Interaction of Bordetella pertussis Adenylate Cyclase with Calmodulin

IDENTIFICATION OF TWO SEPARATED CALMODULIN-BINDING DOMAINS*

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The structural organization of Bordetella pertussis adenylate cyclase was examined by limited proteolysis with trypsin and/or cross-linking with azido-calmodulin, a photoactivatable derivative of its activator, calmodulin (CaM). Adenylate cyclase (which consists of three structurally related peptides of 50, 45, and 43 kDa as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) formed a 1:1 complex with CaM or azido-CaM. CaM-bound adenylate cyclase was cleaved by trypsin into two separate trypsin-resistant fragments of 25 and 18 kDa which both interacted with CaM as judged by their ability to be cross-linked with azido-CaM. These two fragments remained associated with CaM in a catalytically active conformation resembling that of the undigested complex. When proteolysis was carried out in the absence of CaM, the adenylate cyclase was completely inactivated in less than 3 min. Sodium dodecyl sulfate-polyacrylamide gel revealed a single 24-kDa trypsin-resistant fragment. Since this fragment cannot be cross-linked with azido-CaM we suggest that the CaM-binding site on the 25-kDa moiety of the adenylate cyclase is located on a short segment of 1 kDa.

Bordetella pertussis and Bacillus anthracis, causative agents of whooping cough and anthrax, respectively, are the only two prokaryotic organisms which are so far known to secrete an adenylate cyclase (1-5). B. anthracis adenylate cyclase, identified as the edema factor (5-9), is a secreted adenylate cyclase, which consists of three structurally related peptides of 50, 45, and 43 kDa. Adenylate cyclase activity was measured using the procedure of White (18), as modified by Hanoune et al. (19). The specific activity of purified enzyme was between 100 and 120 units/mg of protein. Adenylate cyclase was purified in a single step by chromatography on Affi-Gel-CaM (16). The purification was facilitated by our recent purification to homogeneity of adenylate cyclase from culture supernatants of B. pertussis (16).

In this paper I examined the CaM-adenylate cyclase interaction using structural probes, such as a photoactivatable derivative of CaM and limited proteolysis by trypsin.

Experimental Procedures

Chemicals—Blue-Sepharose, ATP, cAMP, TPCK-trypsin, and soybean trypsin inhibitor were from Sigma. Affi-Gel-CaM was from Bio-Rad. Urea (fluorimetrically pure) was a product of Schwarz/Mann. Methyl-4-azido-benzimidate was purchased from Pierce Chemical Co. [γ-32P]ATP (3000 Ci/mmol), and Na232P (1000 Ci/mmol) were obtained from the Radiochemical Center (Amersham Corp.). [1H]cAMP (40 Ci/mmol) was purchased from CEA (Saclay). Bovine brain CaM was a kind gift from Professor E. Carafoli (Swiss Federal Institute of Technology, Zürich). Microcrystalline cellulose precoated thin-layer plates (CEL 400-10) were obtained from Macherey-Nagel. Ultragel ACA-44 came from LKB.

CaM was azidated essentially as described by Zurini et al. (17). To 1 mg of CaM dissolved in 0.5 ml of 50 mM sodium borate, pH 9.8, 0.2 mM CaCl2, 0.1 mM NaCl, 0.5 mg of methyl-4-azido-benzimidate dissolved in 0.5 ml of the same buffer were added. After 2 h of stirring at room temperature, the reaction mixture was desalted on a 10-ml Sephadex G-25 column equilibrated with 25 mM Tris-HCl, pH 7.4, 0.1 mM NaCl, 0.2 mM CaCl2. Incorporation of azido groups into CaM was estimated spectrophotometrically: a value of 1.3 mol of azido groups/mol of CaM was found.

Azido-CaM was stored at −20 °C protected from light.

Purification and Assay of Adenylate Cyclase—Concentrated culture supernatant of B. pertussis, 18523, phase I (type strain ATCC 9797) was obtained as described previously (16). The specific activity of concentrated culture supernatant was between 100 and 120 units/mg of protein. Adenylate cyclase was purified in a single step by chromatography on Affi-Gel-CaM (16). Purified enzyme in buffer A (50 mM Tris-HCl, pH 8, 0.1% Nonidet P-40, 0.1 mM CaCl2) with a specific activity of 1600 units/mg of protein could be stored at −80 °C for several weeks with no loss of activity. SDS-polyacrylamide gel revealed three structurally related bands (see "Results") corresponding to 50, 45, and 43 kDa.

