RESEARCH LETTER

Esterase activity of *Bordetella pertussis* CyaC-acyltransferase against synthetic substrates: implications for catalytic mechanism *in vivo*

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Abstract

Adenylate cyclase-hemolysin toxin (CyaA) produced from the human respiratory tract pathogen *Bordetella pertussis* requires fatty-acyl modification by CyaC-acyltransferase to become an active toxin. Previously, the recombinant CyaA pore-forming (CyaA-PF) fragment expressed in *Escherichia coli* was shown to be hemolytically active upon palmitoylation *in vivo* by cosynthesized CyaC. Here, the 21-kDa CyaC enzyme separately expressed in *E. coli* as an inclusion body was solubilized in 8 M urea and successfully refolded into an enzymatically active monomer. In addition to the capability of activating CyaA-PF *in vitro*, CyaC showed esterase activity against *p*-nitrophenyl acetate (pNPA) and *p*-nitrophenyl palmitate (pNPP), with preferential hydrolysis toward pNPP when compared with chymotrypsin. A homology-based CyaC structure suggested a conceivable role of a catalytic triad including Ser^{30}, His^{33} and Tyr^{66} in substrate catalysis. Alanine substitutions of these individual residues caused a drastic decrease in specific activities of all three mutant enzymes (S30A, H33A and Y66A) toward pNPP, signifying that CyaC-acyltransferase shares a similar mechanism of hydrolysis with a serine esterase in which Ser^{30} is part of the catalytic triad.

Introduction

Bordetella pertussis, an important human pathogen causing whooping cough, secretes a 1706-amino acid-long adenylate cyclase-hemolysin toxin (CyaA), which is a key virulence factor of this bacterium (Carbonetti *et al*., 2005). CyaA, a bifunctional repeat-in-toxin (RTX), consists of adenylate cyclase (AC) and pore-forming (PF) domains (Sebo & Ladant, 1993). The CyaA protoxin (proCyaA) is converted intracellularly to the mature toxin by palmitoylation (Hackett *et al*., 1994) that is catalyzed by the coexpressed acyltransferase (CyaC) using the acyl–acyl carrier protein (acyl-ACP) as the fatty acid donor (Westrop *et al*., 1996).

Primary targets of CyaA are myeloid phagocytic cells expressing CD11b/CD18 ($\alpha_M\beta_2$ integrin) as a toxin receptor (Guermonprez *et al*., 2001). CyaA delivers its catalytic AC domain into target cells directly, which causes an uncontrolled increase in cAMP leading to cell death by apoptosis (Khelef *et al*., 1993). CyaA can also exert hemolytic activity against sheep erythrocytes as it forms small cation-selective channels in cell membranes, causing colloid-osmotic cell lysis (Bellalou *et al*., 1990). It has been shown that CyaA requires palmitoylation for both cytotoxic and hemolytic activities (Hackett *et al*., 1994). The conjugated palmitoyl group was suggested to increase membrane affinity of CyaA for efficient attachment to target membranes by acting as either a mediator of membrane association or a determinant of specific protein–protein interactions (Masin *et al*., 2005).

In our previous studies, the recombinant CyaA-PF protein (residues 482–1706) coexpressed with CyaC in *Escherichia coli* was found to be palmitoylated *in vivo* at Lys^{983} to become hemolytically active (Powthongchin & Angsuthanasombat, 2008). However, the precise mechanism of CyaA acylation by CyaC-acyltransferase has not yet been fully elucidated. Although it has been reported that CyaC was able to convert the inactive proCyaA *in vitro* into an active toxin exerting biological activities, its enzymatic behavior has not been clearly characterized (Westrop *et al*., 1996). In

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this study, we demonstrate that the recombinant CyaC-acyltransferase, overexpressed in *E. coli* and successfully refolded *in vitro*, was able to hydrolyze two synthetic substrates [*p*-nitrophenyl acetate (pNPA) and *p*-nitrophenyl palmitate (pNPP)] and activate proCyaA-PF *in vitro* to become hemolytically active. In addition, a plausible three-dimensional (3D) CyaC structure built by homology-based modeling suggested a conceivable role of a catalytic triad (Ser^{30}, His^{33} and Tyr^{66}) in comparison with chymotrypsin. Single-alanine substitutions of the proposed catalytic residues suggest that these residues are essential for acyl-enzyme intermediate reaction. We thus report a novel finding of serine esterase activity of CyaC-acyltransferase against the substrate analogs through a possible mechanism related to the known hydrolytic reaction via a catalytic triad.

Materials and methods

Expression of proCyaA-PF, CyaA-PF and CyaC

Escherichia coli BL21(DE3)pLysS harboring the recombinant plasmids pCyaAC-PF encoding CyaA-PF with CyaC, pCyaA-PF (without CyaC) or pCyaC (Powthongchin & Angsuthanasombat, 2008), was grown at 30 °C in Luria–Bertani medium containing 100 μg mL^{-1} ampicillin. Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h. Cultured cells were harvested by centrifugation at 5000 ***g*** (4 °C, 15 min), resuspended in 25 mM HEPES (pH 7.0) and disrupted via French Pressure Cell at 10 000 psi. Soluble and insoluble fractions of cells were separated by centrifugation at 10 000 ***g*** (4 °C, 20 min) and analyzed by sodium dodecyl sulfate (12% w/v) polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were measured via Bradford microassay (Bio-Rad).

