# Characterization of ATP and Calmodulin-Binding Properties of a Truncated Form of *Bacillus anthracis* Adenylate Cyclase†

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ABSTRACT: The *Bacillus anthracis cya* gene encodes a calmodulin-dependent adenylate cyclase. A deletion *cya* gene product obtained by removing 261 codons at the 5′ end was expressed in a protease-deficient *lon*⁻ *E. coli* strain and purified to homogeneity. This truncated enzyme (CYA 62) exhibits catalytic and calmodulin-binding properties similar to the properties of wild-type adenylate cyclase from *B. anthracis* culture supernatants, i.e., a $k_{cat}$ of 1100 $s^{-1}$ at 30 °C and pH 8, an apparent $K_m$ for ATP of 0.25 mM, and a $K_d$ for bovine brain calmodulin of 23 nM. The calmodulin-binding domain of the CYA 62 truncated enzyme was labeled with a cleavable radioactive photoaffinity cross-linker coupled to calmodulin. The labeled CYA 62 protein was then cleaved with cyanogen bromide and *N*-chlorosuccinimide. We show that the calmodulin-binding domain of *B. anthracis* adenylate cyclase is located within the last 150 amino acid residues of the protein. A further deletion at the 3′ end of the CYA 62 coding sequence yielded an adenylate cyclase species (CYA 57) lacking 127 C-terminal amino residues. CYA 57, still sensitive to activation by high concentrations of calmodulin, exhibits less than 0.1% of the specific activity of CYA 62. Binding of 3′dATP (a competitive inhibitor) to CYA 62 was determined by equilibrium dialysis. In the absence of calmodulin, binding of the ATP analogue to this truncated protein was severely impaired, which explains, at least in part, the absolute requirement for calmodulin for the catalytic activity of *B. anthracis* adenylate cyclase.

The exotoxin produced by the Gram-positive pathogen *Bacillus anthracis* is composed of three distinct proteins of 83 kDa (lethal factor, LF), 85 kDa (protective antigen, PA), and 89 kDa (edema factor, EF) (Stanley & Smith, 1961; Leppla, 1984). Individually, these proteins are not toxic, but in combination they give different responses in experimental animals. Thus, PA and EF produce localized edema after intradermal injections, whereas PA and LF cause death (Fish et al., 1968,a,b). PA is therefore thought to be a receptor-binding component that mediates entry of LF and EF in target cells (Leppla, 1984). EF has been described as an adenylate cyclase activated by calmodulin (CaM)[1] (Leppla, 1982, 1984). *Bordetella pertussis*, a Gram-negative pathogen, also secretes a CaM-dependent adenylate cyclase (Wolff et al., 1980). By use of the interaction between adenylate cyclase and CaM as a tool, the corresponding genes from *B. pertussis* and *B. anthracis* have been cloned and their nucleotide sequences determined (Glaser et al., 1988; Mock et al., 1988; Escuyer et al., 1988; Tippets & Robertson, 1988). The two enzymes of 800 (*B. anthracis*) and 1706 (*B. pertussis*) amino acid residues displayed sequence similarity in only three regions. One of these regions, between residues 342–358 in *B. anthracis* adenylate cyclase and residues 54–70 in *B. pertussis* adenylate cyclase, contains a sequence that resembles the generally accepted ATP-binding site (Escuyer et al., 1988).

A striking peculiarity of *B. anthracis* adenylate cyclase, when compared to other CaM-activated enzymes, is its absolute requirement for CaM. Brain cAMP phosphodiesterase and the $Ca^{2+}$ pump of human erythrocytes exhibit measurable activities in the absence of CaM, which indicates that binding of the substrate molecule occurs in both CaM-free and CaM-complexed forms (Krinks et al., 1984; James et al., 1988).

In this paper, we describe the construction of a 5′-end deletion removing 261 codons of the *B. anthracis cya* gene. The corresponding protein, CYA 62 expressed in *Escherichia coli*, has been purified, and the substrate- and CaM-binding properties of this truncated *cya* gene product have been studied. We show that the CaM-binding domain of the enzyme is located at the C-terminal end of the protein molecule. The lack of catalysis in the absence of CaM may be explained, at least in part, by a much weaker binding of nucleotide substrate to the active site of adenylate cyclase.

## Experimental Procedures

*Chemicals.* Adenine nucleotides, restriction enzymes, and T4 DNA ligase were from Boehringer, Mannheim. DNA polymerase (Sequenase) was from USBC. Oligodeoxyribo-

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[1] Abbreviations: CaM, calmodulin; VU-1 CaM, engineered calmodulin encoded by a synthetic gene (SYNCAM, GenBank 1); VU-8 CaM, derivative of VU-1 CaM in which three glutamic acid residues (residues 82–84) have been substituted with three lysine residues; Blue-Sepharose, Cibacron blue 3G-A Sepharose CL-6B; SASD, sulfosuccinimidyl 3-[[2-(*p*-azidosalicylamido)ethyl]dithio]propionate; ASD, [[2-(*p*-azidosalicylamido)ethyl]dithio]propionate; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CNBr, cyanogen bromide; NCS, *N*-chlorosuccinimide; EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; $P_{342-358}$, synthetic peptide that corresponds to residues 342–358 of *Bacillus anthracis* adenylate cyclase having the sequence Gly-Val-Ala-Thr-Lys-Gly-Leu-Asn-Val-His-Gly-Lys-Ser-Ser-Asp-Trp-Gly; CYA 62, deletion derivative of wild-type adenylate cyclase of *B. anthracis*, lacking the first 261 residues; CYA 57, derivative of CYA 62 created by gene fusion between codons 262 and 673 of *B. anthracis* adenylate cyclase and the α domain of β-galactosidase.