Adenylate cyclase activity was measured using the procedure of White (18), as modified by Hanoune et al. (19). The reaction was performed at 30 °C in 100 μl of a medium containing 50 mM Tris-HCl, pH 8, 1 mM [γ-32P]ATP (5 × 106 cpm/assay), 6 mM MgCl2, 100 μg of bovine serum albumin, 0.13 mM [1H]cAMP (1.5 × 106 cpm/assay), 0.12 mM CaCl2, and 0.1 μM CaM (when added). One unit of adenylate cyclase corresponds to 1 nmol of cAMP formed in 1 min at 30 °C at pH 8.

Inductions—Five ml of concentrated culture supernatant containing about 30 units of adenylate cyclase were mixed with 70 μl of packed Affi-Gel-CaM and shaken overnight at 4 °C. The gel which retained more than 75% of enzyme activity was then washed several times with 0.5 M NaCl in buffer A and further with 50 mM Tris-HCl, pH 8. Induction of adenylate cyclase bound to Affi-Gel-CaM was performed with chloramine T at room temperature for 5 min with occasional shaking. The reaction was quenched by addition of sodium metabisulphite, followed by addition of 0.7 ml of 8.5 M urea in buffer A. After 30 min of stirring at room temperature, the mixture was

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loaded onto a 10-ml Sephadex G-25 column equilibrated in buffer A, to remove both urea and free iodine. The iodinated adenylate cyclase (0.3–1× 10^6 cpn/μg of protein corresponding to 0.08–0.25 mol of iodine/mol of adenylate cyclase) was fully active and stimulated by CaM 20–50-fold. Its specific activity was expressed in units per counts per minute of ^125I.

CaM and azido-CaM were iodinated by the chloramine-T method at room temperature to a specific activity of about 20 Ci/mmol (0.02 mol of iodine/mol of CaM) (20).

Binding of Adenylate Cyclase to Blue-Sepharose—Adenylate cyclase was diluted in buffer B (50 mM Tris-HCl, pH 8, 0.1 mM Ca"^2+", 0.1% Nonidet P-40, 20% glycerol) to 0.1 unit/ml. Two-hundred μl of this solution were gently shaken at 4 °C in an Eppendorf tube with 10 μl of a Blue-Sepharose suspension containing 2 μl of packed resin and with different concentrations of CaM or azido-CaM. After different times of incubation (between 30 min and 24 h), tubes were centrifuged and the activity of adenylate cyclase in the supernatant was measured. The percentage of enzyme bound to Blue-Sepharose was calculated by subtracting the activity remaining in the supernatant from the initial activity.

Appropriate runs were made in the absence of Blue-Sepharose to account for enzyme inactivation or adsorption to Eppendorf tubes. It should be noted that in the presence of 20% glycerol adsorption was less than 2% in 3 h.

Isolation of the 43-, 45-, and 50-kDa Polypeptides on SDS-Polyacrylamide Gel Electrophoresis—The iodinated adenylate cyclase preparation was run on a 7.5% SDS-polyacrylamide gel (21), and the proteins were fixed in 10% acetic acid; the gel was further washed extensively against buffer A. Each peptide was assayed for adenylate cyclase in buffer A, and with different concentrations of CaM or azido-CaM. After incubation time (30 min), tubes were centrifuged and the activity of adenylate cyclase in the supernatant was measured. The percentage of enzyme bound to Blue-Sepharose was calculated by subtracting the activity remaining in the supernatant from the initial activity.