Preparations of soluble and refolded CyaC

For preparation of soluble CyaC, the protein was initially purified using a cation-exchange FPLC system (8-mL Mono S column; GE Healthcare). The column was equilibrated with buffer A [25 mM HEPES (pH 7.0), 1 mM 1,4-dithiothreitol]. Chromatographic separations were achieved with an increased step gradient of buffer B (1 M NaCl in buffer A) via 20% B (5-column volume), 20–30% B (2.5-column volume) and 30–100% B (2.5-column volume). Elution fractions across the 700 mM NaCl peak were pooled and subjected to further purification by hydrophobic interaction chromatography (HIC, 5-mL HiTrap™Phenyl HP column). Separation was achieved via a stepwise decrease of 2 M NaCl concentrations in buffer A. Subsequently, the eluted fraction at 2 M NaCl was loaded onto gel filtration (25-mL Superdex™75 column) equilibrated with buffer A at flow rate of 0.4 mL min^{-1}. Peak fractions containing the 21-kDa protein were pooled and concentrated by ultrafiltration using 50-mL Centriprep column (10-kDa cutoff).

For preparation of refolded CyaC, insoluble inclusions were washed with 80 mM K_2HPO_4 (pH 6.5) containing 0.8 M NaCl and 0.1% Triton X-100, followed by washing twice with cold distilled water. CyaC inclusions (1–5 mg mL^{-1}) were solubilized in 20 mM Tris-HCl (pH 8.0) containing 8 M urea at 37 °C for 1 h. After centrifugation at 18 000 ***g*** for 20 min, the unfolded CyaC protein was initially refolded in Superdex™75 column equilibrated with refolding buffer [20 mM Tris-HCl (pH 8.0), 2 M urea, 150 mM NaCl]. The eluted monomeric CyaC fraction was dialyzed against 300 volumes of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1 M urea at 4 °C for 4 h, and finally dialyzed twice against the same buffer without urea.

Identification of CyaC by LC/MS/MS

Purified CyaC separated by SDS-PAGE (12% gel) was eluted out from the excised gel by soaking with 0.1 M NH_4HCO_3 and subsequently digested with trypsin at a substrate : enzyme ratio of 10 : 1. Trypsin-generated fragments were separated on a 0.18 × 100-mm C18 column (Thermo Electron) and analyzed by LC/MS/MS (Finnigan LTQ Linear Ion Trap Mass Spectrometer).

In vitro activation of proCyaA-PF by CyaC and assessment for hemolysis

Toxin activation *in vitro* mediated by CyaC was performed by mixing 10 μg of purified CyaC monomer with *E. coli* cell lysate (1 mg total protein) containing 10 μg proCyaA-PF and cytosolic acylating factors (fatty acids and acyl-ACP). The mixture was adjusted to 1-mL reaction with hemolysis buffer [25 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM $CaCl_2$] and incubated at 37 °C for 30 min.

For hemolysis assay, 10 μL sheep erythrocytes (10^9 cells) were added to 1-mL activation mixture. The assay mixture was incubated at 37 °C for 5 h and then centrifuged at 10 000 ***g*** for 5 min to remove unlysed cells. Hemoglobin released in the supernatant was measured at $OD_{540\,nm}$. Background hemolysis of the untreated control sample was determined by incubating cells in buffer alone. An $OD_{540\,nm}$ value corresponding to complete hemolysis was obtained by lysing the erythrocytes with 0.1% Triton X-100. The percentage of hemolysis was calculated by $[(OD_{540\,nm}$ sample $- OD_{540\,nm}$ negative control)/($OD_{540\,nm}$ of 100% hemolysis $- OD_{540\,nm}$ negative control)$] \times 100$.

Assays for esterase activity of CyaC

Two chromogenic substrates, pNPA and pNPP, were used for assaying ester-bond hydrolysis. Purified CyaC (4.5 μg) was added to 300 μL of 50 mM Tris-HCl (pH 7.4) containing

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1 mM pNPA (1% v/v final acetonitrile concentration) or 100 μM pNPP (5% v/v final isopropanol concentration). Esterolytic reaction was determined from the formation of *p*-nitrophenol (pNP) product by measuring $OD_{400\,nm}$ ($\varepsilon = 11.6\,mM^{-1}\,cm^{-1}$) (Elbaum & Nagel, 1981) with a Soft-Max Pro spectrophotometer (0.7-cm light-path). The reaction was performed simultaneously with a CyaC-free blank. The amount of enzyme liberating 1 μmol pNP min^{-1} was defined as one enzyme unit (U). Specific activity (μmol min^{-1} mg^{-1} protein or U mg^{-1} protein) of ester-bond hydrolysis was used to determine the activity of each sample in comparison with α-chymotrypsin (TLCK-treated, type VII from bovine pancreas, Sigma).

Circular dichroism (CD) analysis of CyaC

CD measurements of the 21-kDa FPLC-purified CyaC protein (0.4 mg mL^{-1} in 20 mM Tris-HCl, pH 8.0) were performed on a Jasco J-715 CD spectropolarimeter in the far UV region (190–280 nm) at 25 °C using a rectangular quartz cuvette (0.2-mm optical path-length). CD spectra were recorded at scanning rate of 20 nm min^{-1} with a 2-nm spectral bandwidth and 2-ms response times. CD signals (mdeg) averaged from five measurements were converted into mean residue ellipticity (deg cm^2 $dmol^{-1}$). Secondary structure contents were estimated from the spectra using CDPRO software (Manavalan & Johnson, 1987).