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nucleotides used as primers in DNA sequencing were purchased from Pharmacia. 3'dATP, coupling enzymes, and bovine brain CaM were from Sigma. Blue-Sepharose, Ultrogel AcA 44, polybuffer exchanger 94, and polybuffer 96 were from Pharmacia LKB Biotechnologies. [$\alpha$-$^{32}$P]ATP (3000 Ci/mmol), [$^3$H]cAMP (40 Ci/mmol), [$^{125}$I]NaI (1000 Ci/mmol), and [$\alpha$-$^{35}$S]2'dATP$\alpha$S (1000 Ci/mmol) were obtained from the Radiochemical Centre, Amersham (U.K.). [$\alpha$-$^{32}$-P]-3'dATP (5000 Ci/mmol) came from NEN Research Product. VU-1 and VU-8 CaM, made according to Craig et al. (1987), were a gift from J. Haiech (Laboratorie de Chimie Bactérienne, UPR, CNRS, Marseille). Rabbit antibodies against synthetic peptide $P_{342-358}$ were obtained as previously described (Goyard et al., 1989).

*Synthesis and Iodination of ASD-CaM.* SASD (0.2 mg) in 0.8 mL of 60 mM sodium borate (pH 9.8), 0.1 mM $CaCl_2$, and 0.1 M NaCl was mixed with CaM (0.8 mg) dissolved in 0.2 mL of $H_2O$. After 16 h of stirring at room temperature, the mixture was desalted onto a Sephadex G25 column (1.6 × 5 cm) equilibrated in 50 mM MOPS (pH 7.5), 0.2 mM $CaCl_2$, and 0.1 M NaCl. ASD-CaM was iodinated by the Iodogen procedure (Pierce Chemical Co.) as described by the manufacturer. [$^{125}$I]ASD-CaM was separated from unreacted [$^{125}$I]NaI by chromatography on a Sephadex G25 column (1.6 × 5 cm) equilibrated in 50 mM Tris-HCl (pH 8), 0.2 mM $CaCl_2$, 0.1 mM NaCl, and 0.1% Nonidet P40 and stored at −20 °C protected from light.

*Bacterial Strains and Growth Media.* Two *E. coli* strains were used: JM105 (Yanish-Perron et al., 1985) and a protease-deficient strain (*lon*$^-$) CAG1139 (Grossman et al., 1983). Bacteria were grown in rich medium LB (Miller, 1972) and reached an $OD_{600nm}$ of 24 when grown under 20-L fermentor conditions. When required, ampicillin was added at 100 $\mu$g/mL.

*DNA Analytical Procedures.* Unidirectional deletions in the *cya* gene cloned in M13mp19 were generated by using the "cyclone" system (IBI) as described by the manufacturer. The nucleotide sequence was determined by a modified dideoxynucleotide chain terminator method, using sequenase as DNA polymerase (Tabor & Richardson, 1987).

*Purification of CYA 62 Protein.* A total of 70 g of a wet frozen paste of bacteria was suspended in 500 mL of 50 mM Tris-HCl (pH 8) and disrupted by sonication at 20 kHz and 100 W (3 × 4 min). Cell debris was removed by centrifugation at 12000*g* for 20 min at 4 °C.

(*A*) *Blue-Sepharose Chromatography.* The bacterial extract was loaded onto a Blue-Sepharose column (5 × 20 cm) equilibrated with 50 mM Tris-HCl (pH 8) at a flow rate of 400 mL/h. The column was washed with 5 volumes of the same buffer for 5 h; then proteins were eluted with 0.5 M NaCl in 50 mM Tris-HCl (pH 8) and precipitated with solid ammonium sulfate (80% saturation).

(*B*) *Chromatofocusing.* Following centrifugation at 10000*g* for 10 min, the precipitated proteins were redissolved in 25 mM Tris-HCl (pH 9) and then desalted by gel filtration on a Sephadex G25 column equilibrated with the same buffer. The desalted sample was then loaded onto a polybuffer exchanger 94 column (1 × 50 cm) at a ratio of 10 mg of protein/mL of swollen gel and a flow rate of 100 mL/h. After being washed with 250 mL of 25 mM Tris-HCl (pH 9) for 1 h, the proteins were eluted with 450 mL of 10-fold diluted polybuffer 96 adjusted to pH 7 with 1 N HCl. Samples of 5 mL were collected at a flow rate of 30 mL/h. Adenylate cyclase activity was eluted between pH 8.3 and pH 8.1. Those samples containing adenylate cyclase activity were pooled and precipitated with solid ammonium sulfate (80%).

(*C*) *Ultrogel AcA 44 Chromatography.* The precipitated enzyme was redissolved in 50 mM Tris-HCl (pH 7.4) and loaded onto a 1.5 × 120 cm column of Ultrogel AcA 44 equilibrated with the same buffer. Enzyme was eluted at a flow rate of 15 mL/h, collecting fractions of 3 mL. Samples containing pure protein were pooled and stored at −20 °C or were lyophilized after extensive dialysis against 50 mM ammonium bicarbonate.

*Photoaffinity Labeling of B. anthracis CYA 62 Protein with ASD-CaM. B. anthracis* enzyme (between 50 and 300 nM) was incubated for 10 min at 4 °C with [$^{125}$I]ASD-CaM (between 70 and 150 nM, $10^9$ cpm/nmol) and then irradiated for 2 min with a "long-wave" mercury lamp (mineral light UVSL 58) without screen, positioned 5 cm from the samples. The samples were then boiled for 2 min with Laemmli sample buffer containing 0.2 M $\beta$-mercaptoethanol and run onto a 10% SDS–PAGE. After electrophoresis the gel was either directly exposed to Kodak X-O-Mat AR film for 1–3 h at 4 °C or fixed in 10% acetic acid and dried before autoradiography at −80 °C.

