Running title: Catalytic mechanism of anthrax edema factor

**Structural and Kinetic Analyses of the Interaction of Anthrax Adenylyl Cyclase Toxin with Reaction Products, cAMP and Pyrophosphate**

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ABSTRACT

Anthrax edema factor (EF) raises host intracellular cyclic AMP (cAMP) to pathological levels through a calcium-calmodulin (CaM) dependent adenylyl cyclase activity. Here, we report the structure of EF-CaM in complex with its reaction products, cAMP and pyrophosphate (PPi). Mutational analysis confirms the interaction of EF with cAMP and PPi, as depicted in the structural model. While both cAMP and PPi have access to solvent channels to exit independently, PPi is likely released first. EF can synthesize ATP from cAMP and PPi and the estimated rate constants of this reaction at two physiologically relevant calcium concentrations are similar to those of adenylyl cyclase activity of EF. Comparison of the conformation of adenosine in the structures of EF-CaM-cAMP-PPi with EF-CaM-3’dATP reveals about 160° rotation in the torsion angle of N-glycosyl bond from the +anti conformation in 3’d-ATP to -syn in cAMP; such a rotation could serve to distinguish against substrates with the N2 amino group of purine. The catalytic rate of EF for ITP is about two orders of magnitude better than that for GTP, supporting the potential role of this rotation in substrate selectivity of EF. The anomalous difference Fourier map reveals that two ytterbium ions (Yb3+) could bind the catalytic site of EF-CaM in the presence of cAMP and PPi, suggesting the presence of two magnesium ions at the catalytic site of EF. We hypothesize that EF could use "histidine and two-metal-ion" hybrid mechanism to facilitate the cyclization reaction.
Keywords: adenylyl cyclase toxin, anthrax edema factor, cAMP, catalytic mechanism

Abbreviations: EF3: catalytic domain of edema factor (aa 291-800); EF3-CaM: catalytic domain of edema factor complexed with calmodulin; 3’dATP: 3’-deoxy-ATP; PMEAPP: adefovir diphosphate; 2’d-3’ANT-ATP: 2’-deoxy-3’-anthraniloyl ATP; cAMP: adenosine 3’,5’-cyclic monophosphate; P Pi: pyrophosphate, ITP: inosine 5’triphosphate; GTP: guanosine 5’ triphosphate; ATPαS: adenosine α-thio 5’ triphosphate.
INTRODUCTION

Cyclic AMP (cAMP), a key intracellular second messenger, is primarily regulated at the level of synthesis by adenylyl cyclase, the enzyme that converts ATP to cAMP and pyrophosphate. Adenylyl cyclase can be categorized into five classes (1,2). Enzymes within a class share sequence similarity but have no homology with members from the other classes. Class II adenylyl cyclase consists of several bacterial toxins that are secreted by pathogenic bacteria and activated upon their entry into host cells (3-5). These include edema factor (EF) from *Bacillus anthracis* (anthrax), CyaA from *Bordetella pertussis* (whooping cough) and ExoY from *Pseudomonas aeruginosa* (various nosocomial infections). Class III is the largest group which includes adenylyl cyclases from bacteria, yeasts, parasites, insects and vertebrates. Class III includes enzymes responsive to a plethora of extracellular signals such as hormones, neurotransmitters, odorants, and chemokines. These enzymes control diverse physiological responses such as sugar and lipid metabolism, fight or flight responses, and learning and memory. The other three classes are found in various prokaryotes including gram-negative bacteria (class I), *Aeromonas hydrophila* (class IV), and *Prevotella ruminicola* (class V). The molecular structures of catalytic domain of EF (class II) and mammalian adenylyl cyclase (class III) reveal no structure similarity between these two members, suggesting the converging evolution of these two classes of enzymes (6,7).

EF, a key virulence factor for anthrax pathogenesis, has two functional domains (8,9). The N-terminal 30 kDa of EF binds anthrax protective antigen (PA) with high affinity (5-10 nM), enabling its entrance into the intracellular space (10). The C-terminal 58 kDa domain of EF is a calmodulin (CaM) dependent adenylyl cyclase and its activity is modulated by
physiological calcium concentrations (5). This domain can be further divided into two
functional entities. The N-terminal 43 kDa portion of the adenylyl cyclase domain of EF forms
the catalytic core which shares 34% and 29% sequence similarity to CyaA and ExoY while the
C-terminal 17 kDa helical domain has no catalytic activity but facilitates CaM activation of EF
(11). Structures of the 58 kDa domain of EF alone and in complex with CaM reveals that one
of the catalytic loops of EF is disordered in the absence of CaM (6). CaM has N- and C-
terminal globular domains, each binding two Ca\(^{2+}\) ions (12). NMR and mutational analyses
suggests that the N-terminal CaM initiates its contact with the C-terminal 17 kDa helical
domain of EF, which leads to the insertion of C-terminal CaM between the catalytic core and
helical domains of EF (5,13). The binding of CaM induces the conformational changes to
stabilize the disordered catalytic loop, leading to over 1000-fold increase in the catalytic rate
(6).