RESULTS

Interaction of B. pertussis Adenylate Cyclase with CaM and Azido-CaM—Preliminary experiments showed that free adenylate cyclase, but not the CaM-complexed enzyme, bound reversibly to Blue-Sepharose at neutral pH. Blue-Sepharose-bound adenylate cyclase was released into the medium by excess CaM, with a half-time varying between several minutes at 30 °C and several hours at 4 °C (Fig. 1). These differences in affinity for Blue-Sepharose between free and CaM-bound adenylate cyclase prompted me to investigate the CaM-adenylate cyclase interaction by a gel competition method. This method is similar in many respects to that reported by Schubert (22) for determination of the metal ions-nucleotide affinity constant.
The stability of the adenylate cyclase-CaM complex in the presence of EGTA. Binding of adenylate cyclase to Blue-Sepharose was measured in buffer B, in the presence of 0.1 mM Ca²⁺ (●) or 2 mM EGTA (■) and 2–50 nM CaM, as described under "Experimental Procedures." After 3 h of incubation, percentage of enzyme remaining in the supernatant was determined and plotted versus the concentration of CaM. Then, 2 mM EGTA were added to the samples which were previously incubated in the presence of Ca²⁺. After another incubation of 3 h the percentage of enzyme remaining in the supernatant was determined and plotted versus the concentration of CaM.

If we take into consideration the following two equilibria:

\[ \text{adenylate cyclase} + \text{Blue-Sepharose} \leftrightarrow \text{adenylate cyclase} \ (\text{Blue-Sepharose}) \]  
\[ \text{adenylate cyclase} + n(\text{CaM}) \leftrightarrow \text{adenylate cyclase} \ (\text{CaM})_n \]

the partition coefficient, i.e. the ratio between enzyme in solution and enzyme bound to the gel, in the presence \( k_e \) or absence \( k_o \) of CaM will be defined by the following equation:

\[ k_e = k_o \left(1 + \frac{1}{K_D(\text{CaM})} \right) \]

Where \( K_D \) is the dissociation constant of the adenylate cyclase-CaM complex. As shown in Fig. 2, \( k_o \) is a linear function of CaM concentration, either in the presence or absence of calcium ions, which is consistent with a stoichiometry of 1:1 for adenylate cyclase-CaM interaction. Dissociation constants calculated from data obtained in several experiments range from 0.09 to 0.17 nM in the presence of Ca²⁺ and from 13 to 23 nM in the presence of a large excess of EGTA. When dissociation constants have been calculated from CaM-adenylate cyclase dose-response curves, the values obtained were similar (not shown). Despite these differences in affinity of \( B. \ pertussis \) adenylate cyclase for CaM, once the complex was formed in the presence of Ca²⁺, addition of excess EGTA did not promote its dissociation (Fig. 3). This is consistent with our previous observation that adenylate cyclase bound to CaM-agarose cannot be eluted by EGTA.

Azido-CaM, a photoactivatable derivative of CaM (23), behaves almost identically to the parent compound, both in activating the bacterial adenylate cyclase (not shown) and binding to the enzyme (Fig. 2). Thus, the half-maximum activating concentrations of azido-CaM are the same as those of CaM either in the presence of Ca²⁺ or EGTA. \( K_D \) values calculated from data shown in Fig. 2 indicated a similar affinity of CaM and azido-CaM for adenylate cyclase.

Solid-phase Iodination of Pure Adenylate Cyclase—Solid-phase iodination of pure adenylate cyclase bound to Affi-Gel-CaM yielded an active \(^{125}\)I-adenylate cyclase preparation which was activated up to 50-fold by 100 nM CaM. Autoradiography after SDS-polyacrylamide gel of the iodinated enzyme revealed the same three peptides of 50, 45, and 45 kDa (Fig. 4) which were also detected by Coomassie Blue staining of the CaM-Affi-Gel-purified enzyme (16). Peptide mapping of these three bands gave similar patterns (not shown).

In order to determine whether all three peptides were endowed with adenylate cyclase activity, they were separated by SDS-polyacrylamide gel and the corresponding bands, revealed by autoradiography, excised; the iodinated polypeptides were extracted with 8 M urea, 1% Nonidet P-40 (see "Experimental Procedures"). Upon dialysis in the presence of CaM, each polypeptide recovered adenylate cyclase activity (Table I).

Moreover, as will be shown below, the 50- and 45-kDa peptides can be converted to the 43-kDa species by limited proteolysis. These results suggest that the three peptides are structurally related. Since adenylate cyclase is released extracellularly, it seems likely that the three peptides arose from a differential processing of a common precursor during secretion.