Homology-based modeling of CyaC structure

Multiple sequence alignments of eight homologous RTX-C proteins were aligned with CyaC and *Bordetella parapertussis* L-2,4-diaminobutyric acid acetyltransferase (DABA) (PDB-3D3S) using the CLUSTALX program. The 3D model of CyaC was generated based on DABA structure using the WHATIF program (Vriend, 1990). Insertion regions in the model relative to the DABA template was accomplished by extracting from short fragment database using loop-search algorithm in the WHATIF program (Vriend, 1990). The entire CyaC structure was subjected to energy minimization using GROMOS96 simulation software (Christen *et al.*, 2005). Packing environments, bond lengths and bond angles were analyzed using the Check option in WHATIF program (Vriend, 1990). Stereochemical parameters of the model were analyzed with the PROCHECK program (Laskowski *et al.*, 1996).

Site-directed mutagenesis of CyaC

The pCyaC plasmid encoding the 21-kDa CyaC-acyltransferase (Powthongchin & Angsuthanasombat, 2008) was used as a template for single-alanine substitutions at Ser^{30}, His^{33} and Tyr^{66}, using a pair of mutagenic oligonucleotides as follows: S30A (f-primer, 5′-GATGAAC**G**CTCCCATGCA**T**CGCGACTGGCCGGT-3′ and r-primer, 5′-GTCGCG**A**TGCATGGGAG**C**GTTCATCCACAGCCAG-3′, with bold letters indicating changed nucleotides and underlined bases indicating a added NruI restriction site); H33A (f-primer, 5′-CCCATG**GC**CCCGCGACTGG-3′ and r-primer, 5′-CGCGG**GC**CATGGGAGAGT-3′, with bold letters indicating changed nucleotides and underlined bases indicating an added NcoI restriction site); Y66A (f-primer, 5′-GTTGCA**GCA**TGCAGCTGGGC-3′ and r-primer, 5′-GCTGCA**TGC**TGCAACCGGCA-3′, with bold letters indicating changed nucleotides and underlined bases indicating a deleted PstI restriction site). All mutant plasmids were generated by PCR-based directed mutagenesis using a high-fidelity Pfu DNA polymerase, following the procedure of the QuickChange™ Mutagenesis Kit (Stratagene). Selected *E. coli* clones with the required mutations were initially identified by restriction endonuclease analysis and subsequently verified by automated DNA sequencing. Each refolded monomeric CyaC mutant was prepared according to the method described above for the wild type.

Results and discussion

Recently, we have shown that only the 126-kDa CyaA-PF fragment (without AC domain) coexpressed with CyaC in *E. coli* was able to be palmitoylated *in vivo* at Lys^{983} to become hemolytically active (Powthongchin & Angsuthanasombat, 2008). Here, further attempts were made to obtain more insights into functional and structural details of CyaC-acyltransferase using the proCyaA-PF fragment as a target of toxin acylation *in vitro*.

Expression and purification of CyaC proteins

Upon IPTG-induced expression at 30 °C via the utility of T7 promoter in *E. coli*, the 21-kDa protein, which is verified to be CyaC by LC/MS/MS, was produced mostly as inclusions (~100 mg L^{-1} of culture) together with small amount of the soluble form (≤ 5 mg L^{-1} of culture) (Fig. 1a). Despite its low expression, the soluble CyaC portion was able to activate proCyaA-PF *in vitro* as shown by toxin activity against sheep erythrocytes (Table 1). Therefore, the soluble CyaC protein presumed to adopt a native-folded form was initially chosen for purification. Using three consecutive chromatographic techniques, CyaC was predominantly eluted at a concentration of 700 mM NaCl by cation-exchanger (Fig. 2a, lane 2), subsequently eluted with 2 M NaCl by HIC (Fig. 2a, lane 3) and finally purified by gel filtration as a single peak at an elution volume corresponding to a 21-kDa monomer, which was obtained with ~90% purity and ~20% yield recovery (~1 mg L^{-1} of culture) as analyzed by SDS-PAGE (Fig. 2a, lane 4).

Attempts were also made to prepare a large quantity of enzymatically active CyaC from high-yield expressed

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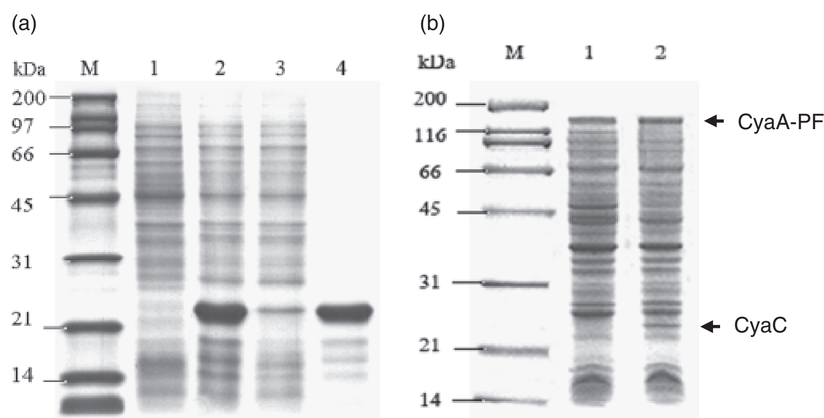