EF has relatively high catalytic rate with a turnover number around 1-2 ms\(^{-1}\). With a
Km around 0.2-1 mM, the catalytic efficiency (\(k_{cat}/K_m\) of EF-CaM) approaches 10\(^7\)/M/s, a
catalytic rate that is at least 100 fold higher than mammalian adenylyl cyclases (mAC)
(5,6,14,15). Structures of EF3-CaM in complex with several non-cyclizable ATP analogs
together with mutational analyses have provided a starting point in building a model of EF
catalysis (5,6,16). The adenine moiety is recognized by a main chain carbonyl while the ribose
is held in position by an asparagine (N583). The triphosphate moiety is coordinated by several
positively charged residues, including Arg 329, Lys 346, Lys 353, and Lys 372. His 351 is
near the putative 3'OH. The homologous residue in CyaA (H63) is postulated to act as a
catalytic base (17). This is based on the observation that the mutation of H63 to arginine
shifted the pH dependency toward a more alkaline optimum. Thus, H351 is proposed to serve
as a catalytic base to generate 3’ oxy anion. EF also has two aspartates, Asp 491 and Asp 493 that could coordinate the catalytic metals similar to mAC and many DNA and RNA polymerases (15,18-24).

Little is known about how EF binds and releases reaction products, cAMP and PPI. Here we report the structure determination of EF-CaM in complex with reaction products, cAMP and PPI as well as a kinetic analysis of EF. These analyses suggest a mechanism for the binding and releasing of reaction products in EF. The structure of EF-CaM in complex with cAMP and PPI also offers evidence suggesting "histidine and two-metal-ion" hybrid mechanism of catalysis.
EXPERIMENTAL PROCEDURES

Materials: Quik-Change Kit was purchased from Biocrest, Bradford reagent from Bio-Rad. Ni\textsuperscript{2+}-NTA resin and anti-H5 antisera were from Qiagen. Pyrophosphate, cAMP, Rp-cAMP\textalpha{}S, Sp-cAMP\textalpha{}S, ITP, and GTP were from Sigma-Aldrich. The racemic mixture of ATP\textalpha{}S was from Jena Bioscience. The purified Rp-ATP\textalpha{}S and Sp-ATP\textalpha{}S diastereomers were gift from Fritz Eckstein at Max-Planck Institute. Hexokinase and type XI glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim and Sigma, respectively.

Protein expression and purification: The plasmids for the expression of mutant forms of the catalytic domain of EF (EF3) is constructed by site-direct mutagenesis and confirmed by DNA sequencing (11). The recombinant proteins expressed and purified from \textit{E. coli} including EF3, EF3 mutants and CaM were performed as described (5,6,11).

Structure determination of EF3-CaM-cAMP-PPi complex: To determine the structure of EF3-CaM-cAMP-PPi, crystals of EF3-CaM complex were grown using vapor diffusion, soaked with 1mM cAMP and 1mM PPI during cryoprotection for overnight, and frozen in liquid nitrogen as described (25). Data were collected at 100K at APS Biocars 14-BM-C and SBC ID-19 and processed with the programs DENZO and SCALEPACK (26). Initial phase was obtained by difference Fourier method using program CNS and the model of EF3-CaM complex (6). The model was refined and built using the programs CNS and O (27). The coordinates for EF3-CaM-cAMP-PPi are available from the Protein Data Bank (accession code 1SK6).
Enzymatic assays for the forward reaction of adenylyl cyclase: The activities were measured at 30 °C in the presence of 10 mM MgCl₂, the indicated ATP concentrations and a trace amount of [³²P]α-ATP for 10 min (28). The reaction is buffered by 100 mM Hepes, pH7.2 and free calcium concentration is controlled by 10 mM EGTA, to 0.1 µM/2 µM free Ca²⁺ based on calculated using the MAXC program, http://www.stanford.edu/~cpatton/max.html. cAMP were separated from ATP by Dowex and alumina columns as described (28). Initial velocities were linear with time, and less than 10% of the ATP was consumed at the lowest substrate concentrations.

Enzymatic assays for the reverse reaction of adenylyl cyclase: Synthesis of ATP from cyclic AMP and PPi by EF3 was measured spectrophotometrically in the presence of glucose, hexokinase, NADP, and glucose-6-phosphate dehydrogenase (14). Reaction velocities were calculated from the linear increase in A₃₄₀ resulting from the reduction of NADP. Reactions contained 100 mM Na-Hepes (pH 7.2), 50 mM glucose, 0.8 mM NADP, 10 mM free MgCl₂, 2.5 units of hexokinase, and 0.5 units of glucose-6-phosphate dehydrogenase in a volume of 500 µl. PPi was always added last to avoid precipitation. Reactions were typically started by the addition of adenylyl cyclase toxin to the reaction mixtures. The reaction is monitored based on the changes in A₃₄₀ for 15-20 min at 30 °C in a Beckman DU640 spectrophotometer with a temperature-controlled cuvette holder. The background for the change in A₃₄₀ in the absence of adenylyl cyclase was subtracted and optical densities of greater than 1.5 were excluded from analysis.
Non-isotopic adenylyl cyclase assays using HPLC: Adenylyl cyclase assays of EF were carried out using 10 μM CaM, 10 mM MgCl₂, 1.1 μM free CaCl₂ as calculated using MAXC program, and the indicated concentrations of EF and nucleotide triphosphate analog at pH 7.2. The reaction was incubated at 30°C (unless stated otherwise) and was stopped with 50 mM EDTA. 0.4 mM GTP or cAMP was added as a tracer to monitor the recovery. Phenol extraction was used to separate the nucleotides from the proteins. The aqueous phase was then loaded on reverse-phase C18 HPLC column and eluted with a linear gradient of 0.1 M triethylammonium acetate and acetonitrile.
RESULTS AND DISCUSSIONS

