High-Affinity Calmodulin Binding Is Required for the Rapid Entry of *Bordetella pertussis* Adenylyl Cyclase into Neuroblastoma Cells†

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Received February 20, 1992; Revised Manuscript Received May 22, 1992

ABSTRACT: *Bordetella pertussis* produces a calmodulin-stimulated adenylyl cyclase that invades animal cells and raises intracellular cAMP levels [Confer, D. L., & Eaton, J. W. (1982) *Science 217*, 948–950; Shattuck, R. L., & Storm, D. R. (1985) *Biochemistry 24*, 6323–6328]. The mechanism for invasion of animal cells by this enzyme has not been defined, but there is considerable evidence that it does not enter by receptor-mediated endocytosis [Gordon, V. M., Leppla, S. H., & Hewlett, E. L. (1988) *Infect. Immun. 56*, 1066–1069; Donovan, M. G., & Storm, D. R. (1990) *J. Cell. Physiol. 145*, 444–449]. In this study, the importance of high-affinity calmodulin (CaM) binding for entry of the enzyme into neuroblastoma cells was evaluated using a mutant enzyme that has significantly lower affinity for calmodulin than the wild-type enzyme. Oligonucleotide-directed site-specific mutagenesis was used to create a point mutant at a critical tryptophan residue (Trp-242) within the proposed CaM binding domain of the *B. pertussis* adenylyl cyclase. Substitution of Trp-242 with Glu lowered the apparent affinity of the enzyme for calmodulin by 250-fold; however, the maximal enzyme activity in the presence of saturating calmodulin was equivalent to the wild-type enzyme. The Glu-242 mutant adenylyl cyclase was returned to *B. pertussis* by homologous recombination, and the enzyme produced by this strain was examined for invasion of neuroblastoma cells. Although the mutant enzyme stimulated the production of intracellular cAMP in neuroblastoma cells, the rate of cAMP accumulation was at least 10-fold lower than that caused by the wild-type enzyme. These differences in the rate of cAMP production were not due to suboptimal stimulation of the mutant enzyme by intracellular calmodulin because extracts prepared from neuroblastoma cells activated the wild-type and mutant enzymes to the same maximal activity. We conclude that high-affinity binding between the catalytic subunit of the *B. pertussis* adenylyl cyclase and calmodulin may be required for the rapid entry of the adenylyl cyclase into neuroblastoma cells.

Bordetella pertussis, the pathogen responsible for whooping cough, produces several virulence factors implicated in the pathogenesis of this disease (Weiss & Hewlett, 1986; Wardlaw & Parton, 1988). One of these factors is an adenylyl cyclase that is released extracellularly into the culture media (Hewlett et al., 1976; Hewlett & Wolff, 1976; Shattuck et al., 1985). This enzyme exhibits two unusual properties; it is strongly stimulated by calmodulin (CaM),[1] and it rapidly invades animal cells (Confer & Eaton, 1982; Shattuck & Storm, 1985; Hanski & Farfel, 1985).

The structural gene for the *B. pertussis* adenylyl cyclase, *cya*A, encodes a 177-kDa polypeptide that has both adenylyl cyclase and hemolysin domains (Glaser et al., 1988a,b). Although the 177-kDa form of the enzyme purified from *B. pertussis* cells can invade animal cells (Rogel et al., 1989; Hewlett et al., 1989; Gentile et al., 1990), it has also been demonstrated that the 177-kDa polypeptide can be proteolyzed to a 45-kDa catalytic subunit and one or more additional subunits that are released into the culture media (Shattuck & Storm, 1985; Masure & Storm, 1989). By itself, the purified 45-kDa subunit cannot enter animal cells (Masure et al., 1988), but reconstitution with other factor(s) from the culture media (invasive factor) yields an invasive adenylyl cyclase (Donovan et al., 1989). Invasive factor has not been extensively characterized; however, it does contain several polypeptides which are derived from the 177-kDa *cya*A gene product.[2] The size of the partially purified 45-kDa catalytic subunit increases in the presence of Ca^{2+}, suggesting that Ca^{2+} promotes complex formation between the catalytic subunit and invasive factor (Masure et al., 1988). The addition of Ca^{2+} to the intact 177-kDa adenylyl cyclase also causes significant conformational changes (Hewlett et al., 1991), which may be due to interactions between these two domains within the intact polypeptide.

