Protein-Protein Docking and Analysis Reveal That Two Homologous Bacterial Adenylyl Cyclase Toxins Interact with Calmodulin Differently

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Calmodulin (CaM), a eukaryotic calcium sensor that regulates diverse biological activities, consists of N- and C-terminal globular domains (N-CaM and C-CaM, respectively). CaM serves as the activator of CyaA, a 188-kDa adenylyl cyclase toxin secreted by Bordetella pertussis, which is the etiologic agent for whooping cough. Upon insertion of the N-terminal adenylyl cyclase domain (ACD) of CyaA to its targeted eukaryotic cells, CaM binds to this domain tightly (~200 pm affinities). This interaction activates the adenylyl cyclase activity of CyaA, leading to a rise in intracellular cAMP levels to disrupt normal cellular signaling. We recently solved the structure of CyaA-ACD in complex with C-CaM to elucidate the mechanism of catalytic activation. However, the structure of the interface between N-CaM and CyaA, the formation of which contributes a 400-fold increase of binding affinity between CyaA and CaM, remains elusive. Here, we used site-directed mutations and molecular dynamic simulations to generate several working models of CaM-bound CyaA-ACD. The validity of these models was evaluated by disulfide bond cross-linking, point mutations, and fluorescence resonance energy transfer experiments. Our study reveals that a β-hairpin region (amino acids 259–273) of CyaA-ACD likely makes contacts with the second calcium binding motif of the extended CaM. This mode of interaction differs from the interaction of N-CaM with anthrax edema factor, which binds N-Cal via its helical domain. Thus, two structurally conserved, bacterial adenylyl cyclase toxins have evolved to utilize distinct binding surfaces and modes of activation in their interaction with CaM, a highly conserved eukaryotic signaling protein.

Protein-protein interactions are integral to many mechanisms of cellular control, including signal transduction, protein localization, competitive inhibition, allosteric regulation, and gene regulation. Calmodulin (CaM) serves as an excellent model to address how a highly conserved and ubiquitously expressed eukaryotic calcium sensor can interact in a Ca2+-dependent manner with over 100 different effectors to transmit calcium-mediated cell signaling (1–3). The molecular structures of Ca2+-bound and Ca2+-free CaM have been determined by x-ray crystallography and NMR (4–8). CaM has two globular domains, termed N-CaM and C-CaM, which are connected by a flexible, central α-helix (9). Each domain is composed of two helix-loop-helix EF-hand calcium binding motifs (4). The binding of calcium to CaM initiates a series of conformational transitions to shift each of these EF-hand domains from a mainly hydrophilic closed state, to an open conformation, exposing a large, hydrophobic binding pocket (10–13). This hydrophobic pocket plays a key role in the binding of CaM to its cellular effectors in a calcium-dependent manner.

Structures of CaM in complex with its effectors have begun to reveal a rich repertoire of dynamic interactions (1–3, 14–20). Many of the structures composed of CaM in complex with CaM-binding effector peptides reveal that compact conformations of either apo- or calcium-loaded CaM use a canonical binding site to make a variety of tight contacts with targets (14, 16, 17, 20). Substantial biochemical analyses reveal that CaM also uses sites outside the canonical CaM-effector interaction region to utilize additional contact regions with its effectors (21–25). Structures of CaM in complex with CaM-activated anthrax adenylyl cyclase toxin, edema factor, and the CaM binding domain of rat small conductance potassium channels further expand our knowledge of the possible contact surfaces used by the extended conformation of partially calcium-loaded CaM to bind its effectors (15, 18, 19).

CyaA, a 188-kDa CaM-activated adenylyl cyclase toxin, is secreted by the pathogenic bacterium Bordetella pertussis, which causes whooping cough, a severe childhood disease

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whose prevalence is on the rise worldwide (26). CyaA serves as an anti-inflammatory and anti-phagocytic factor, facilitating respiratory tract colonization by *B. pertussis*. Upon binding to its cellular surface receptor, α_{5}β_{2} integrin, CyaA becomes an integral membrane protein by insertion of its N-terminal ACD into the cytosol. After binding to CaM, CyaA-ACD raises intracellular cAMP of the host cells to pathogenic levels. Our laboratory has recently solved the x-ray structure of the adenylly cyclase domain of pertussis CyaA in complex with C-CaM (16). Interestingly, although the ACD of CyaA shares substantial similarity in its tertiary structure and catalytic mechanism with the ACD of anthrax edema factor, their respective CaM contact surfaces have completely diverged.

CyaA binds CaM with a high affinity (0.2 nM), which is necessary for CyaA-ACD to efficiently compete with other cellular proteins that bind CaM located at or near the cell surface (26). Although C-CaM can bind and activate CyaA-ACD, N-CaM contributes a 400-fold increase to the already high binding affinity of CaM to CyaA (27). Interestingly, this 400-fold increase in activation can take place even when N-CaM is locked by a disulfide bond in the closed conformation or when N-CaM is unable to bind calcium. Furthermore, the extended conformation of CaM is used to bind and activate CyaA, thus the binding region of N-CaM to CyaA-ACD is likely distinct from those of C-CaM (15). The structural basis of how the binding of N-CaM contributes to the activation of CyaA by CaM remains elusive.