Structure of EF3-CaM in complex with cAMP and PPi: We have determined the structure of the catalytic domain of EF (EF3) and CaM with and without the non-cyclizable ATP analogs (6,16). To better understand the catalytic mechanism, we have solved the structure of EF3-CaM in complex with its reaction products, cAMP and PPi. To do so, EF3-CaM crystals were soaked with cAMP and PPi (1 mM each) and the crystal diffracted at best to 3.2Å resolution (Table 1). The structure model of EF and CaM in the EF3-CaM-cAMP-PPi structure is similar to those in EF3-CaM. EF3 consists of the catalytic core (CA and CB) and helical domains and the extended conformation of CaM inserts in between these two domains of EF3 (Figure 1A).

There are three EF-CaM molecules in each asymmetric unit of I222 crystal lattice and the cAMP and PPi molecules are clearly visible in the active site of all three EF molecules based on the simulated annealing omit map contoured at 3.5σ (Figure 1B). We have also soaked EF3-CaM crystals with 1 mM cAMP. However, the simulated annealing omit map reveals no visible electronic density of cAMP in the catalytic site of EF, suggesting that PPi is required for the high occupancy of cAMP.

The conformation of cAMP in all three EF3-CaM-cAMP-PPi model of the asymmetry unit is roughly similar. The ribose of cAMP is best fit to C2’-exo puckering with the torsion angle of N-glycosidic bond approximately -40° so that adenine is in the -syn conformation relative to ribose. The adenosine ring of cAMP forms van der Waals contacts with the main chains of Thr 548, Gly 578, Thr 579, Asp582, and Asn583. The N6 atom of adenine is within hydrogen-binding distance from the main-chain carbonyl of Thr548. The O4’ atom of ribose forms hydrogen bond with the side chain of Asn 583; such interaction is postulated to hold ribose in place during the catalysis (6). The 3’O of cAMP is within hydrogen bonding distance
(2.7Å in model B) from the side chain of H351, which is proposed to serve as a catalytic base (6). PPI is about 4.3Å - 5.1Å away from cAMP and is coordinated by salt bridges with several positively charged residues including Lys372 and Lys346.

Ytterbium ion is one of the additives that promote the growth of EF-CaM crystal. We have found that only one ytterbium ion occupies the catalytic site of EF in the structure of EF-CaM in complex with the non-cyclizable ATP analog, 3’deoxy-ATP (3’dATP) (6). This ion is coordinated by Asp 491 and Asp 493. Surprisingly, the anomalous difference Fourier map reveals the presence of more than one ytterbium ion (supplemental figure 1) in the active site of EF-CaM-cAMP-PPI. In particular, the alternation of one Yb³⁺ ion and two Yb³⁺ ions in a ratio of four to one reduces the residual electron density (Fₒ-Fc) to be less than 2.5σ. This suggests that two metal binding states exist in the structure of EF-CaM-cAMP-PPI: one with a single Yb³⁺ ion and the other with two Yb³⁺ ions. In the single Yb³⁺ binding state, the metal ion is coordinated by Asp491, Asp493, and His577. This coordination pattern is similar to that of the Yb³⁺ ion in the structures of EF-CaM-3’dATP and EF-CaM-2’d3’-ANT-ATP complexes (5,6). In the state with two Yb³⁺ ions, the two ions are about 4Å apart from each other. The first ion is coordinated by His577 and Asp493, and also interacts with 3’O of cAMP (3.7Å in model B). The second ion is coordinated by Asp491 as well as the phosphate of both cAMP and PPI.

**Mutational analysis of EF to validate the crucial interactions of EF with its products:**

Mutational analysis was used to evaluate whether the EF3-CaM-cAMP-PPI model accurately depicts the interaction of EF with PPI and cAMP. Lys372 forms a salt bridge with 3’dATP (6), and also appears to make a crucial contact with PPI in the structure of the reaction product. Thus, we made the EF3-K372A mutant, in which Lys372 is mutated to alanine. This mutation...
reduced the catalytic rate constant of EF3-K372A 30-fold, and increased the Km value of ATP 3-fold (EF3, 6.0 ms\(^{-1}\), 0.6 mM; EF3-K372A, 0.2 ms\(^{-1}\), 2.0 mM) with minimal effect on the EC\(_{50}\) value for CaM activation (EF3, 12 nM and EF3-K372A, 6 nM). We then tested the ability of cAMP and PPi to inhibit EF3-K372A. Consistent with the structural model, EF3-K372A had at least a 20-fold increase in the IC\(_{50}\) value for the inhibition by PPi while its ability to be inhibited by cAMP was not affected (Figure 2A, B).