The mechanism by which the *B. pertussis* adenylyl cyclase enters animal cells has not been elucidated, but appears to involve a pathway independent of receptor-mediated endocytosis (Gentile et al., 1988; Gordon et al., 1988; Donovan & Storm, 1990). The unusual high affinity of this enzyme for CaM, both in the presence and in the absence of Ca^{2+} (Greenlee et al., 1982), and the high concentrations of CaM within animal cells suggest that high-affinity interactions between the catalytic subunit and CaM may be important for cell entry. In order to test this hypothesis, we produced a point mutant of the *B. pertussis* adenylyl cyclase catalytic subunit that has a greatly reduced affinity for CaM and have

† This work was supported, in part, by National Institutes of Health Grants GM 31708 (to D.R.S.) and AI 08138 (to M.K.G.) and by U.S. Public Health Service National Research Service Award T32 GM07270 from the National Institute of General Medical Sciences (to D.J.O.).

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[1] Abbreviations: CaM, calmodulin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Nal, nalidixic acid; Str, streptomycin; Kan, kanamycin; Gen, gentamycin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BCA, bicinchoninic acid; HPLC, high-pressure liquid chromatography; BG, Bordet–Gengou.

[2] M. G. Donovan, M. K. Gross, and D. R. Storm, unpublished observations.

0006-2960/92/0431-8884$03.00/0

Table I: Bacterial Strains and Plasmids

strain/plasmid	description	reference/source
B. pertussis		
Nalr11	Tahoma phase I; Nal^r, Str^r	Gross et al. (1992)
S7c2	derived from Nalr1; 1.6-kb Kar^r cassette inserted at *Bcl*I site in *cya*A	Gross et al. (1992)
BP8-8	derived from Nalr11; Trp-242 to Glu mutation in *cya*A	this study
E. coli		
SG21173	strain sued for expression of cloned *cya*A; triple protease minus (TPM) strain: htpR$^-$, lon$^-$, clp$^-$	Marizzi
TPM-ACW	SG21173 carrying plasmid pMG2; Amp^r	this study
TPM-ACE	SG21173 carrying plasmid pDO5; Amp^r	this study
TPM-ACL	SG21173 carrying plasmid pDO6; Amp^r	this study
SM10	donor strain for homologous recombination with *B. pertussis*; RP4::Mu, Kan^r	Simon et al. (1983)
SM10(pDO5V)	SM10 carrying vector pDO5V; Kan^r, Amp^r, Gen^r, Str^s	this study
plasmids		
pUC19	cloning and expression plasmid, Amp^r	
pMG2	pUC19 with 2.7-kb *Bam*HI/*Eco*RI *cya*A fragment, stop codon introduced at amino acid 436	this study
pDO5	pMG2 with condon for Trp-242 replaced with codon for Glu in *cya*A	this study
pDO6	pMG2 with codon for Trp-242 replaced with codon for Leu in *cyaA*	this study
pSS1129	vector for replacement of cloned sequences into *B. pertussis*; similar to pRTP1 except also has Gen^r cassette; Amp^r, Gen^r, Str^s	Stibitz et al. (1986)
pDO5V	pSS1129 vector with 2-kb upstream fragment and 2.7-kb *Bam*HI/*Eco*RI *cyaA* fragment with Trp-242 to Glu mutation	this study

characterized the cell entry properties of this mutant enzyme. Our data indicate that high-affinity CaM binding may be a requirement for the rapid entry of the catalytic subunit into animal cells.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. B. pertussis strain Nalr11 is an Nal^r and Str^r variant of the Tohama phase I strain (Table I). S7c2 is a mutant strain that was derived from the parental strain Nalr11 by homologous recombination. S7c2 has a 1.6-kb Kan^r cassette inserted into a *Bcl*I restriction enzyme site within the *cya*A gene (Gross et al., 1992). BP8-8 is a mutant strain of *B. pertussis* in which the wild-type *cya*A gene was mutagenized to replace Trp-242 with Glu. This mutant *cya*A gene was introduced into *B. pertussis* by homologous recombination.