In this study, we used mutational analysis to define a key region in CyaA crucial for activation by N-CaM. N-CaM was then docked onto the structure of the complex of CyaA-ACD and C-CaM. Our flexible docking protocol considered three variants of the protein-protein interface, which were compatible with our initial mutational analysis. Structures of the resulting complexes were obtained by calculating nanosecond molecular dynamic (MD) trajectories, guided toward the bound state by flat-bottom harmonic constraints. The best theoretical structural model, which featured the lowest calculated binding-free energy, was subsequently confirmed using additional mutational studies. Both native CyaA and CaM lack cysteine residues. We introduced cysteine residues to CyaA and CaM and confirmed the model by the formation of disulfide bonds. To further validate this model, average distance measurements of fluorescent tags placed on chosen residues of CyaA and CaM were performed using fluorescence resonance energy transfer (FRET) analysis.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids for the Expression of CyaA-ACD and CaM; Purification and Labeling of CyaA-ACD and CaM for FRET Measurement**

Mutant forms of CyaA-ACD and CaM were generated using the QuickChange mutagenesis kit (Agilent Technologies, Inc.). The expression and purification of N-terminal hexahistidine-tagged CyaA-ACD, CaM, and their mutants were performed as described (16). To label CyaA-ACD or CaM, the purified protein was first incubated with 10 mM dithiothreitol (DTT) in a 20 mM Tris (pH 7.7) buffer at 4 °C overnight. After DTT was removed by PD-10 desalting column (Sephadex G-25, GE Healthcare), 1,5-IAEDANS (FRET donor), and DABMI (FRET acceptor/non-fluorescent quencher) were added to the concentration of 250 nM, and the reaction mixture was stirred in the dark at 4 °C overnight. The reaction was stopped by the addition of an excess of DTT. The PD-10 column was used to remove excess of fluorescence probe and DTT. Fractions containing the labeled protein were pooled and concentrated using Amicon YM10 membranes and stored in the dark to prevent photo-degradation of 1,5-IAEDANS. The labeling efficiency was calculated using the molar extinction coefficients of 1,5-IAEDANS (5,700 cm⁻¹ M⁻¹ at 336 nm) and DABMI (34,000 cm⁻¹ M⁻¹ at 419 nm) (28, 29).

**In Vitro Adenylyl Cyclase Assay**

The activities of the adenylyl cyclase were measured at 30 °C in the presence of 10 mM MgCl₂, indicated ATP concentrations, and a trace amount of α-ATP for 10 min as described (30). The reaction was buffered in 100 mM HEPES (pH 7.2), and the free calcium concentration was maintained at 1 µM using 10 mM EGTA based on calculated values using the MAXC program (www.stanford.edu/~c Patton/max.html). cAMP was separated from ATP by Dowex and alumina columns.

**MD Simulations**

The goal of our computational analysis was to predict the structure of the CyaA-ACD/full-CaM complex within the structural limits imposed by the crystal structure of the CyaA-ACD/C-CaM complex and the finite length of the linkage connecting the studied N-domain to the anchored C-domains. Because these additional structural requirements decreased the number of docked configurations that needed to be evaluated, we were able to subject both CyaA and N-CaM proteins and surrounding solvent to all-atom MD simulations. The actual simulations consisted of four stages, which included structure preparation, equilibration, a forced docking using a constraint-guided MD, and simulated annealing. The viability of the docked structures was examined by calculating the average electrostatic and van der Waals interactions of the docked helix of N-CaM and its environment, in the framework of the linear interaction energy (LIE) approximation (31). Each of these stages is described in detail below.

**Initial Structure Preparation**—Starting structures for classic MD simulations were generated from the crystal structure of CyaA-ACD/C-CaM (PDB code: 1YRT (16)) and from the solution structures of apo-CaM (PDB code: 1FC (10)). The N-terminal domain of apo-CaM was appended to CyaA-ACD/C-CaM to build a CyaA-ACD/full-length CaM structure. The linkage between the two domains was built by aligning CyaA-ACD/C-CaM and apo-N-CaM with helix IV (residue 83–92) and the ligation of N- and C-CaM. Three different conformations from the NMR solution structures of N-CaM were chosen as the initial models of CaM-bound CyaA-ACD complex (Fig. 2A). Charged residues were identified as Glu, Asp, Lys, and Arg based on their pK_{a} values in water. All residues farther than 30 Å from the C_alpha atom of Lys-26 were kept in their electroneutral forms. The total charge of the simulated system was 0 atomic units. All crystallographic water molecules were removed. New
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Water molecules were added by immersing the simulated protein molecules in a sphere (30-Å radius) of TIP3P water molecules modeled by a transferable intermolecular potential with three atom-centred point charges (TIP3P) (32) subjected to the surface-constraint all-atom solvent type boundary conditions. These constraints were designed to mimic infinite aqueous solution. Those water molecules that were not sterically overlapping with the atoms present in the crystal structure were retained in the starting structure for the MD simulations. Protein atoms lying outside the 30-Å sphere were fixed at their crystallographic positions, and their non-bonded interactions were turned off.