Lys353 forms a salt bridge with Glu588. This salt bridge forms a “lid” over the catalytic site of EF. In addition, Lys353 is in proximity to form a salt bridge with PPi and phosphate of cAMP. We have previously reported that the mutation of Lys353 to alanine resulted in 500-fold reduction in catalytic rate and 7-fold increase in Km value of ATP without affecting the interaction of EF with CaM (6). Our present kinetic analysis reveals that EF3-K353A also exhibits a 20-fold increase in IC\(_{50}\) value for the inhibition by PPi. In contrast, the propensity of this mutant to be inhibited by cAMP is unaltered (Figure 2A, B). Lys346 also forms a salt bridge with PPi. The mutation of Lys346 to alanine results in a reduction of catalytic rate greater than four orders of magnitude, making a more accurate kinetic analysis impractical.

In the EF3-CaM-cAMP-PPi structure model, adenosine moiety participates in numerous main chain interactions with EF. Here, the most prominent interaction is the hydrogen bonding of its O’4 atom with Asn583. Our previous analysis showed that the mutation of Asn583 to alanine decreased the catalytic rate constant 150-fold. The same mutation had only a minimal effect on the Km value of ATP or the IC\(_{50}\) value for CaM activation (6). Consistent with our structural model, EF3-N583A increases the IC\(_{50}\) value for the inhibition by cAMP about 10-fold, while the sensitivity of this mutant to the inhibition by
PPi is the same as that of the wild type enzyme (Figure 2C, D). Our structures show that Glu588 and Asp590 contribute the organization of the catalytic site of EF (6) but they are not directly involved in the binding of cAMP and PPi. The mutation of these residues to alanine resulted in minimal alteration of the inhibition by cAMP or PPi (Figure 2C, D). Thus, our mutational data confirms the structural model of EF3-CaM-cAMP-PPi.

**Product inhibition of the EF3-CaM complex:** Patterns of inhibition of enzymatic activity by products can be used to determine whether the release of product is an ordered or random event. To do so, we examined the inhibition of adenylyl cyclase activity of EF3 by cAMP and PPi (Figure 3). As reported, calcium not only affects the binding of CaM to EF to facilitate activation but also binds directly to EF to inhibit catalysis (5). Thus, we performed our assays in the presence of two free Ca$^{2+}$ concentrations, 0.1 µM and 2 µM (Figure 3A,B). With the large excess of 10 µM CaM, most of EF should be tightly associated with CaM in both calcium concentrations. However, the activity of EF-CaM is minimally affected by calcium ion at the 0.1 µM free Ca$^{2+}$ while its activity is significantly reduced at the 2 µM free Ca$^{2+}$. At the 0.1 µM free Ca$^{2+}$, kinetic data could be interpreted as an ordered product release with PPi being released first. This is because the kinetic of inhibition of EF activity by cAMP was competitive while that by PPi was mixed. However, such ordered release became random at 2 µM free Ca$^{2+}$ when the kinetics of inhibition of enzymatic activity by either cAMP or PPi were mixed. The kinetic parameters are listed in Table 2.

This kinetic data is consistent with the structure of EF3-CaM-cAMP-PPi. Upon CaM binding, the catalytic site of EF changes from an open channel to a closed tube that is open at both ends (6). The EF3-CaM-cAMP-PPi structure reveals that cAMP and PPi have access to...
different solvent channels, allowing them to exit independently (Figure 1C). Why does the kinetic analysis show the apparent ordered release of products at the 0.1 µM free Ca²⁺? We hypothesize that this is due to the preferential binding of EF-CaM to cAMP over PPi at the 0.1 µM free Ca²⁺. Consequently, PPi has much higher probability to be released first. Although we could not measure the binding affinity of EF-CaM to cAMP or to PPi directly due to their low affinity, we found 7-fold decrease in the IC₅₀ value for the inhibition by PPi when the free calcium concentration was raised from 0.1 µM to 2 µM (Figure 3C). However the same change in the calcium concentration did not alter the IC₅₀ value for the inhibition by cAMP (Figure 3C). This is consistent with the notion that calcium can increase the affinity of PPi for EF.

**Kinetic analysis of the reverse reaction of EF-CaM:** The rate of the reverse reaction of EF3, which converts cAMP and PPi to ATP, has been examined by kinetic experiments presented in Figure 4 and Table 2. We performed an analysis of the rate of ATP synthesis at varying concentrations of cAMP and PPi. An ATP-coupled reaction was used to monitor the ATP concentration. We found that EF3 could readily convert cAMP and PPi to ATP, and that this reaction was CaM dependent. The dissociation constants for the binding of individual substrates to free enzyme can be determined from plots of the slopes versus 1/[cAMP] or 1/[PPi] (Table 2, Figure 4 inset). The plots of the apparent 1/Vₘₐₓ versus 1/[cAMP] or 1/[PPi] provide the Vₘₐₓ of the system at infinite substrate concentrations (2000 s⁻¹ at 0.1 µM free Ca²⁺ and 200 s⁻¹ at 2 µM free Ca²⁺) which is similar with the rate constant of the forward reaction (1200 s⁻¹ at 0.1 µM free Ca²⁺ and 147 s⁻¹ at 2 µM free Ca²⁺) (Table 2, Figure 4 inset).
Stereo-selectivity of EF for α-thio-adenosine triphosphate (ATPαS): The preferential conversion of Sp-ATPαS over Rp-ATPαS is the hallmark for several class III adenylyl cyclases (29,30). The reaction proceeds with the inversion of configuration on Pα, resulting in Rp-cAMPαS from Sp-ATPαS. The stereo selectivity of EF for ATPαS has not been examined. To address this question, we have established an HPLC elution profile to ensure the identity of Rp- and Sp-ATPαS as well as Rp- and Sp-cAMPαS. We then performed an adenylyl cyclase assay of CaM-activated EF using the mixture of Rp- and Sp-ATPαS (Figure 5A, C). We found that only Sp-ATPαS was converted to the product and the reaction product was Rp-cAMPαS based on its elution profile. We also performed the same reaction with Rp-ATPαS and observed no product that had the same retention time as either Sp- or Rp-cAMPαS (Figure 5B, D).

