests that the Glu570 and Glu581 residues are unlikely to be part of a single α-helical segment, and the two residues appear to be contributing to toxin activities by rather independent and differing mechanisms. If the two glutamate residues are part of different secondary structures, however, both of them would be involved and cooperating during association of the toxin with cellular membrane, AC translocation, and pore formation.

It has, indeed, been shown that translocation of the AC domain across target cell membrane depends on negative membrane potential (40) and that a net positive electrostatic charge of a segment between residues 222 and 242 of the AC domain is critical for its effective translocation into cell cytosol (41). Our previous work also supports the assumption that the AC domain needs to bear an overall net positive charge in order to translocate across target cell membrane, possibly being conducted along the negatively charged hydrophilic surface formed by amphipathic transmembrane segments of the hydrophobic domain of CyaA (34). In fact, reversal of the charge of the predicted α-helix502–522 from −2 to +2 in the CyaA-E509K/E516K abolished the capacity of the double mutant to translocate the AC domain into cells (34). A similar interfering effect on AC translocation activity was observed here for the substitution of Glu581 by the positively charged lysine residue (E581K) that altered the charge of the second predicted α-helix565–591 from −2 to 0. Moreover, translocation of the AC domain of the CyaA-E516K and CyaA-E570K mutants, harboring the same charge alteration as the CyaA-E581K mutant, was partly reduced too (Table 1), and a minor reduction of AC translocation was also observed for the CyaA-E509K mutant (34).

Aside from the change of the negative net charge, also the impact of the disruption of the α-helical character of the

![Image](https://example.com/image1.png)

**FIGURE 7.** Schematic representation of the model of the interaction of CyaA with target membrane. A, two-level model of the CyaA interaction with target membrane. In solution, two conformational isomers of CyaA exist in equilibrium that, upon membrane insertion, yield either a translocation precursor competent for subsequent translocation of the AC domain across target cell membrane or a pore precursor that enters an association-dissociation reaction involved in formation of oligomeric CyaA pores. Factors such as low temperature and/or low calcium loading selectively favor insertion of pore precursors (28, 31), whereas properties of human red blood cell membrane favor insertion of AC translocation precursors (32). B, proposed model for the activity of the CyaA-E509K/E516K or CyaA-E581K mutant. The charge alterations of the α-helix502–522, of CyaA-E509K/E516K, or α-helix565–591, of CyaA-E581K selectively ablate formation and membrane insertion of AC translocation precursors, thereby reducing the total amount of membrane-inserted toxin (34) (Table 1). Membrane-inserted pore precursors of both mutants, however, associate with high frequency, thereby giving rise to the highly increased pore-forming and hemolytic activity, as compared with intact CyaA (34) (Fig. 4, Table 1). Moreover, the pore precursors of CyaA-E581K exhibit not only an increased propensity to form membrane pores but also form much more stable pores with importantly prolonged lifetimes (Fig. 4, Table 1). Although the cation selectivity of CyaA-E581K pores is unaffected (Table 1), the pores formed by CyaA-E509K/E516K exhibit significantly reduced selectivity due to charge-reversing substitutions of glutamates involved in cation filtering within the pore (34). C, model for the activity of the CyaA mutants harboring an E509P, E516P, or E581P mutation. These CyaA mutants are capable of membrane insertion in the form of translocation precursors, but contrary to wild-type CyaA, they are unable to fully translocate the AC domain into cell cytosol. The membrane-trapped translocation intermediates thus formed are able to catalyze the conversion of the intracellular ATP to cAMP, with the catalytic and calmodulin-binding sites being already exposed at the cytosolic side of the membrane while still having some portions loopsing out of the cell membrane. The latter remain accessible to cleavage by externally added trypsin, yielding enzymatically inactive fragments when extracted from cell membrane.
mutagenized segments on overall structure of CyaA yielded loss or reduction of AC translocation capacity of the mutant constructs. This interpretation of data is supported by the observed sharp effects on toxin activity of α-helix-breaking proline substitutions for glutamates at position 516, 570, or 581. Indeed, the AC translocation activity on erythrocyte membrane was abolished (CyaA-E570P) or substantially (CyaA-E516P) or partially (CyaA-E581P) reduced. In turn, despite the same reduction of negative net charge of the α-helical segments as for the proline mutants, glutamine substitutions of the same glutamate residues affected CyaA activities only marginally. CyaA constructs having the glutamate residues 516, 570, or 581 replaced by glutamine residues exhibited the same AC translocation capacities as intact CyaA (cf. AC translocation activities of CyaA-E516Q, CyaA-E570Q, and CyaA-E581Q in Table 1). Moreover, a complete loss of AC translocation capacity was previously observed also for CyaA-E509P, whereas the AC translocation capacity of the CyaA-E509Q mutant remained unaffected (34). Altogether, these observations suggest that both the net negative charge and the conformation of the α-helices predicted to form between residues 502 and 522 or 565 and 591 play a critical role in translocation of the AC domain into cell cytosol.