Limited Proteolysis of Purified \(^{125}\)I-Adenylate Cyclase by Trypsin—Incubation of free \(^{125}\)I-adenylate cyclase with trypsin at 4°C (at a 1:1 (w/w) ratio) resulted in complete inactivation of the enzyme within 3 min (Fig. 5A); the 50-, 45-, and 43-kDa polypeptides were converted to a 24-kDa fragment.
FIG. 5. Limited proteolysis of purified $^{125}$I-adenylate cyclase by trypsin. A. 0.1 units of $^{125}$I-adenylate cyclase in 0.1 ml of buffer A supplemented (AC-CaM) or not (AC) with 0.4 μM CaM were submitted to trypsin proteolysis at 4 °C as described under “Experimental Procedures.” After different times of digestion (indicated at the bottom of the figures), aliquots were withdrawn and diluted in buffer A containing soybean trypsin inhibitor in a 20-fold molar excess over TPCK-trypsin. Adenylate cyclase activity (expressed in percentage of initial activity) was determined; the different samples corresponding to the different times of digestion were then run on a 12.5% SDS-polyacrylamide gel and the gel was autoradiographed. Molecular mass standards: bovine serum albumin (67 kDa), glyceraldehyde-3-phosphate dehydrogenase (38 kDa); soybean trypsin inhibitor (20 kDa), and lysozyme (14 kDa). The autoradiograph was overexposed for better visualization of T18. B, after autoradiography, the bands corresponding to the native polypeptides (50, 45, and 43 kDa) (●) or to the T25 (■), and T18 (○) fragments were sliced from the dried gel and the radioactivity was measured.

FIG. 6. Autoradiograms of two-dimensional tryptic peptide maps of T18, T25, and T24. The two-dimensional tryptic peptide maps were performed as described under “Experimental Procedures.” ○ designates the origin of migration.
**FIG. 7.** Limited proteolysis of the isolated 50-, 45-, and 43-kDa iodinated polypeptides. The 50-, 45-, and 43-kDa polypeptides were separated on SDS-polyacrylamide gel and renatured as described under "Experimental Procedures." Each peptide (15 × 10⁻³ units/ml in buffer A containing 3 μM of CaM) was digested with 0.5 μg/ml of trypsin at 4°C. At the indicated times 100-excess of soybean trypsin inhibitor was added over trypsin and adenylate cyclase activity was assayed; then the corresponding samples were run on a 9% (A) or a 12.5% (B) SDS-polyacrylamide gel and autoradiographed. Lanes a-d: the 50-kDa polypeptide was digested for 0, 0.5, 5, and 10 min, respectively (corresponding units/ml in buffer A containing 3 μM of CaM) was digested with 0.5 μg/ml of trypsin at 4°C; the digestion was stopped by addition of 10 μg of soybean trypsin inhibitor (remaining activity: 6 × 10⁻² units). The sample was then diluted in 100 μl of buffer A containing 200 μg of bovine serum albumin, 30 μg of adenylate kinase, 1 μg of CaM, and 20 μg of soybean trypsin inhibitor and loaded onto an Ultrogel AcA-44 column (0.6 × 28 cm) equilibrated in buffer A containing 20 μg/ml of soybean trypsin inhibitor. Fractions of 0.1 ml were collected at a flow rate of 0.5 ml/h and analyzed for activity (O) and radioactivity (●). Insert shows an autoradiograph of the two-dimensional tryptic peptide map (Fig. 6). The generation of the minor iodinated peptide T18, obtained only in the presence of CaM, was highly reproducible with the same ¹²⁵I-adenylate cyclase preparation. The apparent yield of this peptide varied with different preparations of ¹²⁵I-adenylate cyclase; for instance in a previous report it was not detected (16). It is likely that differences in intensity could be accounted for by differences in iodination of the regions that yield T25 and T18 due to the number of exposed tyrosines in each fragment.

To elucidate the origin of T25 and T18, the bands corresponding to these peptides (Fig. 5A) have been excised for quantification. As shown in Fig. 5B, the kinetics of appearance of the two tryptic peptides was similar and in addition, their ratio remained constant. Thus, it is unlikely that T18 is derived from T25. Moreover, the two-dimensional tryptic peptide map of T25 was significantly different from that obtained for T18 (Fig. 6), suggesting that T25 is not related to T18. Thus, T25 and T18 might represent two different domains of adenylate cyclase.