These results suggest that the cyclization reaction of EF is initiated by the direct attack of 3’ OH to the α phosphate and that the catalytic reaction does not involve pseudorotation of the pentavalent phosphorane intermediate (i.e. the interchange of two non-bridging phosphorane oxygens). This lack of pseudorotation is indicated by the observed inversion of configuration on Pα phosphorus upon going from the reactant to the products. The fact that the pseudorotation does not occur during the catalytic reaction could be explained either by phosphorane corresponding to a transition state rather than to a reaction intermediate, or by a phosphorane intermediate in which the barrier for pseudorotation is larger than the barrier for the departure of the leaving group (PPi). Because strong interactions of one of the non-bridging phosphorane oxygens with the metals bound in the EF active site can significantly increase the pseudorotation barrier compared to its magnitude observed in solution reactions, the observed inversion of configuration cannot be used to assess directly the character and relative stability
of the elusive phosphorane structure. On the other hand, the observed preference for the Sp stereoisomer of the ATPαS substrate clearly indicates that the interactions stabilizing the rate-limiting transition state structure are highly asymmetric. More specifically, interactions of the pro-Sp non-bridging oxygen are contributing significantly more to the transition state stabilization than interactions involving the pro-Rp oxygen.

**Structural comparison of EF-CaM-cAMP-PPi and EF-CaM-3’d-ATP:** Comparing the structure of EF3-CaM in complex with 3’dATP versus cAMP and PPi has provided new insights in the similarity and difference in how EF3 binds its substrate and reaction products (Figure 6). The ribose of both structures is held in place by the hydrogen bonding between O4’ ribose and the side chain of Asn583 while the adenine moiety is held by the van der Waals contacts with the main chain of EF as well as the hydrogen bonding of N6 adenine with main chain of Thr548. However, the conformations of adenosine moiety in these two structures are quite different. The ribose is shifted from 3’C-endo in 3’dATP to 2’C-exo in cAMP while the N-glycosidic bond is rotated from the +anti conformation (ac, 160°) in 3’dATP to -syn (-sc, -40°) in cAMP.

The nearly 160° rotation of adenine moiety may represent an important step in the overall reaction mechanism of EF catalysis, which may serve to prevent EF from using GTP as its substrate. While the EF3-3’dATP crystal structure can accommodate equally well anti conformers of both the ATP and GTP substrates, the N2 amino group of syn-GTP would sterically crash into the main chains of EF (Gly578/Thr579), which constitute a part of a loop crucial for catalysis. If this steric clash is indeed important, ITP which lacks the N2 amino should be a better substrate for EF than GTP. To test this hypothesis, we first examined
whether GTP and ITP could serve as the inhibitors for cyclization reaction of EF using ATP as the substrate (Figure 7A). We found that GTP acted as a competitive inhibitor of EF with Ki value of 0.3 mM. However, ITP inhibited the reaction of EF with Ki value of 3.2 mM in the manner that was mixed inhibition.

We then tested whether ITP and GTP could serve as the substrate for the cyclization reaction of CaM-activated EF. To do so, we performed the cyclization reaction with variable amounts of EF and quantified the production of cyclic nucleotide by HPLC (Figure 7B). The resulting catalytic rate from this assay is 250 s\(^{-1}\), which is similar to the catalytic rate observed in the α-\(^32\)P-ATP based assay. The production of cIMP could only be observed with 200 µg EF for 5 min (Figure 7B). The estimated catalytic rate of EF for the conversion of ITP to cIMP was 0.01 s\(^{-1}\). Under the same assay condition, no cGMP was observed (Figure 7B). We only observed the production of cGMP with prolonged incubation (14 hours) at the elevated temperature (37 °C instead of 30 °C). Thus, the estimated catalytic rate of EF for the conversion of GTP to cGMP was 250-fold lower than that for ITP (not shown). This suggests that the rotation of adenine may indeed play an important role in the substrate selectivity of EF, although other explanations of this selectivity, for example the binding of purine moiety to the non-productive, alternative binding site cannot be excluded (16).