Fig. 1. SDS-PAGE (Coomassie blue-stained 12% gel) of cell lysate from *Escherichia coli* containing the 21-kDa CyaC and 126-kDa CyaA-PF proteins. (a) Soluble and insoluble fractions of CyaC. Lanes 1 and 2, lysate harboring pET-17b and pCyaC, respectively. Lanes 3 and 4, supernatant and pellet fractions, respectively, obtained after centrifugation of the lysate from lane 2. (b) Soluble fractions of proCyaA-PF and CyaA-PF. Lanes 1 and 2, soluble fractions of lysate harboring pCyaA-PF and pCyaAC-PF, respectively. M, molecular mass standards.

Table 1. *In vitro* activation of CyaA-PF via hemolytic activity assays

Reaction mixture	Hemolytic activity* (% hemolysis ± SEM)
Lysate containing proCyaA-PF+soluble CyaC	31.4 ± 4.6
Lysate containing proCyaA-PF+refolded CyaC	29.4 ± 2.9
ProCyaA-PF-free lysate+soluble CyaC	2.8 ± 0.3
Lysate containing proCyaA-PF	5.0 ± 1.4
Lysate containing CyaA-PF	88.1 ± 6.8

*Percentage values were averaged from three independent experiments performed in duplicate.

inclusions for further crystallization and X-ray crystallographic studies. CyaC inclusions were completely dissolved in 8 M urea at 37 °C for 1 h (Fig. 2b, lane 1). A fast removal of urea in the refolding step using a reciprocal dialysis or a high dilution (10–100-fold) of the unfolded CyaC solution resulted in a large fraction (≥ 80%) of sediment aggregates. It has been shown that certain aggregation suppressors (e.g. NaCl) added to the refolding solution at an intermediate-denaturant concentration can induce denatured proteins to refold into globular shape favoring a native conformation (Lairez *et al.*, 2003). Herein, one-step reduction of urea to an intermediate concentration (2 M) of the denatured CyaC solution supplemented with 150 mM NaCl was found to recover a high proportion of refolded monomers (Fig. 2b, lane 2) as observed by size-exclusion chromatography. Thus, this cardinal step allowed us finally to obtain the urea-free refolded CyaC protein with ~90% purity and ~70% yield recovery (~70 mg L^{-1} of culture) as analyzed by SDS-PAGE (Fig. 2b, lane 3). It should be noted that the 21-kDa purified proteins obtained from both soluble and insoluble fractions were reverified to be CyaC-acyltransferase as their part of trypsin-generated peptide sequence (DWPVHLLARNTLAPIQLGQYILLR) analyzed by LC/MS/MS, perfectly matching the corresponding CyaC sequence (residues Asp^{35}-Arg^{58}).

Activities of CyaC via CyaA-PF activation and hydrolysis of synthetic substrates

As mentioned earlier, the CyaA-PF fragment (Fig. 1b, lane 2) can be acylated *in vivo* by coexpressed CyaC to exhibit hemolytic activity (Powthongchin & Angsuthanasombat, 2008). By this activation analogy, we initially used this fragment as an acylated target for testing the activating activity of CyaC. When the cell lysate containing proCyaA-PF (Fig. 1b, lane 1) was mixed with the purified CyaC protein, it showed high hemolytic activity against sheep erythrocytes (~30%). In contrast, the lysate containing proCyaA-PF alone or the proCyaA-PF-free lysate mixed with CyaC exhibited very weak activity (≤ 5%) (Table 1). These results indicate that the proCyaA-PF fragment could be acylated by CyaC *in vitro*. It was also observed that both soluble and refolded CyaC could activate the proCyaA-PF fragment *in vitro* to show comparable hemolysis of ~30%, suggesting that the refolded CyaC is likely to exist as an active monomer corresponding to the native-folded protein in soluble fraction. Thus, this hemolytic activity could be inferred as the CyaC capability in transferring acyl group to the proCyaA-PF acceptor. Further attempts were therefore made to assay its catalyzing capability of acyl group, as this has not been characterized thus far for any RTX-acyltransferases.

It has been shown that homoserine acyltransferase (Ziegler *et al.*, 2007) and arylamine *N*-acetyltransferase (Pluvinage *et al.*, 2007) also catalyze a related reaction *in vitro* – namely, the hydrolysis of oxygen–ester bond of a nonphysiological substrate (i.e. pNPA). Herein, a spectrophotometric assay was developed for detection of esterase activity using two indolyl substrates, pNPA and pNPP, which contain acetyl and palmitoyl (C16:0) units, respectively. As the best-known feature of enzyme-catalyzed ester hydrolysis (Hardman *et al.*, 1971) chymotrypsin was used as a control in the reaction. Table 2 shows that specific