*Cleavage of [$^{125}$I]ASD-Labeled CYA 62 Protein with Cyanogen Bromide and N-Chlorosuccinimide.* Peptide bands identified by SDS–PAGE and autoradiography were sliced from the gels, rehydrated when necessary, and then washed with either urea/water/acetic acid (1:1:1 w/v/v) (Trp cleavage) or 70% formic acid (Met cleavage) for 20 min. Peptides were cleaved by immersion of the gel slices at room temperature or at 37 °C in 15 mM NCS in urea/water/acetic acid for 30 min (Trp cleavage) (Lischwe & Ochs, 1982) or in 4% CNBr in 70% formic acid for 90 min (Met cleavage) (Nikodem & Fresco, 1979). After neutralization with 1 M Tris-HCl (pH 11) and several washes with water, slices were equilibrated in electrophoresis buffer (4% SDS, 12% glycerol, 2% $\beta$-mercaptoethanol, 0.01% bromophenol blue, and 50 mM Tris, pH 6.8), loaded onto a 12.5% glycerol/SDS–PAGE according to the procedure of Schägger and von Jagow (1987), and allowed to migrate overnight at 20 mA. Following migration, gels were dried and autoradiographed.

*Analytical Procedures.* Unless otherwise stated, the adenylate cyclase activity was measured as previously described (Ladant et al., 1986) in 100 $\mu$L of medium containing 50 mM Tris-HCl (pH 8), 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 $\mu$M bovine brain CaM, 0.5 mg/mL bovine serum albumin, 0.1 mM cAMP ($10^4$ cpm/assay), and 2 mM ATP ($5 \times 10^5$ cpm/assay). One unit of adenylate cyclase activity corresponds to 1 $\mu$mol of cAMP formed in 1 min at 30 °C and pH 8. Binding of [$\alpha$-$^{32}$P]3'dATP to adenylate cyclase was investigated by equilibrium dialysis at 6 °C in chambers of 0.5-mL capacity. Aliquots of 0.25 mL of a solution having a final concentration of 0.05 mM adenylate cyclase, 0.06 mM CaM, 50 mM Tris-HCl (pH 8), 50 mM KCl, 0.5 mM $CaCl_2$, and 2 mM $MgCl_2$ were equilibrated for 12 h with an equal volume of the same solution in which enzyme was replaced by increasing concentrations of radiolabeled nucleotide (from 25 to 250 $\mu$M). Equilibrium data were analyzed by Scatchard plots. The N-terminal amino acid sequence of the purified protein was determined by using the Applied Biosystems automated protein sequencer. SDS–PAGE was performed as described by Laemmli (1970). In some experiments proteins separated by SDS–PAGE were transferred to nitrocellulose sheets and incubated with antibodies directed against synthetic peptide $P_{342-358}$ as described by Monneron et al. (1988). The immunochemical detection was performed with $^{125}$I-labeled protein A. Recombinant adenylate cyclase was quantitated in crude

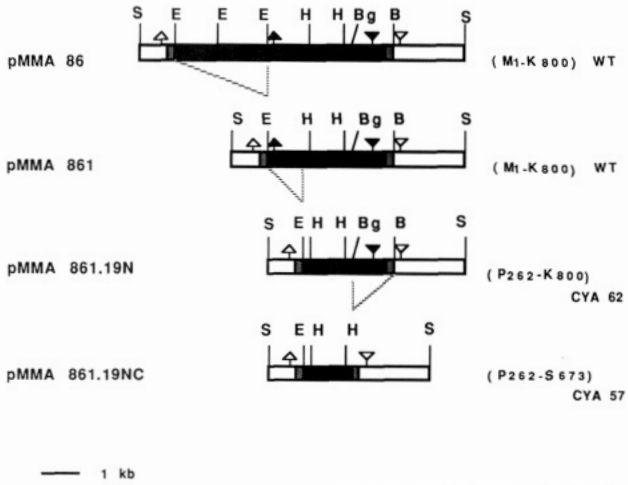


FIGURE 1: Schematic representation of pMMA861 deletion derivative plasmids encoding part or all of the *cya* locus of *B. anthracis*. Wild-type (WT) or truncated forms (CYA 62, CYA 57) of the adenylate cyclase protein encoded by each plasmid are given in the right margin. The first and last amino acid residues of the adenylate cyclase sequence present in each protein are given by the standard one-letter code. $M_1$–$K_{800}$ corresponds to the wild-type protein. For convenience, all plasmids are represented linearized at the unique *Sac*I site of pUC8. *B. anthracis* DNA is represented by a solid box. The start (▲) and stop (▼) codons of the *cya* gene are indicated. The open box represents pUC8 DNA. The start (△) and stop (▽) codons of *lac Z'* gene are indicated. The dotted box represents the vector polylinker sequence. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Bg, *Bgl*II; S, *Sca*I.

bacterial extracts after SDS–PAGE and immunochemical detection by autoradiography; the radioactive bands cut from the nitrocellulose sheets were analyzed with a $\gamma$ counter. Purified CYA 62 served as the standard; a linear relationship between radioactivity and protein quantity within 0.1 and 1 $\mu$g was found.