The occupancy of the metal ion(s) is another difference in structures of EF-CaM in complex with either 3’dATP or reaction products. The anomalous difference Fourier map reveals a possible binding state with two ytterbium ions when EF-CaM is associated with cAMP and PPi. These two metal ions are coordinated by the side chains of same residues (His577, Asp 491, and Asp493) which coordinate the ytterbium ion in the EF-CaM-3’d-ATP structure. Magnesium is the catalytic ion in EF. Mg\(^{2+}\) is 20% smaller than Yb\(^{3+}\) and has a
smaller positive charge. Even though only one ytterbium is found in the catalytic site of EF in the EF-CaM-3’d-ATP structure, Klenow fragment of *E. coli* DNA polymerase I, proven to use two-metal-ion catalysis, can only bind one lanthanide ion at its catalytic site of (31). Thus, it is reasonable to assume that the catalytic site of EF could accommodate more than one magnesium ion. This pair of Mg$^{2+}$ ions could be arranged in a similar way as the ytterbium ions in the structure of EF3-CaM-cAMP-PPi with two Yb$^{3+}$ ions.

*Model for the mechanism of catalysis of EF:* From our analysis of the stereo-specificity of the ATP$\alpha$S cyclization reaction, we hypothesize that the reaction leading to the cAMP formation is mediated by the nucleophilic attack of the 3’-oxygen atom to the α phosphate. This attack generates a bipyramidal, penta-coordinated phosphorus, which may correspond to either transition state or high energy intermediate. To promote this mechanism, EF needs to bind its substrate, facilitate the generation of 3’-oxy anion and the formation of the penta-coordinated phosphorane transition state or intermediate, and promote the departure of PPi from this intermediate, as well as effectively release cAMP and PPi (Figure 8). Since the α phosphate of ATP belongs to the phosphate diester class of substrates, the reaction should be associative or concerted, rather than dissociative. This is because for the phosphate diester hydrolysis, the metaphosphate intermediate, which is a characteristic intermediate in the dissociative mechanism, is significantly less stable than its penta-coordinated phosphorane counterpart that defines the associative pathway (32). In contrast, phosphorane and metaphosphate intermediates or transition states are of similar energy for reactions involving phosphate monoester class of substrates (e.g. γ-phosphate of ATP) (33).
From the structure of EF3-CaM-3’d-ATP, we have proposed the following model for the mechanism of catalysis of EF (Figure 8) (6). The deprotonation of 3’OH is mediated by a catalytic base, histidine 351. A magnesium ion coordinated by D491, D493, and H577 as well as several positively charged residues including R329, K346, K372, are involved in stabilizing the transition state by neutralizing the negative charge developed during the inline attack of 3’oxy anion on the α phosphate. These groups also facilitate the dissociation of PPi from the transition state intermediate. Based on the comparison of structures of EF3-CaM-3’d-ATP and EF-CaM-cAMP-PPi described above, we propose one major revision to our previous model (Figure 8). While one magnesium ion serves to stabilize the developing charges on the oxygen bridging the α- and β-phosphates, an additional magnesium ion coordinating nearby the 3’hydroxyl group could serve to lower the pKa of 3’hydroxyl group by stabilizing the 3’oxy anion. This does not exclude a role for His351 as a catalytic base. A hybrid mechanism of catalysis using a catalytic base, His 351, as well as a catalytic metal ion should ensure rapid deprotonation of the 3’hydroxyl group. The additional metal ion could also work to lower the transition state energy of subsequent steps, including the stabilization of penta-covalent phosphorane intermediate and the departure of PPi from this intermediate. A similar hybrid mechanism which utilizes an aspartate residue and two metal ions has recently been proposed for T7 DNA polymerase based on the quantum mechanical/molecular mechanical calculations (32).

**Comparison of the mechanism of catalysis between EF and mAC:** From the structural and kinetic analyses, there are at least three major differences in the mechanism of catalysis between EF and mAC models. First is the recognition of adenine moiety. In mAC, the N6 and
N1 of adenine forms hydrogen bonds with the side chain of conserved aspartate and lysine, respectively, while only N6 of adenine forms a hydrogen bond with the main chain carbonyl of Thr548 in EF. The additional hydrogen bonding with N1 of adenine in mAC serves to distinguish ATP from GTP. This interaction also prevents a significant rotation of N-glycosyl bond of the substrate. This interpretation is supported by the fact that the interaction of adenine moiety with the mAC model, 5C1-2C2, is nearly identical in the six structures of 5C1-2C2-Gsα in complex with analogs which mimic the substrate and the reaction products (7,15,34).

Another difference between these two classes of adenylyl cyclases is the mechanism of deprotonation of the 3’ hydroxyl group. In the mAC model, the deprotonation is attributed to a metal ion only whereas in EF3, both histidine and a metal ion could work in concert. The third difference is the order of product release. Kinetic analysis reveals that cAMP has a high propensity to be released first in mAC. This allows adenosine analogs (P-site inhibitor), which mimic product, to cooperate with PPi to effectively inhibit the catalysis of mAC (14,34,35). In contrast, kinetic data indicate that PPi tends to be released before cAMP in EF. These three differences in the mechanism of catalysis and product release could contribute to the reasons why EF has at least two orders of magnitude higher catalytic activity than mAC (6,14).