Homologous recombination of the *B. pertussis cya*A gene was performed by the method of Stibitz et al. (1986) using *Escherichia coli* strain SM10 (Simon et al., 1983) as the donor strain and the gene replacement vector, pSS1129, generously provided by S. Stibitz. The acceptor strain, S7c2, and the donor strain, SM10(pDO5V), were used for production of the mutant strain BP8-8. SM10(pDO5V) carries the plasmid vector pDO5V, which is the pSS1129 vector containing a 2-kb *Bam*HI fragment directly upstream of *cya*A and a 2.7-kb *Bam*HI/*Eco*RI *cya*A fragment with a Trp-242 to Glu mutation. Initial selection for *B. pertussis* strains with the Trp-242 to Glu mutation was based on kanamycin sensitivity and hemolytic phenotype to verify loss of the Kan cassette within *cya*A. Several colonies with the appropriate phenotype (Kan^s, Hly$^+$) were then analyzed for the secretion of CaM-sensitive adenylyl cyclase, and the relative CaM affinity of the adenylyl cyclase was determined. The BP8-8 strain produced an adenylyl cyclase that had a significantly lower affinity for CaM compared to the wild-type enzyme.

B. pertussis strains were grown on Bordet–Gengou (BG) agar supplemented with 15% defibrinated sheep blood. Nal (50 μg/mL) and Str (300 μg/mL) were added to BG agar plates and to liquid media when cultures were grown for freezing and storage at –80 °C. Liquid cultures were grown in either Cohen–Wheeler media or Stainer–Scholte (SS) minimal media (Stainer & Scholte, 1971). One-liter flasks containing 600 mL of Stainer–Scholte media were inoculated either directly from cultures grown on BG plates or with 30 mL of a previously frozen culture. Following growth overnight at 37 °C and 200 rpm, 12-mL aliquots of this starter culture (A_{650} = 1.0) were used to inoculate 10 2.5-L flasks, each containing 800 mL of SS media. The bacteria were grown to an A_{650} of 0.5–0.6 and then harvested by centrifugation. The wild-type or Glu-242 mutant adenylyl cyclases were then partially purified from the conditioned media obtained from strain Nalr11 or BP8-8, respectively.

E. coli strain NM522 (Gough & Murray, 1983) was used for the general manipulation of plasmids carrying portions of the wild-type *cya*A and mutant *cya*A sequences. For expression of the recombinant adenylyl cyclases, a 2.7-kb *Bam*HI/*Eco*RI *cya*A fragment (wild type or mutant) was inserted into the polylinker site of the plasmid pUC19 (Table I) and oriented for expression under control of the *lac* promoter. Plasmid pMG2 contains the wild-type *cya*A fragment with a stop codon at amino acid 436. Plasmid pDO5 contains the *cya*A fragment with the Trp-242 to Glu mutation and the stop codon. Plasmid pDO6 contains the *cya*A fragment with the Trp-242 to Leu mutation and the stop codon. The triple protease minus *E. coli* strain, SG21173 (htpR$^-$, lon$^-$, clp$^-$), was used for the expression of the wild-type and mutant recombinant adenylyl cyclase proteins. Competent SG21173 cells were prepared by the $CaCl_2$ procedure and transformed with the plasmids pMG2, pDO5, and pDO6. SG21173 was generously provided by M. Maurizi. *E. coli* strain SM10 was used for biparental matings in experiments to return mutations in *cya*A to *B. pertussis* by homologous recombination. *E. coli* strains were grown on LB agar or in liquid LB media with appropriate addition of antibiotics. For maximum production of the recombinant wild-type and mutant adenylyl cyclases, the SG21173 strains were grown in EnYT media at 30 °C. The CJ236 dut$^-$ung$^-$ strain was used for oligonucleotide-directed mutagenesis of the *cya*A gene with M13 bacteriophage. General manipulations of plasmid DNA including plasmid isolation and restriction enzyme digests were by procedures described by Maniatis et al. (1990).