## Results

*Expression and Purification of a Truncated B. anthracis cya Gene Product in E. coli.* Expression of *B. anthracis* adenylate cyclase in *E. coli* carrying recombinant plasmid pMMA86 (Mock et al., 1988) was too low (0.03 unit/mg of protein) for a large-scale purification procedure. A 3-kb *Eco*RI–*Bam*HI fragment from pMMA861 (derivative of pMMA86, Figure 1), encoding the *cya* locus, was cloned into M13mp19, and unidirectional deletions were generated from the *Eco*RI site in order to remove the DNA sequences upstream from the 5′ end of the *cya* gene. DNA fragments carrying the different deletions were recovered from an *Eco*RI–*Pst*I digestion of the phage replicative form. These fragments were subcloned into pUC8, thus bringing expression of the *cya* gene under *lac* promoter control. Clones carrying the recombinant plasmids were analyzed for adenylate cyclase expression. One of these, pMMA861.19N, was found to express significantly higher amounts of adenylate cyclase (1 unit/mg of protein). DNA sequencing at the *Eco*RI site of the corresponding M13mp19 subclone indicated that the first 261 codons of the adenylate cyclase gene had been removed, thereby expecting a truncated gene product with Pro 262 as the N-terminal amino acid. However, the N-terminal sequence of the purified enzyme has been found to be Asn-Ser-Pro-Asp-Met-Phe-Glu-Tyr-Met-Asn-Lys-Leu-Glu-Lys-Gly-Gly-Phe-Glu. The first two residues have no equivalent in the *B. anthracis* wild-type protein since they result from the fusion of the adenylate cyclase coding sequence to the M13mp19 polylinker nucleotide sequence (Norrander et al., 1983). Transformation of pMMA861.19N into a protease-deficient strain, CAG1139, and growth of bacteria in a 20-L fermentor strongly enhanced adenylate cyclase production, which could be raised to 20 units/mg of protein.

Table I: Purification of *B. anthracis* Truncated Adenylate Cyclase (CYA 62) Expressed in *E. coli*[a]

| step | protein (mg) | total act. ($\mu$mol/min) | sp act. [$\mu$mol min$^{-1}$ (mg of protein)$^{-1}$] | yield (%) |
|---|---|---|---|---|
| soluble cell extract | 9800 | 206 000 | 21 | 100 |
| Blue-Sepharose chromatography | 749 | 142 000 | 190 | 69 |
| chromatofocusing | 76 | 72 200 | 950 | 35 |
| Ultrogel AcA 44 | 44 | 45 300 | 1020 | 22 |

[a] From 70 g of wet cells.

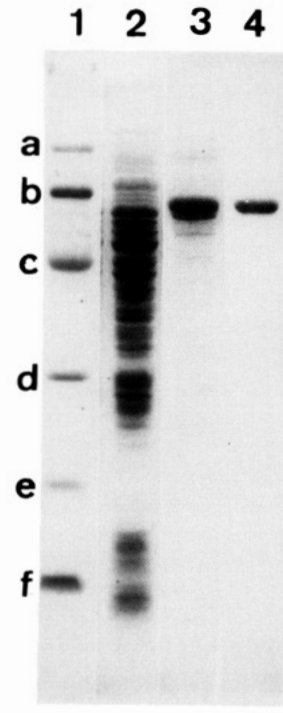


FIGURE 2: SDS–PAGE (12%) of fractions obtained during the purification of the *B. anthracis* CYA 62 adenylate cyclase expressed in *E. coli*. Lane 1: standard proteins, (a) phosphorylase *b* (94 000), (b) bovine serum albumin (67 000), (c) ovalbumin (43 000), (d) carbonic anhydrase (30 000), (e) soybean trypsin inhibitor (20 300), and (f) lysozyme (14 400). Lane 2: cell extract, 40 $\mu$g of protein. Lane 3: Blue-Sepharose, 6 $\mu$g of protein. Lane 4: Ultrogel AcA44, 2.4 $\mu$g of protein.

Truncated *B. anthracis* adenylate cyclase CYA 62 expressed in *E. coli* represented approximately 2% of the total bacterial proteins. Because the number of basic residues (Lys + Arg = 87) exceeds significantly the number of acidic residues (Asp + Glu = 79), we expected a rather strong adsorption of the enzyme on Blue-Sepharose at pH 8. Under these conditions more than 90% of *E. coli* proteins do not interact with the matrix. Elution of Blue-Sepharose-bound enzyme with 0.5 M NaCl yielded an 8-fold purification of adenylate cyclase with a 70% recovery rate. Almost pure protein was obtained by chromatofocusing between pH 9 and pH 7, the peak of enzyme eluting at pH 8.2. Gel permeation chromatography in 50 mM Tris-HCl (pH 7.4) was used to remove the ammonium sulfate, the ampholine, and some low molecular weight contaminants. Table I and Figure 2 summarize the purification steps. Pure CYA 62 protein at a concentration above 1 mg/mL was stable for several weeks at −20 °C. Because lyophilization of enzyme does not alter its catalytic properties, we used this procedure for long-term storage or for further spectroscopic studies.

Table II: Kinetic Parameters of *B. anthracis* Truncated Adenylate Cyclase (CYA 62) Activation by Bovine Brain, VU-1, and VU-8 Calmodulin

| calmodulin | max act.[a] | $K_d$ (nM) | Hill no. |
|---|---|---|---|
| bovine brain | 1100 | 23 | 1.08 |
| VU-1 | 1080 | 25 | 1.02 |
| VU-8 | 980 | 280 | 0.98 |

[a] $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$.