To determine further whether all three native polypeptides (50, 45, and 43 kDa) contain both T25 and T18 domains, limited proteolysis was performed on the separated iodinated polypeptides complexed with CaM. As shown in Fig. 7, upon exposure to trypsin, the 50- and 45-kDa bands were rapidly converted into a 43 kDa one, which was further cleaved into T25 and T18. These results demonstrate that each polypeptide of 50, 45, and 43 kDa contains a common region of 43 kDa which can be proteolytically cleaved into two peptides of 25 and 18 kDa.

**FIG. 8.** Gel filtration chromatography of trypsin cleaved adenylate cyclase/CaM complex. 3 × 10⁵ cpm of ¹²⁵I adenylate cyclase (9 × 10⁻³ units) in 50 μl of buffer A containing 0.5 μg of CaM were digested 10 min with 75 ng of trypsin at 4°C; the digestion was stopped by addition of 10 μg of soybean trypsin inhibitor (remaining activity: 6 × 10⁻² units). The sample was then diluted in 100 μl of buffer A containing 200 μg of bovine serum albumin, 30 μg of adenylate kinase, 1 μg of CaM, and 20 μg of soybean trypsin inhibitor and loaded onto an Ultrogel AcA-44 column (0.6 × 28 cm) equilibrated in buffer A containing 20 μg/ml of soybean trypsin inhibitor. Fractions of 0.1 ml were collected at a flow rate of 0.5 ml/h and analyzed for activity (O) and radioactivity (●). Insert shows an autoradiograph of the two-dimensional tryptic peptide map (Fig. 6). The generation of the minor iodinated peptide T18, obtained only in the presence of CaM, was highly reproducible with the same ¹²⁵I-adenylate cyclase preparation. The apparent yield of this peptide varied with different preparations of ¹²⁵I-adenylate cyclase; for instance in a previous report it was not detected (16). It is likely that differences in intensity could be accounted for by differences in iodination of the regions that yield T25 and T18 due to the number of exposed tyrosines in each fragment.

To elucidate the origin of T25 and T18, the bands corresponding to these peptides (Fig. 5A) have been excised for quantification. As shown in Fig. 5B, the kinetics of appearance of the two tryptic peptides was similar and in addition, their ratio remained constant. Thus, it is unlikely that T18 is derived from T25. Moreover, the two-dimensional tryptic peptide map of T25 was significantly different from that obtained for T18 (Fig. 6), suggesting that T25 is not related to T18. Thus, T25 and T18 might represent two different domains of adenylate cyclase.

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**Gel Filtration Chromatography of Trypsin-cleaved Adenylate Cyclase-CaM Complex—Analysis of the trypsin-cleaved ade-
Cross-linking of azido-CaM to tryptic fragments of adenylate cyclase. A. 0.1 units of pure $^{125}$I-adenylate cyclase in 50 $\mu$L of buffer A supplemented with (lanes 1 and 2) or without (lane 3) 0.4 $\mu$M of azido-CaM were incubated with 50 ng of trypsin at 4°C for the indicated time. Proteolysis was stopped by addition of 5 $\mu$g of soybean trypsin inhibitor, and adenylate cyclase activity was determined (expressed in % of initial activity). Sample 3 was supplemented with 0.4 $\mu$M azido-CaM, and then all samples were photolysed (+) or not (-), run on a 10% SDS-polyacrylamide gel, and the dried gel was autoradiographed. B. 0.1 units of pure adenylate cyclase in 50 $\mu$L of buffer A supplemented with (samples 1 and 2) or without (sample 3) 100 nM azido-$^{125}$I-CaM were submitted to trypsin proteolysis as above. Proteolysis was stopped with an excess of soybean trypsin inhibitor, and adenylate cyclase activity was determined. To sample 3 100 nM azido-$^{125}$I-CaM were added, and then all samples were treated as described in A.

Identification of CaM-binding Sites in Adenylate Cyclase by Photoaffinity Labeling with Azido-CaM—Preliminary experiments revealed that photolysis of a mixture of azido-$^{125}$I-CaM and pure or crude preparations of adenylate cyclase resulted in one major cross-linked product exhibiting a $M_r$ of 70 kDa on SDS-polyacrylamide gel (Fig. 9) which was not observed when photolysis was carried out in the presence of a 25-fold molar excess of unmodified CaM. In the absence of photolysis no cross-linked product could be revealed. In a similar way, photolysis of a mixture of $^{125}$I-adenylate cyclase and azido-CaM resulted, again, in one major cross-linked product with a $M_r$ of 70 kDa on SDS-polyacrylamide gel (Fig. 9). Since the apparent $M_r$ of CaM or azido-CaM on SDS-polyacrylamide gel was 20 kDa, the 70-kDa cross-linked product corresponds most likely to covalent attachment of one adenylate cyclase polypeptide to one azido-CaM molecule.