**Conclusion:** From our structural and kinetic analyses, we have revised our model of the mechanism of catalysis in EF. Several hypotheses can be derived from this model that are suitable for evaluation both by computer simulation and empirical studies (32,36-38) Better understanding of the catalysis of class II enzymes will advance our understanding of the structural basis of the transition state stabilization by the enzyme environment and, importantly,
provide a molecular basis for identifying small molecule inhibitors that can specifically block the activity of bacterial adenylyl cyclase toxins. Such small molecule inhibitors can serve both as an experimental tool to address the role of adenylyl cyclase toxins in anthrax, whooping cough, hospital-acquired infections, and plague and as potential therapeutics against infections of several pathogenic bacteria (39,40).
FIGURE LEGEND

Figure 1  Structure of EF3-CaM-cAMP-PPi. (A) Secondary structure of EF3-CaM in complex with cAMP and PPi. The catalytic domain (CA and CB) of EF is in green, the helical domain is in yellow, CaM is in red, cAMP and PPi is by their atom color (C atom-grey, N atom-blue, O atom - red, and P atom - yellow). (B) The active site of EF3. cAMP and PPi in complex with either 2 Yb^{3+} ions (top) or one Yb^{3+} ion are shown with the simulated annealing omit map contoured at 3.5σ. (C) The surface representation of EF3-CaM that interacts with cAMP and PPi. The surface is colored by electrostatic potential. The binding surface for PPi is shown after 180° rotation along the vertex axis from the view of cAMP.

Figure 2  The inhibitions of EF3 mutants by cAMP and PPi. The activity was measured in the presence of 0.3nM EF3, 10µM CaM, 2mM ATP and 0.1µM free Ca^{2+} with the variable concentrations of cAMP and PPi. The specific activities of wild type EF3 and EF3 mutants, EF3-K353A, EF3-K372A, EF3-N583A, EF3-E588A, and EF3-D590A were 1197 s^{-1}, 2 s^{-1}, 30 s^{-1}, 8 s^{-1}, 50 s^{-1}, and 188 s^{-1}. Mean ±SE are representative of at least two experiments.

Figure 3  Product inhibition of adenylyl cyclase activity of EF3. Activities were measured with the indicated concentrations of cAMP and PPi under 0.1µM (A) or 2 µM (B) free Ca^{2+}. (C) Inhibition of adenylyl cyclase activity of EF-CaM by cAMP and PPi at the 0.1 µM (●) or 2 µM (■) free Ca^{2+}. Assays were performed in the presence of 10 mM free MgCl₂, 10 µM CaM, 1 nM EF3 and 0.125 to 2 mM ATP. The specific activities of EF3-CaM with 0.1 µM and 2 µM free Ca^{2+} were 1230 s^{-1} and 169 s^{-1}, respectively. Data are representative of at least two experiments.
Figure 4  ATP synthesis by EF3/CaM. Assays (3nM EF3) were performed in the presence of 10 mM MgCl$_2$ and 10 μM CaM with the indicated concentrations of cAMP and PPi at the 0.1 μM (A) and 2 μM free Ca$^{2+}$(B). Absorbance at 340nm was monitored as described under “Experimental Procedures” and rates were determined by linear fits to 20 data points. Data are representative of at least two experiments. Insets are the plots of intercept (●) or slope (■) with the variable concentrations of PPi or cAMP.

Figure 5  The stereo selectivity of ATPαS by EF based on the HPLC elusion profile. (A) Racemic mix of ATPαS. (B) Rp ATPαS. (C) 0.5 mM racemic mix of ATPαS was incubated with 10 μg EF, 10 μM CaM, 10 mM MgCl$_2$, 1.1 μM CaCl$_2$, and 1 mM EDTA for 1 hour at 30°C. GTP was used as a tracer. (D) 0.5 mM Rp ATPαS was incubated same as in (C).

Figure 6  The comparison of active sites of EF between EF-CaM-cAMP-PPi (top) and EF-CaM-3’d-ATP (bottom). The backbone of EF is colored green while residues in the catalytic site of EF, 3’d-ATP and cAMP-PPi of EF3-CaM-cAMP-PPi and EF-CaM-3’d-ATP are colored in grey for carbon, red for oxygen, nitrogen in blue and yellow for phosphorus (yellow).

Figure 7  The catalysis of EF for ITP and GTP. A. The Lineweaver-Burke plot for the inhibition of EF by ITP and GTP. B The HPLC elusion profile of nucleotides after the cyclization reaction of EF. Samples are incubated for 5 minutes in the 30°C with 10 μM CaM, 0.1 μM free Ca, 10 mM Mg, 10 mM EGTA and 1 mM of nucleotide triphosphate. For ATP as
the substrate, three low EF3 concentrations, 2 ng (dot line), 20 ng (dash line) and 200 ng (solid line) were used. For ITP or GTP as the substrate, much higher concentrations of EF3, 20 µg (dash line) and 200 µg (solid line) were used.