*Interaction of Truncated B. anthracis Adenylate Cyclase with CaM.* Adenylate cyclase of *B. anthracis* is activated by CaM in both the presence and absence of $Ca^{2+}$ (Leppla, 1984). The degree of enzyme activation as a function of CaM concentration may be described by the Hill equation $f = CaM^n/K_d + CaM^n$, where $f$ is the fraction of maximal activation of the enzyme ($f = V/V_{max}$), $V$ is the reaction rate at a given concentration of CaM, and $V_{max}$ is the reaction rate at saturating activator concentration. The affinity of bovine brain CaM for CYA 62 enzyme ($K_d$ = 23 nM; Table II) is very close to that shown for the whole protein excreted by *B. anthracis* in culture supernatants ($K_d$ = 26 nM). The maximal activity of CYA 62 enzyme at saturating concentrations of ATP and CaM, pH 8 and 30 °C, was 1100 $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$, which corresponds to a $k_{cat}$ of about 1100 s$^{-1}$. This value is close to that calculated for adenylate cyclase purified from *B. anthracis* culture supernatants if correction for temperature (23 °C versus 30 °C) is made (Leppla, 1984). Under identical experimental conditions *B. pertussis* adenylate cyclase exhibited $k_{cat}$ values of between 1700 and 2300 s$^{-1}$ (Ladant et al., 1986; Rogel et al., 1988; Glaser et al., 1989). VU-1 CaM, which is a genetically engineered CaM, did not differ essentially from the bovine brain protein both in its affinity for and in maximal activation of truncated *B. anthracis* adenylate cyclase (CYA 62). VU-8 CaM, a modified variant of VU-1 CaM in which three glutamic acid residues at positions 82–84 were replaced by three lysine residues, showed a 10-fold lower affinity for CYA 62 and 90% of maximal activation exhibited by VU-1 CaM or bovine brain CaM (Table II). It is worthwhile to note that VU-8 CaM showed a 1000-fold lower affinity and only 16% of maximal activation of VU-1 or bovine brain CaM when tested on *B. pertussis* adenylate cyclase (Haiech et al., 1988). These results already suggest a different structural organization of the CaM-binding domain in *B. pertussis* and *B. anthracis* adenylate cyclase. Whereas the presence of negatively charged side chains at residues 82–84 in the central $\alpha$ helix that links the two halves of CaM is critical for activation of *B. pertussis* adenylate cyclase, the *B. anthracis* enzyme does not exhibit such a stringent structural requirement for binding and activation.

*Photolabeling of Truncated B. anthracis Adenylate Cyclase with [$^{125}$I]ASD-CaM.* The CaM-binding domain or domains of the CYA 62 protein were labeled with a cleavable radioactive photoaffinity cross-linker coupled to CaM. CaM was derivatized with the heterobifunctional reagent SASD (Knutson, 1987) via displacement of the *N*-hydroxysuccinimide moiety of SASD. The ASD-CaM was as effective an activator of *B. anthracis* adenylate cyclase as was the native CaM. Upon iodination of ASD-CaM, most of the $^{125}$I radioactivity was incorporated into the azidohydroxybenzoyl moiety of ASD (Figure 3, lane 1), which can be released from CaM by cleavage of the disulfide bond (Figure 3, lane 2). [$^{125}$I]-ASD-CaM was cross-linked to CYA 62 by photoactivation; then CaM was cleaved with $\beta$-mercaptoethanol leaving the radioactive label on the bacterial enzyme (Figure 3, lane 3). The cross-linking of [$^{125}$I]ASD-CaM to the bacterial enzyme is specific. Thus, when an excess of CaM or EGTA was present in the incubation mixture or when the iodinated azidohydroxybenzoyl moiety was released from ASD-CaM before irradiation, very little radioactivity, if any, was associated with *B. anthracis* CYA 62 (Figure 3, lanes 4–6).

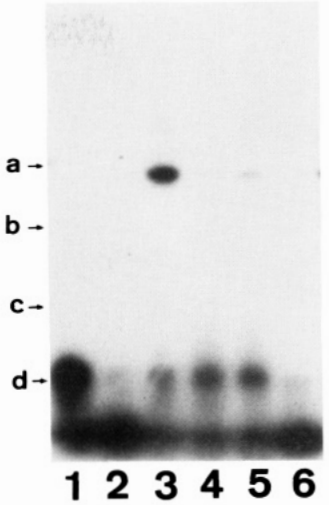


FIGURE 3: Photoaffinity labeling of CYA 62 protein with [$^{125}$I]-ASD-CaM. CYA 62 protein (100 nM) and [$^{125}$I]ASD-CaM (70 nM) in 50 $\mu$L of Tris-HCl (pH 8) containing 0.1% NP40, 0.2 mM $CaCl_2$ (lanes 3, 4, and 6), or 5 mM EGTA (lane 5) were photolyzed as described under Experimental Procedures, then treated with 0.2 M $\beta$-mercaptoethanol, run on a 10% SDS–PAGE, and autoradiographed. Lane 1: control, run in the absence of adenylate cyclase; sample not treated with $\beta$-mercaptoethanol. Lane 2: control, run in the absence of adenylate cyclase and treated with $\beta$-mercaptoethanol. Lane 3: complete reaction medium. Lane 4: complete reaction medium plus 10 $\mu$M cold bovine brain CaM. Lane 5: complete reaction medium in which EGTA replaced $Ca^{2+}$. Lane 6: complete reaction medium; $\beta$-mercaptoethanol was added before photolysis. The arrows on the left side indicate the standard proteins: (a) bovine serum albumin, (b) ovalbumin, (c) carbonic anhydrase, and (d) soybean trypsin inhibitor.