Support for this conclusion comes also from additional observations. Indeed, the AC translocation capacity of CyaA is typically assessed in two different ways, by in vitro assay for AC enzyme activity that was protected from inactivation by cleavage with trypsin added to toxin-treated cells or by quantifying the amounts of cAMP generated by internalized AC that catalyzed conversion of intracellular ATP to cAMP. In the first assay, cells are incubated with CyaA for a given time, and after washing-out of unbound toxin, an excess of trypsin is added to cells to digest the AC domain of the toxin that was not translocated into cellular membrane or into cytosol of cells and was therefore not protected from inactivation by cleavage with trypsin. After the addition of trypsin inhibitor and repeated washing out of trypsin, cells are solubilized by detergent, and the protected (remaining) AC enzyme activity is determined in an assay for conversion of radioactively labeled ATP to cAMP. In the second, indirect assay for translocation of AC domain into cells, erythrocytes or other cells are incubated with CyaA, and after washing out of excess unbound toxin and lysis of cells with detergent in hydrochloric acid, the amount of cAMP formed in cells due to catalytic action of AC enzyme translocated into the cytosol of cells is determined using a competition immunoadsorbent assay. In principle, the relative cell-invasive activities of CyaA mutants in percentage of cell-invasive activity of intact CyaA are expected to be the same when determined by the two approaches.

Data summarized in Fig. 6, indeed, show that this was the case for most of the constructs, where the trypsin accessibility assay and determination of intracellular cAMP levels in toxin-treated cells yielded very similar relative capacities of the mutant CyaA to deliver the AC domain across the cell membrane. The striking exceptions were the results obtained for constructs with proline residue substitutions at positions 509, 516, and 581. The CyaA-E516P mutant repeatedly exhibited about 4-fold lower cell-invasive activity when assessed by the trypsin accessibility assay, whereas its capacity to elevate cellular cAMP levels was fully retained (Fig. 6). A similar difference in cell-invasive activity was also observed for the CyaA-E581P mutant, which exhibited about half of the activity of intact CyaA to penetrate cells when assessed by the trypsin inaccessibility assay, whereas it exhibited the same activity as intact toxin when assessed by the assay for intracellularly accumulated cAMP (Fig. 6). Most intriguingly, examination of cell-invasive activity of the previously characterized CyaA mutants (34), having the glutamate residue Glu509 replaced by proline, valine, glutamine, or lysine residues, respectively, revealed that although the CyaA-E509P mutant exhibited a nil cell-invasive activity in the trypsin inaccessibility assay, it still exhibited a substantial capacity to catalyze the conversion of the intracellular ATP to cAMP and elevate its levels, which was about one-third of the activity of intact CyaA (Fig. 6). These seemingly contradictory results led us to propose that CyaA mutants harboring the proline substitutions E516P, E581P, and E509P, respectively, were unable to translocate the AC domain into cell cytosol fully, leaving some portions of the incompletely translocated AC domain extracellularly exposed and thus amenable to cleavage by trypsin. Although this membrane-trapped translocation intermediate(s) of the AC domain would still be able to bind calmodulin and to catalyze conversion of intracellular ATP to cAMP on the cytosolic side of the cell membrane, externally added trypsin would digest those parts of the AC domain that would remain protruding out of the cell. Once the nicked AC domain is extracted from cells in form of protein fragments, these would not be able to assemble into active enzyme anymore and would not catalyze conversion of radioactively labeled ATP to cAMP in the in vitro assay. This would further imply that cleavage by trypsin occurred outside of the loop linking the T25 and T18 subdomains of AC, since the cleavage in this loop yields fragments still able to form an enzymatically active ternary complex with calmodulin (35).