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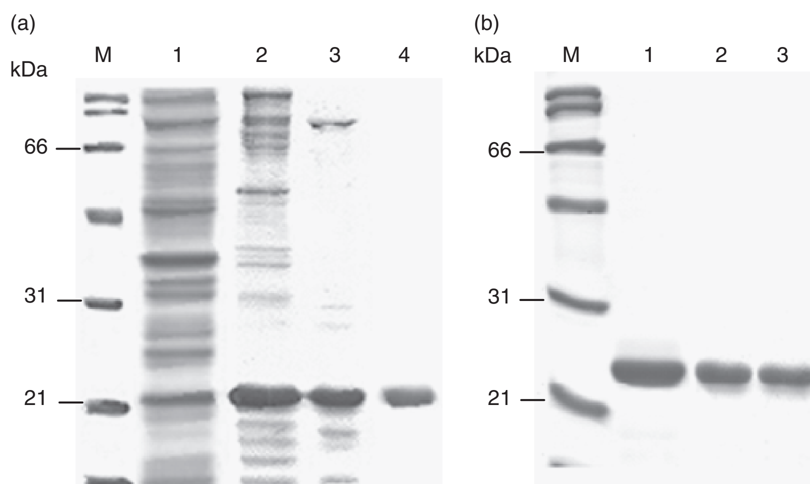

Fig. 2. SDS-PAGE (Coomassie blue-stained 12% gel) of CyaC purification. (a) Purification profiles of soluble CyaC: lane 1, supernatant fraction before purification; lane 2, CyaC eluted from cation exchanger across 700 mM NaCl; lane 3, eluted CyaC fraction at 2 M NaCl from HIC; lane 4, CyaC purified from gel filtration. (b) Unfolding–refolding of CyaC inclusions: lane 1, solubilized protein in 8 M urea; lane 2, refolded CyaC monomer eluted from size-exclusion column with 2 M urea + 150 mM NaCl; lane 3, final pure-refolded CyaC after removal of residual urea. M, molecular mass standards. Part (b) contains only lanes M, 1, 2 and 3.

Table 2. Specific activity of CyaC toward pNPA and pNPP

	Specific activity* (U mg^{-1} protein ± SEM)	
Substrate	pNPA	pNPP
Chymotrypsin	68.5 ± 1.9	16.2 ± 2.0
Refolded CyaC	46.6 ± 1.5	289.4 ± 1.2
Soluble CyaC	49.0 ± 3.2	ND
Bovine serum albumin	3.0 ± 0.7	4.2 ± 1.1

*Results were averaged from three independent experiments performed in duplicate. One unit of enzyme activity is defined as the amount of enzyme liberating 1 μmol of pNP min^{-1} at 37 °C.
ND, not determined.

activities of the purified CyaC enzyme in catalyzing pNPA and pNPP are ~49 U mg^{-1} and ~289 U mg^{-1}, respectively, indicating that CyaC exerted a much higher esterase activity toward a palmitoyl group, which has been shown to be a preferred physiological substrate (Havlicek *et al.*, 2001). Conversely, pNPA was preferred over pNPP for the chymotrypsin activity under the conditions used. We noted that both soluble and refolded CyaC showed relatively the same specific activity in catalyzing pNPA that was consistent with the CyaA-PF hemolytic activities upon *in vitro* activation by either form of CyaC. Despite the fact that CyaC-acyltransferase and chymotrypsin exhibit different substrate preferences, their reactions toward these analogs may share a common feature regarding the hydrolysis of oxygen–ester bond. Therefore, structural insights into the mechanistic basis for the esterolytic reaction of CyaC in comparison with this serine esterase are of great interest.

Homology-based CyaC structure with implications for its catalysis

As the crystal structure of CyaC-acyltransferase has not been yet resolved, a plausible 3D structure of this enzyme was built instead by modeling based on the known DABA structure, which is the best-fit template available so far in the acetyltransferase group. As shown in Fig. 3, although pairwise alignment between DABA and CyaC displays only ~30% sequence similarity, multiple alignments show relatively high similarity (~50%) among all the nine related RTX-acyltransferases with the same template, implying a common 3D-folded structure for these enzymes. Validating the model, its stereochemical quality showed an overall G-factor value of − 0.15, which is in the range of good quality (the best model displaying a value close to 0) (Laskowski *et al.*, 1996). The Ramachandran plot of the CyaC model revealed that over 90% of nonglycine and nonproline residues possess ϕ/ψ backbone-dihedral angles in energetically favorable and allowed regions. This indicates that the modeled structure has most of the sterically favorable main-chain conformations. As also assessed by CD spectroscopy, secondary structural contents of purified CyaC were found to be 25% helix and 27% β-strand, comparable to those estimated from the derived model (26% helix and 22% β-strand), supporting the validity of this model.

As shown in Fig. 4a, the CyaC structure (Leu^{26}-Ala^{185}) comprises of a single domain with a β-sheet core of six strands (βA, βB, βC, βD, βE and βF) connected by five α-helices (αA, αB, αC, αD and αE) to form a two-layer α/β sandwich, which is a typical fold of α/β hydrolase family (Holmquist, 2000). Using molecular surface analysis, a hydrophobic groove was clearly visible in the CyaC structure (Fig. 4b). This putative substrate-binding groove could likely serve as an acyl-binding pocket. This might explain the highly efficient catalysis of pNPP by this enzyme as the hydrophobic interactions would contribute more significantly to the palmitate-binding affinity in this apolar cavity.