To identify the labeled domain(s) after cross-linking with [$^{125}$I]ASD-CaM, the protein was cleaved with CNBr and then with NCS. The fragments, separated by SDS–PAGE, were assigned by comparing their molecular mass calculated from a calibration curve of six molecular weight markers to the molecular mass expected from the primary structure of the truncated enzyme (Figure 4). As shown in Figure 5 (lane 2), three iodinated CNBr fragments of $^{125}$I-photolabeled adenylate cyclase were resolved on SDS–PAGE. From the apparent molecular mass, two were assigned as fragments 478–800 and 557–800, respectively. The third radioactive peptide was assigned as fragment 611–800 only after further cleavage with NCS (Figure 5, lane 6). The smallest $^{125}$I-labeled NCS-generated peptide with an apparent molecular mass of 18 kDa was common to all three fragments and corresponds to the C-terminal segment (residues 646–800) of the recombinant enzyme.

From these results we conclude that the CaM-interacting domain of *B. anthracis* adenylate cyclase photolabeled by [$^{125}$I]ASD-CaM is located within the last 150 amino acids of the protein. In further experiments we removed 127 codons from the 3′ end of the *cya* gene (Figure 1) by deletion of the *Bgl*II–*Bam*HI fragment of pMMA861.19N. This construction (pMMA861.19NC) created a gene fusion between codons 262–673 of *B. anthracis* adenylate cyclase and the $\alpha$ domain of $\beta$-galactosidase. The resulting chimeric protein (CYA 57) of 502 residues (apparent molecular mass on SDS–PAGE of 57 kDa) was expressed in *E. coli* ten times less efficiently than the truncated form CYA 62 (Figure 6, lanes 4 and 5). CYA 57 protein still exhibited a detectable CaM-stimulated activity

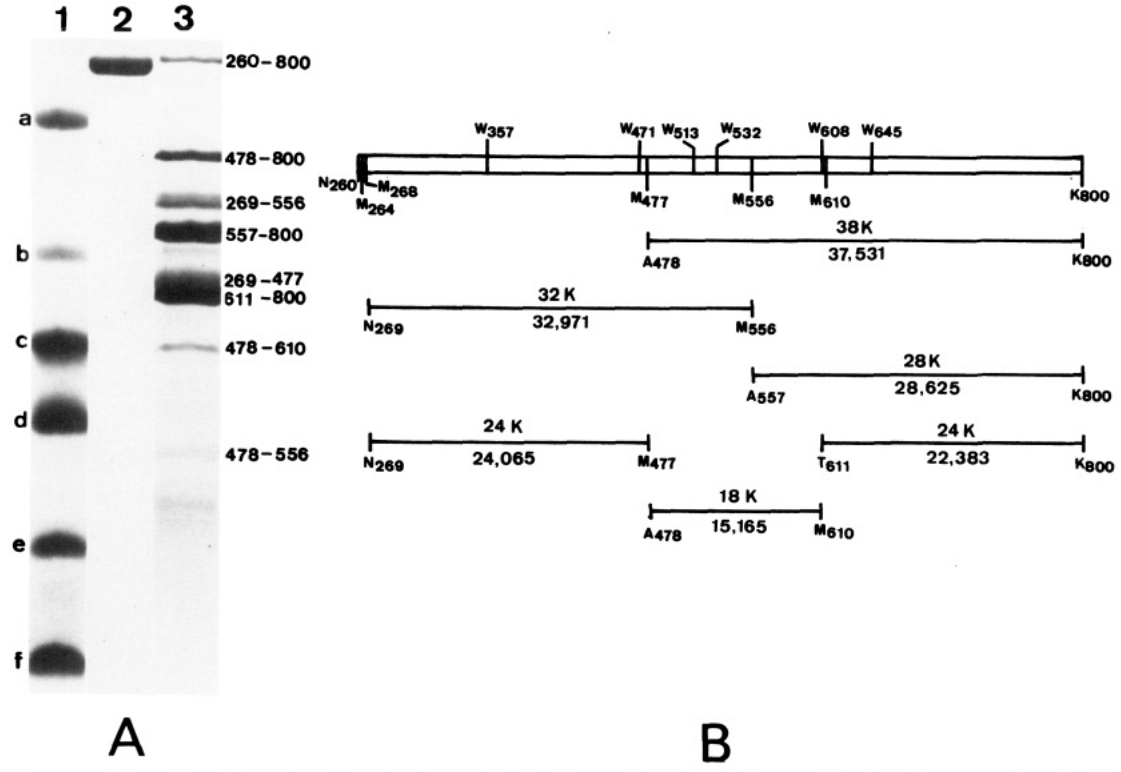


FIGURE 4: Cleavage of *B. anthracis* CYA 62 protein by CNBr and assignment of fragments according to their apparent molecular mass. (A) Truncated CYA 62 protein (0.2 mg) was solubilized in 50 μL of 70% formic acid containing 1 mg of CNBr. After 4-h incubation in the dark at room temperature, the sample was diluted 10-fold with $H_2O$ and then lyophilized in aliquots of 100 μL. The lyophilized material was solubilized in 50 mM Tris-HCl (pH 6.8), 4% SDS, 12% glycerol, 2% β-mercaptoethanol, and 0.01% bromophenol blue and loaded onto a 12.5% glycerol/SDS–PAGE according to the procedure of Schägger and von Jagow (1987). Lane 1: molecular weight standards, (a) ovalbumin (43 000), (b) α-chymotrypsinogen (25 700), (c) β-lactoglobulin (18 400), (d) lysozyme (14 400), (e) bovine trypsin inhibitor (6200), and (f) insulin (3000). Lane 2: uncleaved adenylate cyclase (2.5 μg of protein). Lane 3: CNBr-cleaved adenylate cyclase. (B) The positions of Met and Trp residues in the truncated 62-kDa form of adenylate cyclase are indicated, keeping the numbering for the entire protein as it was deduced from the nucleotide sequence of the corresponding gene. The main fragments resulting from partial or complete cleavage of truncated adenylate cyclase after treatment with CNBr are also indicated. The molecular mass (shown above each fragment), determined by using proteins of known molecular weight, was compared to the $M_r$ calculated from sequence data.