The observation that the AC domain of CyaA proteins harboring the E516P, E581P, and E509P substitutions is trapped at least in part in the cellular membrane as an incompletely translocated intermediate raises the question of whether the AC domain of intact CyaA is translocated into cell cytosol completely or whether it also remains partly inserted in the target cell membrane although inaccessible to digestion by extracellular trypsin. Experiments are in progress to address this issue and to determine which parts of the AC domain of the CyaA-E509P, CyaA-E516P, and CyaA-E581P mutants are looping out of the cell membrane.

Recently, we proposed a model of interaction of CyaA with the target membrane and suggested a role of the mutagenized α-helical segment comprising glutamate residues 509 and 516 in this interaction (34). This model, schematically summarized in Fig. 7, predicts that two conformational isomers of CyaA might exist in solution, one yielding membrane-inserted AC translocation precursors and the other CyaA pore precursors. The equilibrium between these two forms can be shifted in favor of the membrane insertion of pore precursors by factors such as low temperature and/or low calcium load (28, 31) or by alterations of the charge of the α-helix502–522, resulting from the replacement of Glu509 or Glu516 by oppositely charged

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lysine residues (Fig. 7B) (34). Although a rise in temperature or calcium concentration still allows the intact pore precursors to translocate their AC into cells (28), the E509K/E516K double mutation appears to lock the pore precursor in a conformation that prevents translocation of the AC domain (34). Moreover, the charge reversal of the predicted \(\alpha\)-helix502–522 from -2 to +2 reduces the total capacity of CyaA to insert into target membrane, possibly by selectively inhibiting membrane insertion of the AC translocation precursors. However, this enhances the propensity of pore precursors to associate within membrane, thereby accounting for the increased frequency of pore formation and exacerbated hemolytic activity of the CyaA-E509K/E516K mutant.

The data obtained with other mutants here are all in good agreement with and lend further support to the previously proposed model. Like the E509K/E516K double mutation, the here described E581K substitution of Glu581 in the \(\alpha\)-helix565–591, led to partial inhibition of the interaction of the AC translocation precursors with the target membrane and resulted in a lower binding capacity of the toxin and a strongly reduced capacity to translocate AC into erythrocytes (Fig. 7B, Table 1). Moreover, as in the case of CyaA-E509K/E516K, the pore precursors of the CyaA-E581K mutant were favored in membrane, exhibiting not only an increased propensity to form membrane pores, but also a capacity to form importantly more stable pores with remarkably prolonged lifetimes (Fig. 4, Table 1).

The results obtained for the CyaA mutants harboring a E509P, E516P, or E581P mutation can also be explained in terms of the above discussed model. In contrast to CyaA-E509K/E516K and CyaA-E581K, all of these mutants were apparently capable of interacting with target membrane in the form of translocation precursors, but contrary to wild-type CyaA, they were unable to fully translocate the AC domain into cell cytosol (Fig. 7C). Instead, the AC domain appeared to remain trapped in the membrane in the form of a translocation intermediate, being in a transmembrane conformation that already allowed it to catalyze the conversion of the intracellular ATP to cAMP, hence having the catalytic and calmodulin-binding site accessible from the cytosolic side of the membrane.

In conclusion, all of the results described here support the model of a parallel interaction of two distinct conformational isomers of CyaA with target cell membrane and document that individual steps of the CyaA action can be specifically blocked by mutations in two predicted and distinct \(\alpha\)-helical segments of the hydrophobic domain of the toxin, both of them comprising pairs of negatively charged glutamate residues 509, 516, 570, and 581.

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REFERENCES