By analogy to the feature of α/β hydrolase-fold enzymes, including acetyltransferases, chymotrypsin-like serine proteases and esterases (Holmquist, 2000), the CyaC model also

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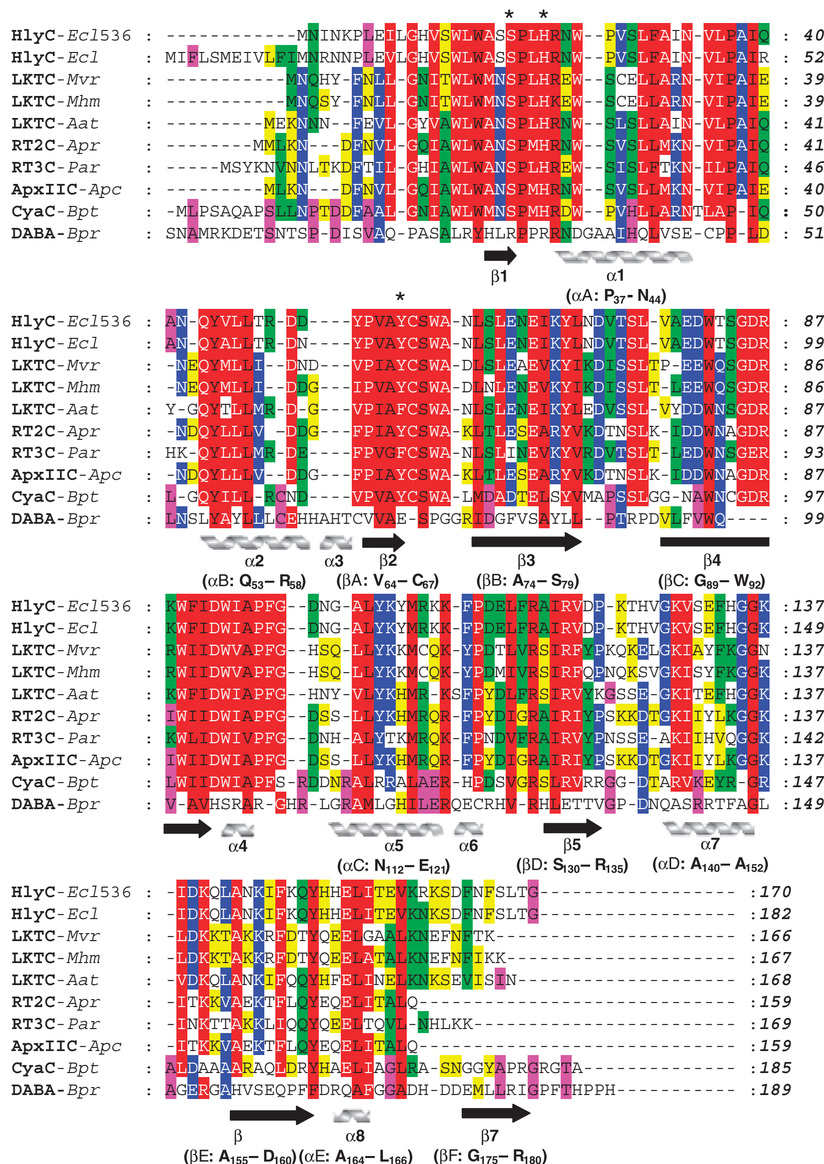

Fig. 3. Multiple sequence alignments of nine RTX-C acyltransferases with DABA. HlyC-*Ecl536*, *Escherichia coli*-536 hemolysin-acyltransferase (NC_008253.1); HlyC-*Ecl*, *E. coli* hemolysin-acyltransferase (AJ488511.1); LKTC-*Mvr*, *Mannheimia varigena* leukotoxin-acyltransferase (Q6TB08); LKTC-*Mhm*, *Mannheimia haemolytica* leukotoxin-acyltransferase (P55121); LKTC-*Aat*, *Actinobacillus actinomycetemcomitans* leukotoxin-acyltransferase (X16829.1); RT2C-*Apr*, *Actinobacillus pleuropneumoniae* RTX-II toxin-acyltransferase (P0A3I3); RT3C-*Par*, *Pasteurella aerogenes* PaxC-acyltransferase (U66588.1); ApxIIC-*Apr*, *A. pleuropneumoniae* ApxIIC-acyltransferase (Q5DI85); ApxIIC-*Apc*, *Actinobacillus porcitonsillarum* ApxIIC-acyltransferase (Q5DI90); CyaC-*Bpt*, *Bordetella pertussis* CyaC-acyltransferase (EF_592556); DABA-*Bpr*, *Bordetella parapertussis* DABA-domain A (PDB-3D3S). Degree of conservation among the sequences is highlighted by shading residues with red (white–black characters), blue, green and yellow for 90–100%, 70–80%, 50–60% and 30–40% homology, respectively. More regions of homology between CyaC and DABA are highlighted in pink. Catalytic triad residues (Ser^{30}, His^{33} and Tyr^{66}) are indicated by *. Positions of secondary structure elements of DABA and CyaC (in blanket) are illustrated under the alignments.