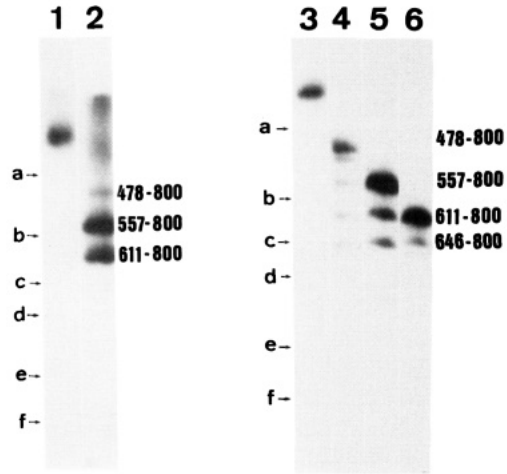


FIGURE 5: Cleavage of truncated adenylate cyclase CYA 62, photolabeled with [$^{125}$I]ASD-CaM, with CNBr and NCS. Truncated adenylate cyclase was photolabeled with [$^{125}$I]ASD-CaM as described in Figure 3 (lane 3), treated with 0.2 M β-mercaptoethanol, run on a 10% SDS–PAGE and autoradiographed. The radioactive protein was sliced from the gel, then cleaved (lane 2) or not (lanes 1 and 3) with CNBr (see Experimental Procedures), and loaded onto a 12.5% glycerol/SDS–PAGE. The three peptides identified by autoradiography on lane 2 were again sliced from the gel and cleaved with NCS and run on a 12.5% glycerol/SDS–PAGE. The fragments resulting from this second cleavage were identified by autoradiography (lanes 4, 5, and 6).

at 1 μM activator. The specific activity of CYA 57 quantitated in crude extracts by immunochemical detection was less than 0.1% of the specific activity of CYA 62. It is unlikely that inactivation of C-terminal-deleted adenylate cyclase is due to a folding problem during expression of fused protein. Fully active *B. pertussis* adenylate cyclase was expressed after fusion to the α domain of β-galactosidase (Glaser et al., 1989).

*Kinetic and Nucleotide Binding Properties of Truncated B. anthracis Adenylate Cyclase.* The apparent $K_m$ for ATP of CYA 62 expressed in *E. coli* is 0.25 mM, similar to that of the wild-type enzyme secreted by toxinogenic strains of *B. anthracis*. With excess $Ca^{2+}$, and in the absence of $Mg^{2+}$, $V_m$ represented only 0.4% of the $V_m$ values in the presence of $Mg^{2+}$. CaATP exhibited a significantly higher affinity (apparent $K_m$ of 45 μM) for CYA 62 than MgATP. 3′dATP, a noncyclizable competitive inhibitor of CYA 62, exhibited $K_i$ values of 86 μM in the presence of excess $Mg^{2+}$ over $Ca^{2+}$ and 22 μM in the presence of $Ca^{2+}$ as the sole divalent cation. This phenomenon was confirmed by equilibrium dialysis experiments in which 3′dATP replaced the natural substrate since binding can be determined in the presence of divalent cations without catalysis. The apparent $K_d$ for 3′dATP of CYA 62 was 30 μM in the presence $Ca^{2+}$ as the sole divalent cation and 130 μM in the presence of excess $Mg^{2+}$ over $Ca^{2+}$. The most important and surprising observation to emerge from equilibrium dialysis experiments is that, in the absence of CaM, binding of 3′dATP to truncated adenylate cyclase CYA 62 is severely impaired (Figure 7).

## DISCUSSION

Genetic, biochemical, and physiopathological studies show that at least two discrete functions can be assigned to *B. anthracis* adenylate cyclase: interaction with PA for binding

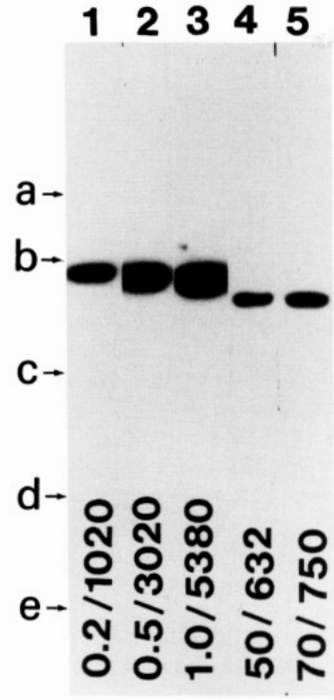


FIGURE 6: Immunochemical detection and quantitation of the CYA 57 deletion derivative of *B. anthracis* adenylate cyclase. Purified CYA 62 (lanes 1, 2, and 3) and crude bacterial extract expressing the CYA 57 deletion derivative of *B. anthracis* adenylate cyclase (lanes 4 and 5) were submitted to SDS–PAGE; then the proteins were transferred to nitrocellulose sheets. After incubation with 1:1000 dilution of rabbit antiserum raised against peptide $P_{342-358}$, truncated enzymes were thus identified by treatment of nitrocellulose sheets with $^{125}$I-labeled protein A and autoradiography. Numbers at the bottom of each lane correspond to micrograms of protein and to radioactivity (cpm), respectively. Assuming that the two truncated forms of *B. anthracis* adenylate cyclase have the same affinity for rabbit antibodies, one may calculate that CYA 57 represents 0.21% of total *E. coli* proteins.