Oligonucleotide-Directed Mutagenesis of the cyaA Gene. Oligonucleotide-directed mutagenesis of *B. pertussis cya*A was performed on a 2.7-kb *Bam*HI/*Eco*RI *Cya*A fragment using the M13mp18 bacteriophage system according to the method of Kunkel et al. (1987). Synthesis oligonucleotides

were obtained from the Howard Hughes Chemical Synthesis Facility at the University of Washington. Replacement of the CAA codon for Gln-436 with a TAA stop codon was achieved using the oligonucleotide 5′-GACCCGGTAAGTCT-TGC-3′. Replacement of the TGG codon for Trp-242 with the GAG codon for Glu-242 was achieved using the oligonucleotide 5′-ATCGACTTGTTGGAGAAAATCGCTCG-3′. The oligonucleotide 5′-TCGACTTGTTGTTGAAAATC-GCTCG-3′ was used to replace the Trp-242 codon with the TTG codon for Leu-242. DNA sequencing was performed to confirm the presence of the desired mutations. The mutagenized *cya*A fragments were removed from the double-stranded M13mp18 vector by restriction digests and ligated into pUC19.

Purification of B. pertussis Adenylyl Cyclase. The adenylyl cyclase from the *B. pertussis* culture supernatant was purified by batch adsorption to QAE-Sephadex followed by elution with a linear salt gradient (Shattuck et al., 1985). Wild-type and Glu-242 mutant adenylyl cyclases produced by *B. pertussis* strains Nalr11 and BP8-8, respectively, were partially purified by this procedure. The elution profiles from QAE-Sephadex were similar for the wild-type and Glu-242 mutant adenylyl cyclases. The peak of enzyme activity eluted at a conductivity between 7 and 8 $m\Omega^{-1}$ for both the wild type and the Glu-242 mutant, and this material was concentrated using an Amicon PM-10 membrane. These partially purified adenylyl cyclase preparations were used for enzyme assays and cell invasion assays. Recombinant adenylyl cyclases expressed in *E. coli* were purified by a similar method following lysis of *E. coli* cells by sonication.

The catalytic subunit of *B. pertussis* adenylyl cyclase has been purified to homogeneity with recovery of catalytic activity using SDS–PAGE (Masure et al., 1988). The recombinant adenylyl cyclases expressed in *E. coli* were also purified to homogeneity by SDS–PAGE. Adenylyl cyclase preparations purified through QAE-Sephadex were run on a 12.5% polyacrylamide–SDS gel, and gel lanes were sliced into 0.25-cm slices. Protein was eluted from gel slices by macerating the gel, immersing it in elution buffer (20 mM Tris, pH 7.4, 40 mM NaCl, 2 mM, $MgCl_2$, and 1% CHAPS), and subjecting the suspension to a freeze–thaw cycle. Residual SDS was removed using ion-exchange resin, AG-1148 (purchased from Bio-Rad), and samples were subsequently assayed for CaM-stimulated adenylyl cyclase activity. This procedure also allowed the determination of the relative molecular mass of the catalytic subunit.

Synthetic Peptides. The synthetic peptides, peptide A and peptide B (amino acid sequences reported in Figure 1), were obtained from the Howard Hughes Chemical Synthesis Facility at the University of Washington. Peptides were purified by reverse-phase high-pressure liquid chromatography on a Varian Model 5000 HPLC using a SynChropak RP-P C18 column eluted with a 10–30% gradient of acetonitrile containing 0.1% trifluoroacetic acid (Bennett et al., 1981; Brown et al., 1983). The concentrations of the peptides were determined by measuring the absorption at 280 nm using the molar extinction coefficients of tryptophan (ϵ = 5600 M^{-1} cm^{-1}) and tyrosine (ϵ = 1300 M^{-1} cm^{-1}) for peptides A and B, respectively. The intrinsic fluorescence of the Trp in peptide A was used to estimate the K_d for interactions with CaM as described by Chapman et al. (1991). All fluorescence measurements were made using an SLM-4800S spectrofluorometer interfaced with a Hewlett Packard 9815A microprocessor.