General Simulation Conditions—MD trajectories were calculated in a 30-Å simulation sphere centered on the Cα atom of Lys-26 using the program Q (33). The Amber 95 force field (34) was used. Residues 1–73 of N-CaM were defined as the “probe” region (1085 total atoms) for LIE calculations. Non-bonding interactions involving one or two probe atoms were explicitly evaluated for all distances, whereas remaining nonbonding interactions were subjected to a 10-Å cutoff. The local-reaction field method (35) was used to treat long-range electrostatic interactions for distances beyond this cutoff. All production trajectories generated constant-temperature ensembles at 310 K. The SHAKE algorithm was used for bonds involving hydrogen atoms. The structure and trajectory analyses were carried out using the program VMD 1.8.4 (36).

Equilibration—Solvated CyaA-ACD/N-CaM complex was equilibrated in a series of MD simulations during which the temperature of the system was gradually increased from 5 to 310 K, and the simulation step was increased from 0.01 to 2 fs. The total time of the equilibration phase was 235 ps.

Protein-Protein Docking—The N-CaM domain was docked onto the surface of the Cα domain of CyaA using a combination of constraint-guided MD and simulated annealing approaches, subject to structural constraints of the flexible five amino acid linkage (Lys-Asp-Thr-Asp-Ser) between the N- and C-terminal domains of CaM. The docking simulation for each model was performed for a total of 1.5 ns. Temperature was changed from 310 K (0.5 ns) to 500 K (0.25 ns) and then returned to 310 K (0.75 ns). Harmonic and flat-bottomed half-harmonic distance-constraint potentials were used during the first 1.25 ns of the docking simulation to bring the surfaces of N-CaM and CyaA into close contact in areas indicated by initial site-directed mutagenesis results. The distance restraints with flat-bottom harmonic well potential (0.1 kcal/mol−1Å−2) were placed on four pairs of amino acid residues between N-CaM and the Cα domain of CyaA to drive docking between N-CaM and the β-hairpin of CyaA. In addition, the internal distance constraints with standard harmonic well potential (10 kcal/mol−1Å−2) were placed on two pairs of residues within N-CaM to ensure its structural integrity during forced docking. All applied constraints are listed in detail in supplemental Fig. S1.

Calculations of the Relative Binding Free Energy—Binding free energies (ΔGbind) of each of the three docked complexes were evaluated using the LIE method (31) as ΔGbind = α(⟨Uvdw⟩L − ⟨Uvdw⟩B) + β(⟨UES⟩L − ⟨UES⟩B), where ⟨UES⟩ and ⟨Uvdw⟩ represent mean electrostatic and van der Waals interaction energies, respectively, of N-CaM (ligand) with its protein and water environment in a docked models A and B, and α and β are standard empirical factors, β = 0.43 and α = 0.18 (37). ⟨UES⟩ and ⟨Uvdw⟩ energies were evaluated for residues 1–73 of N-CaM from 3-ns MD trajectories of the docked CyaA-N-CaM complexes. A 2-fs integration step was used. Energies were sampled every 10 fs.

Disulfide Cross-linking

A cysteine mutant of CyaA was incubated with an equimolar amount of a cysteine mutant of CaM in the presence of 10 mM DTT for 1 h on ice to allow for the interaction. After removing DTT using a PD-10 column, samples were allowed to be oxidized for 10 min before the addition of 10 mM N-ethylmaleimide to quench any remaining free cysteines. Samples were then concentrated by trichloroacetic acid precipitation and analyzed by SDS-PAGE.

Steady-state FRET

The steady-state fluorescence measurements were performed at 4 °C on a Fluoromax-3 spectrofluorometer (Horiba Jobin Yvon Inc.). CyaA mutants (A94C and A225C) were labeled with fluorescent reagent 1,5-IAEDANS (N-iodoacetylnaphthyl-5-sulfo-1-naphthylethylendiamine, Invitrogen) as the fluorescence donor. CaM mutants (Q41C and D50C) were labeled with DABMI (4-dimethylaminophenylazophenyl-4’-maleimide, Invitrogen) as the acceptor. The reduction of 1,5-IAEDANS donor fluorescence emission by the DABMI acceptor was recorded between 420 and 580 nm and corrected for the buffer blank. The protein concentrations of CyaA and CaM were kept at 2 μM. The experiments were conducted in a buffer containing 20 mM Tris-HCl (pH 7.7) and 1 μM CaCl2.