reveals a putative catalytic triad (Ser^{30}, His^{33} and Tyr^{66}) with good geometric relationships corresponding to that of chymotrypsin (Ser^{195}, His^{57} and Asp^{102}) (Fig. 4c and d). Interestingly, the catalytic triad Ser^{30}–His^{33}–Tyr^{66} proposed for CyaC-acyltransferase is highly conserved among the RTX-acyltransferase family (Fig. 3). We have, therefore, performed single-alanine substitutions at these individual residues to validate their contribution to the CyaC esterolytic mechanism. The results revealed that all three mutations (S30A, H33A and Y66A) caused a severe loss in esterolytic activity of the mutant enzymes toward pNPP (see Fig. 5), signifying a vital role in the catalytic behavior for these three conserved residues. This is in agreement with the previous study that a nearly complete loss in acyltransferase activity of CyaC was observed for S30R, S30W, H33S and H33D mutants (Basar *et al.*, 2001). Also for HlyC-acyltransferase, Ser^{20}, His^{23}, Tyr^{70} and Tyr^{150} have been identified to be involved in acyl-transfer catalysis (Trent *et al.*, 1999). As also inferred from the model, Tyr^{66} is likely to help orient the imidazole ring of His^{33} and make a better proton acceptor through hydrogen bonding, similar to Asp^{102} in the catalytic triad of chymotrypsin (see Fig. 4c and d). We thus propose that CyaC-acyltransferase is conceivably a serine esterase in which Ser^{30} is part of a catalytic triad that also includes His^{33} and Tyr^{66}, forming a hydrogen-bonding network.

In conclusion, we have provided pivotal evidence for the first time that the purified recombinant CyaC-acyltransferase, which exists as a monomer clearly exhibits an esterase

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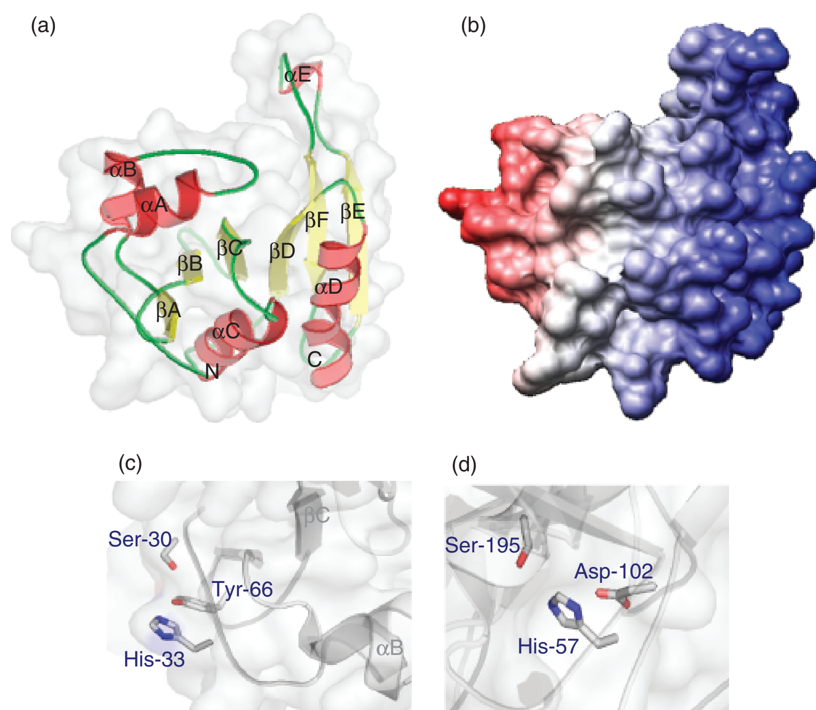

Fig. 4. (a) Ribbon diagram of the homology-based CyaC structure. Helices, strands and coils are colored in red, yellow and green, respectively. (b) Molecular surface electrostatic potential of CyaC model as analyzed by CHIMERA program as surface is colored according to electrostatic potential contoured from − 10 (red) to 10 (blue) kT/e. (c) Proposed catalytic triad of CyaC consisting of Ser^{30}, His^{33} and Tyr^{66} in comparison with that of (d) chymotrypsin (PDB-1ACB) (Ser^{195}, His^{57} and Asp^{102}). (a), (c) and (d) are generated using the PYMOL program.

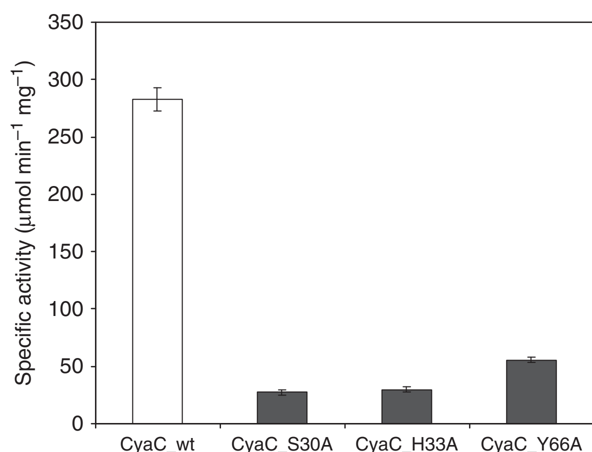

Fig. 5. Specific activities of wild-type and mutant CyaC enzymes toward pNPP substrate. Error bars indicated SEMs from three independent experiments. Shaded boxes represent the specific activity of the mutants that are significantly different (*P*-values < 0.001) from that of the wild-type enzyme.

activity toward the substrate analogs. Based on our 3D CyaC model together with mutagenesis studies, three highly conserved residues, Ser^{30}, His^{33} and Tyr^{66}, were proposed to be a catalytic triad essentially required for enzyme catalysis corresponding to a serine esterase. Nevertheless, the challenge remains of determining the CyaC crystal structure, which would provide more structural and functional details of its mechanistic basis for esterolytic reaction.