Adenylyl Cyclase Enzyme Assay. Adenylyl cyclase activity was assayed by the general method of Salomon et al. (1974). Each assay contained 20 mM Tris-HCl, pH 7.4, 1 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 1 mM EDTA, and 0.1% bovine serum albumin. Samples were assayed in the presence of 2.4 μM CaM unless described otherwise. Bovine brain CaM was prepared as previously described (Masure et al., 1984). One unit of adenylyl cyclase catalyzes the synthesis of 1 nmol of cAMP per minute.

Increases in Intracellular [cAMP] Caused by Treatment of Neuroblastoma Cells with B. pertussis Adenylyl Cyclases. Mouse neuroblastoma N1E-115 cells were grown in DMEM supplemented with 5% FBS. Cells were grown on 60-mm tissue culture plates at 37 °C in a humidified 5% CO_2 environment. Monolayer cultures grown to 80–90% confluency were used for cell invasion assays. Duplicate plates of neuroblastoma cells were incubated with adenylyl cyclase preparations at 37 °C for various periods of time as indicated. The elevation of intracellular cAMP levels in these cells following treatment with the different preparations of adenylyl cyclase was determined as described previously (Shattuck & Storm, 1985). Briefly, the enzyme preparation was removed by decanting, and the cells were washed twice with 2 mL of DMEM. Cells were lysed with a 5% trichloroacetic acid solution and scraped, and the plate contents were centrifuged to remove cell debris and precipitated proteins. Intracellular cAMP levels in the cell supernatant were quantified by the method of Gilman (1970). The total protein per plate of cells was determined to be 1.5–2 mg using the BCA protein assay from Pierce.

Preparation of N1E-115 Mouse Neuroblastoma Cell Extracts. Extracts from N1E-115 neuroblastoma cells were prepared and examined for stimulation of adenylyl cyclase activity in order to determine whether there was sufficient endogenous CaM present in these cells to maximally activate both the wild-type and Glu-242 mutant adenylyl cyclases. N1E-115 mouse neuroblastoma cells were grown to 80–90% confluency on 60-mm plates in DMEM + 5% FBS. The medium was removed from four plates of cells, and the cells were washed. Cells were scraped off the plates and transferred to a small test tube using a minimal amount of buffer (20 mM Tris, pH 7.4 20 mM NaCl, 2 mM $MgCl_2$, and 1 mM EDTA). The total volume of cells and buffer was approximately 1.2 mL. The cells were disrupted with a probe sonicator at 30% of maximum for 3 × 30 s bursts. A 10-μL aliquot of the sonicated cells was added to the enzyme assay, and the cell protein was determined to be 7 mg/mL. The ratio of adenylyl cyclase to cell protein was determined to be 0.046 unit/μg of cell protein for the cell entry assay and 0.042 unit/μg of cell protein for the enzyme assay with cell extracts.

RESULTS

Identification of the CaM Binding Domain of B. pertussis Adenylyl Cyclase. The objectives of this study required the identification of the CaM binding domain(s) of the enzyme so that appropriate point mutants could be introduced into this sequence. Although no consensus sequence for CaM binding domains has been observed, CaM binding regions share some common features including a preponderance of basic and hydrophobic amino acid residues and one or more aromatic amino acids forming the general structure of an amphipathic α-helix (DeGrado et al., 1987; O'Neil & DeGrado, 1990). A potential CaM binding domain, designated domain A (Figure 1), which possesses several of these characteristics was identified in the sequence of the *B. per-*