The distance between residues of CyaA-ACD and CaM was estimated spectroscopically by FRET. The distance is given by: R = R0(E−1 − 1)1/6, where R is calculated in Å, R0 is the Förster critical distance, and E is the FRET efficiency. R0 is given by: R0 = 9.79 × 103(k2/3η4)1/6, where k2 is the orientation factor, η is the refractive index of the buffer, ΦD is the quantum yield of the donor, and J is the overlap integral in cm3/M given by: J = f(D(λ)e(λ)λ2dλ/Fp(λ)dλ, where λ is the wavelength in cm, fD(λ) is the corrected fluorescence of the unquenched donor, and e(λ) is the acceptor molar absorption coefficient in M−1 cm−1. J was obtained by numerical integration of normalized spectra. InstruView v0.5 software (Columbia University) was used to calculate the R0 values using the defined parameters.

Time-domain FRET

Time-domain fluorescence lifetime measurements were performed on a custom time-correlated single photon counting (TCSPC) setup with a SPC-140 TCSPC board and a PMD-100-0 cooled PMT (Becker-Hickl) (38). A frequency-doubled titanium:sapphire femtosecond laser and pulse picker (Spectra-Physics Mai Tai HP, Del Mar Photonics ATsG-3-0.8-P, Brimrose FSCD-400-80-BR-800, and FFJ-400-B4-F10, respectively) provided excitation at 370 nm with a repetition rate of 8 MHz. Samples were placed in a 100-μl microcuvette and 1-format fluorescence collected with a 10-cm lens, 450 nm long-pass
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filter, and polarizer at the magic angle. Count rates were maintained below ~1% of the excitation rate to avoid counting artifacts. Each decay curve contained at least 10,000 counts in its maximal channel. Instrument response functions were measured with a nonresonant 0.01% Ludox solution and were ~220 ps full width at half-maximum. Measured lifetime decays were fit to one-, two-, and three-component exponential models with optimization routines in the SPCImage analysis software package (Becker-Hickl). Optimal fits were determined by minimization of $\chi^2$ parameters and visual inspection of fits and residuals. FRET efficiencies were calculated according to $E = 1 - \left( \tau_{DA}/\tau_D \right)$, where $\tau_{DA}$ is the measured lifetime with donor and acceptor present, $\tau_D$ the lifetime without acceptor present, and $E$ the FRET efficiency. For multicomponent fits, the amplitude-weighted lifetimes $\langle \tau \rangle = \sum \alpha_i \tau_i$ were used for the FRET efficiency calculation (39). Binding distances $R$ were then calculated from FRET efficiencies as above for the steady state.

**Frequency-domain FRET**

Frequency-domain fluorescence lifetime measurements were carried out with an MF2 multilifetime fluorescence spectrophotometer (HORIBA, Jobin Yvon). The excitation source was a 365 nm light-emitting diode simultaneously modulated in an eight-frequency logarithmic series from 2 to 90 MHz. A 450 nm long-pass filter was used to isolate donor fluorescence emission from scattered excitation light. A Hamamatsu R928P PMT operating at 760 v detected the donor fluorescence via cross-correlation, and the resulting signal was digitally unmixed to give the individual frequency contributions. The phase shift and modulation ratio of each frequency component was recorded during a 10-s integration time and five averaging periods. A POPOP (1,4-bis(5-phenyloxazole-2-yl)benzenes) solution with a lifetime of 1.35 ns was used as the lifetime reference. Phase and modulation curves were fit to 1, 2, and 3 component exponential decay models with the FluorEssence analysis software (HORIBA) by minimizing $\chi^2$ parameters and optimizing visual inspection of fits and residuals. Constant error weights of 0.2 and 0.005 were assumed for the phase and modulation curves, respectively. Data were also analyzed in a similar manner using the VINCI software package (ISS). FRET efficiencies were calculated using measured donor lifetimes in the same manner as the time-domain measurements.

**RESULTS**

**Mutational Analysis to Identify the Regions in CyaA That Are Involved in N-CaM-dependent Activation of CyaA—CyaA-ACD** consists of Cα and Cγ domains, which form the catalytic site at their interface (16). Multiple regions of CyaA-ACD make substantial contacts with C-CaM, and these include helix F (aa 197–206), helix H (aa 235–246), the joining loop between helices G and H (aa 250–259) and the C-terminal end of CyaA-ACD (aa 346–360) (Fig. 1A). CaM has been shown to adopt an extended conformation upon binding to CyaA-ACD (15). Furthermore, CaM mutants that could not bind calcium ions to the EF-hand motifs of N-CaM or CaM with N-CaM locked in the closed conformation by a disulfide bond are fully potent for activation of CyaA (27). However, C-CaM alone cannot activate CyaA as potently as full-length CaM. Based on the fact that N- and C-CaM are joined by a flexible linker, which enables them to bind to two distinct sites of a target molecule cooperatively, we placed the N-terminal calcium-free closed conformations of calmodulin models from NMR structures (PDB code 1CFC) into the C-CaM-bound CyaA-ACD structure model (PDB code 1YRT) (3, 9). We found several models that did not physically clash with the C-CaM bound CyaA-ACD structure and could have an acceptable match between the shapes and electrostatic potential energy surfaces of both proteins.