Figure 8  Proposed mechanism of catalysis of EF. For clarity, several key residues for catalysis such as R329 (salt bridges with the α and β phosphates), N583 (hydrogen bonding with O4’ ribose), H577 (coordinating both metal ions) are omitted.
### Table 1  Statistics of the EF3-CaM-cAMP-PPi complex data

<table>
<thead>
<tr>
<th>Data collection</th>
<th></th>
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<tbody>
<tr>
<td>Beamline</td>
<td>APS SBC</td>
</tr>
<tr>
<td>Space group</td>
<td>/222</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
<td></td>
</tr>
<tr>
<td>$a$</td>
<td>116.9</td>
</tr>
<tr>
<td>$b$</td>
<td>166.4</td>
</tr>
<tr>
<td>$c$</td>
<td>342.1</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>15-3.2</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.4 (96.1)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Redundancy&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.5 (4.3)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>$R_{\text{sym}}$(%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.2 (50.2)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>$I/\sigma$</td>
<td>16.6 (2.3)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
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</table>

<table>
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<tr>
<th>Refinement</th>
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<tr>
<td>$R_{\text{cryst}}$ (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.250</td>
</tr>
<tr>
<td>$R_{\text{free}}$ (%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.307</td>
</tr>
<tr>
<td>$\text{Rms}_{\text{bond}}$(Å)</td>
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</tr>
<tr>
<td>$\text{Rms}_{\text{angle}}$(º)</td>
<td>1.35</td>
</tr>
</tbody>
</table>

<sup>a</sup> $N_{\text{obs}}/N_{\text{unique}}$.

<sup>b</sup> $R_{\text{sym}} = \Sigma_i |<I>/ - I_i|/\Sigma <I>$ where $I_i$ is the intensity of the $j$th reflection and $<I>$ is the average intensity.

<sup>c</sup> the outer resolution shell.

<sup>d</sup> $R_{\text{cryst}} = \Sigma_{hkl}|F_{\text{obs}} - F_{\text{calc}}|/\Sigma_{hkl}F_{\text{obs}}$.

<sup>e</sup> $R_{\text{free}}$, calculated the same as for $R_{\text{cryst}}$ but on the 10% data excluded from the refinement calculation.
Table 2 Kinetic parameters of EF at two different calcium concentrations.

<table>
<thead>
<tr>
<th></th>
<th>0.1µM free Ca^{2+}</th>
<th>2µM free Ca^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward reaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>1200 s(^{-1})</td>
<td>147 s(^{-1})</td>
</tr>
<tr>
<td>( K_m )</td>
<td>1.3±0.4 mM</td>
<td>0.3±0.05 mM</td>
</tr>
<tr>
<td>( K_{i(cAMP)} )</td>
<td>3.7±0.6 mM*</td>
<td>1.3±0.3 mM*</td>
</tr>
<tr>
<td>( K_{i(PPi)} )</td>
<td>0.3±0.03 mM*</td>
<td>0.06±0.02 mM*</td>
</tr>
<tr>
<td><strong>Reverse reaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>2000 s(^{-1})</td>
<td>100-300 s(^{-1})</td>
</tr>
<tr>
<td>( K_{i(cAMP)} )</td>
<td>1.5±0.3 mM*</td>
<td>2.0±0.4 mM*</td>
</tr>
<tr>
<td>( K_{i(PPi)} )</td>
<td>1.0±0.2 mM*</td>
<td>1.2±0.1 mM*</td>
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</tbody>
</table>

*Ki is the apparent Ki determined by kinetic analysis, not by direct binding assays.
ACKNOWLEDGEMENTS

We are grateful to Dan Lu for purifying EF mutants, to Fritz Eckstein for Rp- and Sp-ATPαS, to Drs Rong-Guang Zhang, Gary Navrotsky, Bill Desmarais and Robert Henning at APS SBC and BioCars for their help in data collection, to Xiaojing Yang, Carmen Dessauer, and Jeff Beeler for the helpful discussions. This research was supported by NIH GM62548 grant. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under contract No. W-31-109-ENG-38.
REFERENCES


Figure 1

A

B

C

cAMP  PPi

Helical

CaM

C_A  C_B

K372  S354  K353  H351  N583  T548  H577  D493  D491  K346
Figure 2

(A) Fractional activity (%) of WT, K353A, and K372A as a function of cAMP concentration.

(B) Fractional activity (%) of WT and K353A, K372A as a function of PPI concentration.

(C) Fractional activity (%) of WT, N583A, E588A, and D590A as a function of cAMP concentration.

(D) Fractional activity (%) of WT, N583A, E588A, and D590A as a function of PPI concentration.
Figure 3

A. 0.1 µM free Ca\(^{2+}\)

B. 2 µM free Ca\(^{2+}\)

C. Fractional activity (%) vs. cAMP (mM) and PPI (mM) concentrations.
Figure 4

A. 0.1 µM free Ca\(^{2+}\)

B. 2 µM free Ca\(^{2+}\)
Figure 5

A. Rp-/Sp-ATPαS

B. Rp-ATPαS

C. Rp-/Sp-ATPαS and EF

D. Rp-ATPαS and EF
Supplemental figure 1

The active site of EF3 in complex with cAMP and PPI. 2 Yb$^{3+}$ ions is shown with the anomalous difference Fourier map contoured at 6.5σ. Residues in EF3, cAMP, and PPI is colored by their atom color (C atom-grey, N atom-blue, O atom - red, and P atom - yellow).
Structural and kinetic analyses of the interaction of anthrax adenyl cyclase toxin with reaction products, cAMP and pyrophosphate
Qing Guo, Yuequan Shen, Natalia L. Zhukovskaya, Jan Florian and Wei-Jen Tang

J. Biol. Chem. published online May 6, 2004

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