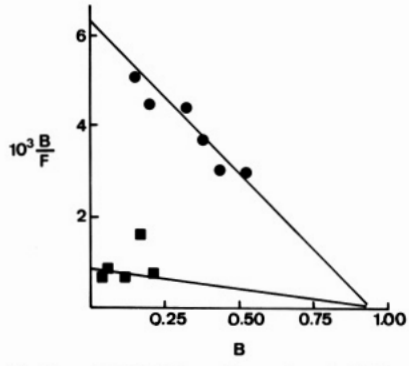


FIGURE 7: Binding of 3′dATP to *B. anthracis* CYA 62 adenylate cyclase complexed (●) or not (■) to CaM as determined by equilibrium dialysis. The experimental conditions are described under Experimental Procedures.

to and translocation into target cells and binding to CaM, which acts as an activator for cyclization of ATP (Leppla, 1982, 1984). These properties suggest that the protein is constructed from different domains. Elucidation of the functional organization of the *B. anthracis* adenylate cyclase has been hampered due to the difficulty in purifying the protein free from the other two toxin components (PA and LF). Cloning and expression of the *cya* gene in *E. coli* were the first steps in the genetic and biochemical investigation of the structure–function relationship of the corresponding protein (Escuyer et al., 1988; Mock et al., 1988; Tippets & Roberston, 1988). We have now constructed, and overexpressed, an N-terminal deletion derivative lacking 261 amino acid residues. This deletion does not affect the binding of CaM or the maximal catalytic activity of adenylate cyclase. Therefore, it is reasonable to assume that the N-terminal region of adenylate cyclase harbors the binding site for PA (Bragg & Robertson, 1989), which probably acts as an independent domain with little or no interaction with the remainder of the molecule. The ease of purification of the truncated adenylate cyclase CYA 62 and its remarkable solubility and stability under various experimental conditions make it a suitable model for physicochemical studies including X-ray crystallography.

*B. anthracis* adenylate cyclase has an absolute requirement for CaM and for the divalent ions $Ca^{2+}$ and $Mg^{2+}$. $Ca^{2+}$ is required to form the adenylate cyclase/CaM complex (Klee & Vanaman, 1982) which then binds with high affinity to the enzyme, whereas $Mg^{2+}$ forms with ATP the MgATP complex, the true nucleotide substrate (Morrison & Heyde, 1972). $Ca^{2+}$ can, however, replace $Mg^{2+}$ in forming the metal ion–nucleotide complex, with 5-fold higher affinity for the active site of adenylate cyclase and with reaction rates 250 times lower. The very low binding of 3′dATP to the *B. anthracis* enzyme in the absence of CaM is a new and interesting observation and could explain why catalysis does not occur in the absence of the activator. We believe that the active site of the bacterial enzyme "closes" or "opens" reversibly for trapping/releasing the nucleotide substrate, a process that is controlled by CaM. Closing of the active site via conformational changes of the adenylate cyclase/CaM complex would favor (i) better contacts between amino acid side chains and different components of the ATP molecule and (ii) more rigid orientation of the polyphosphate group in the vicinity of those side chains that are involved in the catalytic step. The CaM-dependent conformational change may be critical as the cyclization of ATP imposes heavier steric constraints to the molecule than hydrolysis or phosphate group transfer.

The adenylate cyclase from *B. anthracis* shares common features with the CaM-activated adenylate cyclase from *B. pertussis*; therefore, we compared their primary structures in an attempt to localize the ATP- and CaM-binding domains of the *B. anthracis* enzyme. The sequences 342–358 in *B. anthracis* and 54–70 in *B. pertussis* contain the consensus motif of ATP-binding proteins, GXXXXGKT (Moller & Amons, 1985; Fry et al., 1986), and are also highly homologous with each other (15 amino acid residues from a total of 17 are identical). Site-directed mutagenesis of Lys 58 and Lys 65 in *B. pertussis* adenylate cyclase yielded an almost inactive enzyme but with apparently normal CaM-binding properties (Glaser et al., 1989). It is probable that corresponding lysine residues (Lys 346 and Lys 353) play the same role in the *B. anthracis* adenylate cyclase.

In contrast to the ATP-binding site, no sequence in *B. anthracis* adenylate cyclase revealed common stretches with the CaM-binding site of *B. pertussis* enzyme. Although the CaM-binding domains of different enzymes examined to date show a limited degree of sequence similarity, a strong predominance of basic and hydrophobic residues organized in $\alpha$-helical segments is observed in all cases (Blumenthal et al., 1985; DeGrado et al., 1987; James et al., 1988).

Photoaffinity labeling of the *B. anthracis* adenylate cyclase with [$^{125}$I]ASD-CaM, followed by sequential cleavage of the protein with CNBr and NCS, indicates that the last 150 amino acid residues of the protein are the target for interaction with CaM. Unambiguous assignment of a shorter peptide fragment upon photolabeling and tryptic digestion of the radioactive enzyme, followed by HPLC separation, failed. Furthermore, attempts to purify CaM-binding peptide(s) after chemical

cleavage of adenylate cyclase or to obtain active CaM-independent species of enzyme by limited proteolysis were unsuccessful. A C-terminal deletion derivative of CYA 62 lacking 127 amino acid residues that still interact with CaM has only 0.1% of the specific activity of the CYA 62. This seems to indicate that binding and activation by CaM of the bacterial enzyme are complex phenomena that cannot be attributed to a single sequence. It is also likely that activation by CaM of both *B. anthracis* and *B. pertussis* adenylate cyclase occurs by a different mechanism, as has been described for other CaM-dependent enzymes such myosin light chain kinase. Location of residues involved in these different processes in both *B. anthracis* and *B. pertussis* adenylate cyclase is currently under investigation in our laboratories.

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