From this crude modeling, as well as the existing knowledge of CaM-effector interactions, we first postulated that N-CaM interacts with helix G and the loop between helices G and H (aa 213–236) (Fig. 1A), because N-CaM is known to interact with the helical regions of cellular effectors, such as myosin light chain kinase (40), CaM kinase II-α (41), CaM kinase kinase (42), the Ca$^{2+}$-sensitive potassium channel (18), and anthrax edema factor (15, 19). By examining the possible interaction of N-CaM with CyaA, we hypothesized that the acidic residues at the second calcium binding loop of N-CaM might form salt bridges with the basic residues of the loop between helices G and H of CyaA-ACD (arginines 223, 224, and 235 (Fig. 1A)), According to this model, mutation of these residues would affect the potency of CaM to activate CyaA-ACD, but would not affect the potency of C-CaM. We tested this hypothesis by site-directed mutagenesis of these specific residues (R223A/R224A and R235A) and asked if these mutations in CyaA had reduced sensitivity to activation by CaM (Fig. 1B). The adenyl cyclase assays revealed no significant reduction, suggesting that these residues do not affect CaM binding. This suggests that N-CaM is not likely to bind to helix G of CyaA.

We also considered the possibility that N-CaM could interact with the β-hairpin after helix H (aa 259–273) of CyaA, which resides at the opposite end of helix G, where it could form salt bridges or hydrogen bonds with Glu-256, Gln-260, or Arg-262 of CyaA (Fig. 1A). Our mutational analysis revealed that CyaA mutants containing mutation(s) from Glu-256 or both Glu-260 and Arg-262 residue(s) to alanine had 10- to 20-fold reduced sensitivity to activation by CaM but not by C-CaM (Fig. 1B). This mutagenesis data suggests that N-CaM indeed binds the β-hairpin region after the helix H of CyaA. Thus, different from the previous structural finding for N-CaM binding to its effectors, N-CaM binding to CyaA does not use the helix grasping mechanism.

The C-terminal helical domain of anthrax edema factor makes extensive contacts with N-CaM, and this interaction serves to anchor CaM onto anthrax edema factor to induce the conformational switch of this toxin (19, 43). We therefore hypothesized that an alternative possibility may be that the C-terminal region of CyaA-ACD (aa 361–373) binds N-CaM, as found in the edema factor activation. Truncation analysis was performed to test this hypothesis (Fig. 1C). The adenyl cyclase activity of the two CyaA truncation mutants showed that the deletion of aa 361–366 caused a 50-fold reduction in the sensitivity of CyaA catalytic activity to CaM-activation, whereas that of aa 367–372 did not. Interestingly, the removal of aa 361–373 of CyaA-ACD did not affect sensitivity to activation by C-CaM, which, as expected, was about three orders of magnitude less potent than CaM (27). These data suggest that
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A, the structural model of C-CaM bound CyaA-ACD (PDB code 1YRT). The Cα and Cβ domains of CyaA-ACD are colored in green and lime green, respectively. Helices F and G of CyaA-ACD are colored in blue, helix H in purple, and the β-hairpin in orange. C-CaM is colored in magenta. B, adenyllyl cyclase activities of CyaA mutants, CyaA1–373, CyaA1–373_R235A (open circle), CyaA1–373_R223A (open square), CyaA1–373_E256A (open triangle), CyaA1–373_R223A/R224A (closed triangle), CyaA1–373_E256A (open triangle), and CyaA1–373_Q260A/R262A (closed diamond) activated by CaM (left) and C-CaM (right). C, adenyllyl cyclase activities of two truncation mutants, CyaA1–361 and CyaA1–367. The assays were measured in the presence of 10 mM MgCl₂, 1 μM free calcium concentration, and the indicated concentrations of CaM at 30 °C for 10 min. These data are representative of two experiments, and the same applies to the following figures.

FIGURE 1. Characterization of CyaA-ACD mutants that have altered sensitivity to activation by CaM. A, the structural model of C-CaM bound CyaA-ACD (PDB code 1YRT). The Cα and Cβ domains of CyaA-ACD are colored in green and lime green, respectively. Helices F and G of CyaA-ACD are colored in blue, helix H in purple, and the β-hairpin in orange. C-CaM is colored in magenta. B, adenyllyl cyclase activities of CyaA mutants, CyaA1–373, CyaA1–373_R235A (open circle), CyaA1–373_R223A (open square), CyaA1–373_E256A (open triangle), CyaA1–373_R223A/R224A (closed triangle), CyaA1–373_E256A (open triangle), and CyaA1–373_Q260A/R262A (closed diamond) activated by CaM (left) and C-CaM (right). C, adenyllyl cyclase activities of two truncation mutants, CyaA1–361 and CyaA1–367. The assays were measured in the presence of 10 mM MgCl₂, 1 μM free calcium concentration, and the indicated concentrations of CaM at 30 °C for 10 min. These data are representative of two experiments, and the same applies to the following figures.

the short C-terminal segment of CyaA-ACD (aa 361–366) is involved in the potency of CyaA to be activated by CaM. However, upon examining the potential interaction of this segment, we found that it is a large distance away from the presumed location of N-CaM. Thus, we postulate that the role of this segment is unlikely to be its direct binding to N-CaM.