When pure $^{125}$I-adenylate cyclase was complexed with
Azido-CaM, then submitted to trypsin proteolysis for 10 min, 69% of enzymatic activity was retained while the enzyme was entirely converted into T25 and T18 polypeptides. When the digested complex was subsequently photolyzed, three new cross-linked species of 63, 45, and 38 kDa were evidenced (Fig. 10A, lane 2). The 45- and 38-kDa bands might correspond to covalent attachment of azido-CaM to T25 and T18, respectively, whereas the 63-kDa band might result from covalent attachment of azido-CaM to both T25 and T18. Digestion of [125I]-adenylate cyclase with trypsin prior to photolysis in the presence of azido-CaM did not yield any cross-linked product (Fig. 10A, lane 3).

Similar experiments using unlated azide adenylate cyclase complexed with azido-[125I]-CaM gave essentially the same results: after 10 min of trypsin digestion, the same cross-linked peptides of 63, 45, and 38 kDa were detected upon photolysis (Fig. 10B, lane 2). Again, trypsin digestion of adenylate cyclase for 2 min prior to addition of azido-[125I]-CaM did not lead to any cross-linked species upon photolysis (Fig. 10B, lane 3).

**DISCUSSION**

CaM stimulation of B. pertussis adenylate cyclase has been widely documented (10-15, 24). The present results confirm that CaM binds strongly to adenylate cyclase even in the absence of Ca**2+** albeit with less affinity than in the presence of the divalent cation. The 1:1 CaM-adenylate cyclase complex did not dissociate upon addition of EGTA or in media of high ionic strength. This is not an unprecedented case since it has been shown that CaM (the a subunit) remained associated with muscle phosphorylase kinase in the absence of Ca**2+** (25).

Controlled proteolysis of adenylate cyclase by trypsin and/or photoaffinity labeling with azido-CaM suggested that adenylate cyclase is composed of two separate domains of 25 kDa (T25) and 18 kDa (T18) which both interact with CaM. After exposure of the adenylate cyclase-CaM complex to trypsin, the CaM molecule would bridge the two cleaved domains in a structure resembling the native complex. T25 and T18 associated with CaM appeared as very resistant species toward further proteolysis; in contrast, when proteolysis was carried out on adenylate cyclase in the absence of CaM, only a 24-kDa trypsin-resistant fragment (T24) was detected. The specific radioactivity of this fragment as well as its trypptic map were similar to that of T25, suggesting that it corresponds to the same region of the native polypeptide. Since this 24-kDa fragment could not be cross-linked with azido-CaM, it is likely that the extra 1-kDa peptide present in T25 is involved in the binding of CaM. Edelman et al. (26) have already described differences in affinity for CaM of chymotryptic fragments of myosin light chain kinase differing by only 2 kDa at their C terminus. These results suggest that adenylate cyclase contains a compact domain of about 24 kDa largely resistant to trypsin proteolysis whether CaM was present or not. In contrast the T18 fragment displays resistance toward proteolysis only in the presence of CaM. This suggests that binding of CaM to adenylate cyclase could induce a conformational change in the T18 domain rendering it more resistant to proteolysis; alternatively, CaM could mask potential cleavage sites on T18 thus making them inaccessible to trypsin. In both cases, the protective effect involves a multisite interaction between CaM and T18 as depicted in the model presented in Fig. 11.

The structure proposed for B. pertussis adenylate cyclase differs significantly from those known for other CaM-dependent enzymes which can be modified by proteolytic cleavage to yield active CaM-independent forms (17, 26-30). Attempts to identify a CaM-independent catalytic domain of B. pertussis adenylate cyclase have failed thus far. It remains to determine whether this could be achieved using other approaches or if it represents a profound structural difference between B. pertussis adenylate cyclase and other CaM-dependent enzymes.

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