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References

Basar T, Havlicek V, Bezouskova S, Hackett M & Sebo P (2001) Acylation of lysine 983 is sufficient for toxin activity of *Bordetella pertussis* adenylate cyclase. Substitutions of alanine 140 modulate acylation site selectivity of the toxin acyltransferase CyaC. *J Biol Chem* **276**: 348–354.

Bellalou J, Sakamoto H, Ladant D, Geoffroy C & Ullmann A (1990) Deletions affecting hemolytic and toxin activities of *Bordetella pertussis* adenylate cyclase. *Infect Immun* **58**: 3242–3247.

Carbonetti NH, Artamonova GV, Andreasen C & Bushar N (2005) Pertussis toxin and adenylate cyclase toxin provide a one-two punch for establishment of *Bordetella pertussis* infection of the respiratory tract. *Infect Immun* **73**: 2698–2703.

Christen M, Hunenberger PH, Bakowies D *et al.* (2005) The GROMOS software for biomolecular simulation: GROMOS05. *J Comput Chem* **26**: 1719–1751.

Elbaum D & Nagel RL (1981) Esterase activity of hemoglobin. Differences between HB A and HB S. *J Biol Chem* **256**: 2280–2283.

Guermonprez P, Khelef N, Blouin E *et al.* (2001) The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the

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alpha(M)beta(2) integrin (CD11b/CD18). *J Exp Med* **193**: 1035–1044.
Hackett M, Guo L, Shabanowitz J, Hunt DF & Hewlett EL (1994) Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis*. *Science* **266**: 433–435.
Hardman MJ, Valenzuela P & Bender ML (1971) Acylation of α- and δ-chymotrypsins by *p*-nitrophenyl acetate. Enzyme–substrate complex formation and pH dependence. *J Biol Chem* **246**: 5907–5913.
Havlicek V, Higgins L, Chen W, Halada P, Sebo P, Sakamoto H & Hackett M (2001) Mass spectrometric analysis of recombinant adenylate cyclase toxin from *Bordetella pertussis* strain 18323/pHSP9. *J Mass Spectrom* **36**: 384–391.
Holmquist M (2000) Alpha/beta-hydrolase fold enzymes: structures, functions and mechanisms. *Curr Protein Pept Sc* **1**: 209–235.
Khelef N, Zychlinsky A & Guiso N (1993) *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. *Infect Immun* **61**: 4064–4071.
Lairez D, Pauthe E & Pelta J (2003) Refolding of a high molecular weight protein: salt effect on collapse. *Biophys J* **84**: 3904–3916.
Laskowski RA, Rullmannn JA, MacArthur MW, Kaptein R & Thornton JM (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J Biomol NMR* **8**: 477–486.
Manavalan P & Johnson WC Jr (1987) Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra. *Anal Biochem* **167**: 76–85.
Masin J, Basler M, Knapp O, El-Azami-El-Idrissi M *et al*. (2005) Acylation of lysine 860 allows tight binding and cytotoxicity of *Bordetella* adenylate cyclase on CD11b-expressing cells. *Biochemistry* **44**: 12759–12766.
Pluvinage B, Dairou J, Possot OM *et al*. (2007) Cloning and molecular characterization of three arylamine *N*-acetyltransferase genes from *Bacillus anthracis*: identification of unusual enzymatic properties and their contribution to sulfamethoxazole resistance. *Biochemistry* **46**: 7069–7078.
Powthongchin B & Angsuthanasombat C (2008) High level of soluble expression in *Escherichia coli* and characterisation of the CyaA pore-forming fragment from a *Bordetella pertussis* Thai clinical isolate. *Arch Microbiol* **189**: 169–174.
Sebo P & Ladant D (1993) Repeat sequences in the *Bordetella pertussis* adenylate cyclase toxin can be recognized as alternative carboxy-proximal secretion signals by the *Escherichia coli* alpha-haemolysin translocator. *Mol Microbiol* **9**: 999–1009.
Trent MS, Worsham LM & Ernst-Fonberg ML (1999) HlyC, the internal protein acyltransferase that activates hemolysin toxin: roles of various conserved residues in enzymatic activity as probed by site-directed mutagenesis. *Biochemistry* **38**: 9541–9548.
Vriend G (1990) WHAT IF: a molecular modeling and drug design program. *J Mol Graphics* **8**: 52–56.
Westrop GD, Hormozi EK, Da Costa NA, Parton R & Coote JG (1996) *Bordetella pertussis* adenylate cyclase toxin: proCyaA and CyaC proteins synthesised separately in *Escherichia coli* produce active toxin *in vitro*. *Gene* **180**: 91–99.
Ziegler K, Yusupov M, Bishop B & Born TL (2007) Substrate analysis of homoserine acyltransferase from *Bacillus cereus*. *Biochem Bioph Res Co* **361**: 510–515.

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