MD Simulations and Mutational Analysis Reveal a Possible Mechanism for the Interaction between CyaA-ACD and N-CaM—Although N-CaM likely binds CyaA weakly, this weak interaction greatly enhances the affinity of CaM to CyaA (16, 27). The relatively low affinity of N-CaM to CyaA-CaM posts a challenge for protein-protein docking, because standard docking algorithms generally works poorly when trying to identify a low affinity complex. Indeed, when we attempted to dock N-CaM onto C-CaM-bound CyaA-ACD using protein docking programs, Z-dock and Auto-dock without any restraints, neither program generated a satisfactory model that was consistent with our mutational data (44, 45). We then used Rosetta dock, which has been successfully used to model protein complexes, such as the binding between anthrax protective antigen and host cell receptor (46, 47). We ran the docking with and without the following restraints: the distance restraints were placed to move the second calcium-binding, EF-hand motif close to the β-hairpin of CyaA-ACD. A restraint was also placed to anchor the C-terminal end of N-CaM to the N terminus of C-CaM, because N-CaM is linked to C-CaM by a flexible linker with a defined degree of freedom (9). Unfortunately, no satisfactory model emerged from our docking analysis.

We then performed MD calculations to construct a model of CaM-bound CyaA-ACD. This method allows docking simulations with fully relaxed protein atoms and explicit solvent as well as a great degree of control in setting up restraints. The main disadvantage of all-atom MD simulations, large demands on computing resources when dealing with large protein-protein complexes, is of a lesser consequence here because the number of possible mutual orientations of CyaA and N-CaM is small. The three docked models were created by starting from three different NMR structures and using different sets of restraints to guide the MD docking (Fig. 2A). After simulation, three models were ranked based on their relative binding free energies calculated using the LIE approach with standard empirical parameters for scaling electrostatic and van der Waals interactions. The model that provided the lowest binding free energy is depicted in Fig. 2 (A and C). A comparison of these models is shown in supplemental Fig. S2.

In this model, N-CaM uses helix III (residues 40–51) to interact with the β-hairpin (residues 255–264) of CyaA (Fig. 2C). This model also reveals that Glu-47 and Asp-50 of N-CaM...
are likely candidates to contact Gln-260, Arg-262, and/or Glu-256 of CyaA-ACD, residues shown to be important for the activation of CyaA-ACD by CaM (Fig. 1B). Consistent with this model, the mutations of these two acidic residues of N-CaM (Glu-47 and Asp-50) to alanine reduced the potency of CaM to activate CyaA-ACD by ~100-fold (Fig. 2D). As a control, a CaM mutant that has N-CaM locked in the closed conformation by disulfide bond linkage, termed Q41C/K75C, had the potency to activate CyaA similar to wild-type CaM (Fig. 2D).

To further evaluate this model, we designed a disulfide bond cross-linking experiment to test whether we could predict the proximity of paired residues from the complex of CaM and CyaA-ACD, because neither CyaA nor CaM contains cysteine. Based on our MD-simulated models, we used the program Disulfide by Design to predict possible disulfide bonds. This program has been used successfully to predict appropriate disulfide bonds that would lock proteases in the closed conformation (48–50). Three candidates of CyaA (Ala-257, Gln-260, and Thr-263) and two of CaM (Gln-41 and Asp-50) were chosen, and the corresponding cysteine mutants were made. Three cysteine mutants of CyaA (A257C, Q260C, and T263C) were incubated with two cysteine mutants of CaM (Q41C and D50C) and the formation of a disulfide cross-link between CyaA Q260C and CaM D50C was visible as a slow mobility band on an SDS gel (Fig. 3A). This cross-linking could be disrupted in the presence of DTT, which is indicative of cross-linking by a disulfide bond (Fig. 3A). As a control, other combinations of cysteine mutants failed to produce any observable cross-linking (supplemental Fig. S3).

As described above, mutants CyaA1–373 Q260A/R262A and CaM E47A/D50A have a reduced potency in CaM-dependent activation of CyaA (Figs. 1B and 2D). We found that the potency of CaM D50C to activate CyaA was decreased about 10-fold while that of CyaA1–373 Q260C to be activated by CaM was decreased ~10^3 times (Fig. 3B). Our mutational studies and a pair of engineered disulfide bonds between CyaA and CaM revealed that our MD-generated model represents a good working model of how the N-CaM portion of CaM binds to the β-hairpin region in the C_A domain of CyaA (Fig. 2C).

Steady-state and Lifetime FRET Experiments to Evaluate the MD-generated Model—To further evaluate our MD-generated model, we performed steady-state, time-domain lifetime, and frequency-domain lifetime FRET experiments to gain three independent distance measurements for optimal accuracy (Figs. 4–6, Table 1, and supplemental Tables S1 and S2) (39). We examined the distances from Ala-94 and Ala-225, residues that are located at the opposite ends of CyaA, to either Gln-41 or Thr-26 of CaM (Fig. 2B). The mutation of these residues to cysteine had minimal effects on the potency of CyaA activation by CaM (Fig. 4A). The donor fluorescence probe chosen for labeling CyaA was 1,5-IAEDANS for several reasons (28, 29). First, its quantum yield is reasonably high (0.61), and its emission spectrum is well separated (red-shifted) from intrinsic protein fluorescence. Second, its fluorescence lifetime is fairly long (12–20 ns), and often monoeponential when attached to a protein. This means that lifetimes can be measured very accurately and changes can be easily detected and quantified. The donor-labeled CyaA had the expected fluorescence absorption and emission profile (28). As an acceptor for labeling CaM, we used the non-fluorescent probe DABMI. This FRET pair has a suitable critical transfer distance R0 of ~45 Å and is well known in the literature. Choosing a non-fluorescent acceptor avoids issues of spectral cross-talk (29).

In steady-state FRET experiments (Fig. 4B) the fluorescence emission of donor-labeled CyaA (both CyaA1–373 A94C and CyaA1–373 A225C) was significantly reduced with the addition of acceptor-labeled CaM (T26C or T41C) (Fig. 4B). The
donor-acceptor distances derived from this data are listed in Table 1. The $R_0$ parameter for FRET was determined as 44.7 Å based on spectral measurements with parameters $\kappa^2 = 0.67$, quantum yield $= 0.61$ and $\epsilon(\lambda_{\text{max}}) = 20,400$ M$^{-1}$ cm$^{-1}$.

Time-domain TCSPC lifetime fluorescence experiments were performed using the same set of labeled CyaA and CaM mutants (Fig. 5). The curve maxima are normalized to allow direct comparison of the decay behavior. The measured fluorescence lifetime decays of all mutants required at least a bi-exponential fitting model to yield a $\chi^2$ near unity. The best three-component fits included relatively short components; however, the amplitude weighted lifetimes (used for calculating FRET efficiency) were fairly invariant across models with different numbers of components. The best-fit measured donor lifetimes and calculated FRET distances under each condition are listed in supplemental Table S1 and distances summarized in Table 1.

Frequency-domain lifetime experiments were also conducted and produced similar results to the time-domain measurements. Again, each CyaA and CaM combination required at least a biexponential fit with an additional shorter component. The fits for the Cya-A94C mutants were less robust due to the presence of scattering components. However, the amplitude weighted lifetimes were again well preserved over models with potentially different numbers of components. Modulation and phase curves are shown in Fig. 6, and the fitting results are given in supplemental Table S2. Distances are summarized in Table 1.

In general, distance measurements based on all three different FRET experiments revealed reasonably consistent values (Table 1). When compared with the MD-generated model, the distances from CaM Thr-26 to CyaA Ala-94 are in an excellent agreement with our FRET measurements. In addition, the distance values from the FRET experiments from CaM Thr-26 to CyaA Ala-225 or CaM Gln-41 to Ala-94 are only slightly larger than those from our MD-generated model. Together with our mutational and cross-linking data, our FRET data again confirm the validity of our MD-simulated model.

The distance values measured by our FRET experiments between CyaA Ala-225 and CaM Gln-41 are significantly larger than those predicted from the MD-generated model, however, suggesting that this model places Gln-41 residue of CaM too close to the helix G of CyaA where Ala-225 resides (Fig. 2B). Interestingly, the loop region connecting helices G and H (aa 226–234) of CyaA is highly sensitive to protease cleavage and is disordered in our C-CaM-bound CyaA-ACD structure (16). This may explain the significant deviation between the modeled and observed distances. Future structural analysis of CaM-bound CyaA will be needed to resolve this discrepancy.

**DISCUSSION**

More than two decades of structural analysis reveals that the evolutionarily conserved eukaryotic calcium sensor, CaM, utilizes diverse surfaces to achieve selective and high affinity interactions in a calcium-dependent manner (1–3, 16). Using protein-protein docking in conjunction with experimental validation by mutational analysis, disulfide bond cross-linking, and FRET analysis, we show that the second calcium binding, EF-hand motif of CaM interacts with a $\beta$-hairpin motif of CyaA-ACD to contribute a 400-fold enhancement of binding and activation. This interaction is distinct from the interaction of N-CaM with the structurally related adenyl cyclase toxin, Adenylyl Cyclase Activity (s$^{-1}$)
Two Homologous Toxins Interact with CaM Differently

binding with the helical domain of anthrax edema factor. Together with previously revealed differences in the interaction of C-CaM with these two toxins, our analysis indicates that two structurally related adenylyl cyclase toxins can evolve to have completely different binding surfaces with each distinct region of CaM (16, 27).

In addition to increasing the affinity of CyaA to CaM via the contacts between the β-hairpin region of CyaA-ACD and N-CaM, N-CaM may also stabilize the structure of β-hairpin to facilitate the binding of ATP for the catalysis. Our structures of ATP analog-bound CyaA and anthrax edema factor reveal the vital role of the β-hairpin of CyaA in the recognition of adenine moiety of ATP (15, 16). This β-hairpin region of anthrax edema factor is a part of switch A, one of three regions that undergoes a substantial conformational switch upon CaM binding (15). The structural analysis indicates that this β-hairpin region in switch A is important for ATP binding (15). Although there is no structure of isolated CyaA-ACD currently available, the fact that N-CaM makes specific contacts with the β-hairpin region of CyaA suggests that the β-hairpin of CyaA-ACD has likely undergone substantial conformational changes upon binding CAM, thereby facilitating ATP binding and catalysis. This role is distinct from the role of C-CaM, which is to lock the C-terminal tail of CyaA-ACD (aa 346–364) in a state that is best able to stabilize the catalytic loop for the activation of CyaA by CaM (16).

Thus, not only are the contact regions used by these two toxins distinct, but the role of N-CaM in its interaction with B. pertussis and anthrax adenylyl cyclase toxins also varies. The binding of N-CaM to the β-hairpin of CyaA is to directly reshape the catalytic chamber. This is different from the role of N-CaM in the activation of anthrax edema factor, which is to promote the insertion of C-CaM into anthrax edema factor by facilitating a crucial conformational switch without which insertion and activation cannot occur (15). This

anthrax edema factor (19). In the case of the interaction between CaM and anthrax edema factor, the first calcium binding motif of CaM (instead of the second one) is involved in

FIGURE 4. Characterization of CyaA-ACD and CaM mutants and steady-state FRET analysis of their interactions. A, adenylyl cyclase activities of CyaA mutants (left) and CaM mutants (right). B, reduction of 1,5-lAEDANS-CyaA donor fluorescence upon binding to the DABMI-CaM acceptor. Fluorescence emission spectra of donor-labeled CyaA mutants, A94C and A225C, in the absence of CaM (solid line) and the presence of DABM-labeled CaM mutants, T26C and Q41C (dashed line). The concentrations of both proteins were 2 μM. λ exc = 335 nm.

FIGURE 5. Time-domain lifetime measurements of the fluorescence decay of 1,5-IAEDANS donor-labeled CyaA mutants (A94C and A225C) in the absence (solid lines) and presence (dotted lines) of DAMBI-labeled acceptor CaM mutants (T26C and Q41C). FRET interactions clearly reduce the lifetime of the donor decay. λ exc = 370 nm, λ det > 450 nm.
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Summary of distance measurements from FRET experiments and models generated from MD simulation

<table>
<thead>
<tr>
<th>Paired residues</th>
<th>Thr-26 (CaM)</th>
<th>Gln-41 (CaM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ala-94 (CyaA)</td>
<td>Ala-225 (CyaA)</td>
</tr>
<tr>
<td>Steady-state FRET</td>
<td>53 ± 4</td>
<td>52 ± 1</td>
</tr>
<tr>
<td>Time-domain lifetime FRET</td>
<td>58</td>
<td>53</td>
</tr>
<tr>
<td>Frequency-domain lifetime FRET</td>
<td>52</td>
<td>59</td>
</tr>
<tr>
<td>MD simulation-generated model</td>
<td>53</td>
<td>45</td>
</tr>
</tbody>
</table>

is consistent with the fact that the deletion of N-CaM significantly reduces the ability of CaM to activate CyaA by three orders of magnitude, whereas the same deletion completely abolishes the activation of anthrax edema factor by CaM (27).

The docking of a weakly interacting but functionally important domain into an x-ray crystallographic structure of a protein-binary complex that was solved in the absence of this domain was accomplished in the present work by subjecting a protein-binary complex that was solved in the absence of this domain into an x-ray crystallographic structure of a protein binary complex containing point mutations (58–61). Here, the LIE approach was used for the first time to distinguish between different binding conformations. Although the fact that the model with the most favorable LIE binding free energy also showed the best agreement with the experimental data is encouraging, further systematic studies and improvements of the combined constraint-guided MD/LIE approach will be necessary. Such analyses should provide a better means to construct full protein complexes from existing partial structures to gain a better mechanistic understanding of their biological functions.

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REFERENCES


FIGURE 6. Frequency-domain lifetime measurements of FRET between of 1,5-IAEDANS donor-labeled CyaA mutants (A94C and A225C) in the absence (solid lines) and presence (dotted lines) of DAMBI-labeled acceptor CaM mutants (T26C and Q41C). Phase (triangle) and modulation (circle) curves clearly show a right-shift, indicative of the FRET interaction. λexc = 365 nm, λdet > 450 nm.
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Protein-Protein Docking and Analysis Reveal That Two Homologous Bacterial
Adenylyl Cyclase Toxins Interact with Calmodulin Differently
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