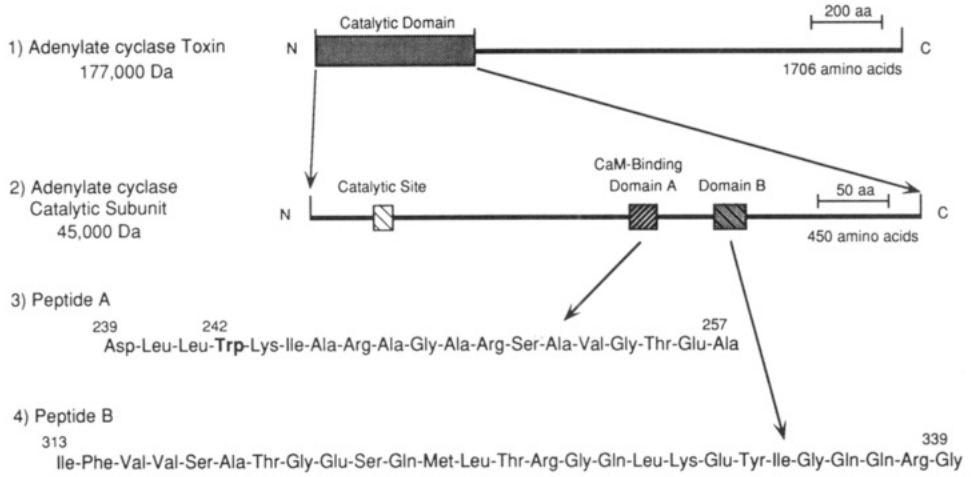

FIGURE 1: CaM binding domain of the CaM-stimulated adenylyl cyclase. Diagram of *B. pertussis* adenylyl cyclase toxin. (1) *B. pertussis* adenylyl cyclase toxin is synthesized as a 177 000-Da protein with a catalytic domain located within the N-terminal 450 amino acids. (2) Within the catalytic subunit, the catalytic domain includes a catalytic site centered at Lys-58 (Au et al., 1989). The relative locations of two potential CaM binding regions, designated domain A and domain B, are shown. (3) Peptide A is a 19 amino acid synthetic peptide that corresponds to the sequence for domain A. (4) Peptide B is a 27 amino acid synthesis peptide that corresponds to the sequence for domain B.

Table II: Amino Acid Sequences of CaM Binding Domains from Several CaM Binding Proteins

B. pertussis adenylate cyclase[a]	
peptide A	DLLWKIARAGARSAVGTEA
peptide B	IFVVSATGESQMLTRGQLKEYIGQQRG
SKMLCK[b]	KRRWKKNFIAVSAANRFKKISSSGAL
Ca^{2+}/CaM ATPase[c]	LRRGQILWFRGLNRIQTQIKVVNAFSSS
CaM protein kinase II[d]	NARRKLKGAILTTMLATRNFS
γ-subunit of phosphorylase kinase[e]	
Phk5	LRRLIDAYAFRIYGHWVKKGQQQNR
Phk13	GKFKVICLTVLASVRIYYQYRRVKP
Neuromodulin[f]	QASFRGHITRKKLKGEK
model peptide[g]	LKWKKLLKLLKKLLKKLLKLG

[a] This study (peptide A was shown be a CaM binding peptide, but peptide B was not). [b] Blumenthal et al. (1985) (SKMLCK, rabbit skeletal muscle myosin light chain kinase). [c] James et al. (1988). [d] Bennett & Kennedy (1987); Hanley et al. (1987). [e] Dasgupta et al. (1989). [f] Alexander et al. (1988). [g] O'Neil et al. (1987).

tussis adenylyl cyclase and had previously been proposed to be a CaM binding domain by Glaser et al. (1988a). Upon examination of the primary sequence of the adenylyl cyclase, we identified a sequence of amino acids, designated domain B (Figure 1), that also contains several basic and hydrophobic amino acids. A comparison of the sequence of these peptides with several known CaM binding domains is given in Table II.

Synthetic peptides corresponding to the primary sequence of these regions were analyzed for inhibition of CaM-stimulated adenylyl cyclase activity (Figure 2). Peptide B, at concentrations up to 0.1 mM, had no effect on CaM stimulation of adenylyl cyclase. However, peptide A completely inhibited CaM-stimulated enzyme activity with an apparent K_i of 10 μM. Neither peptide affected basal adenylyl cyclase activity. Direct binding between peptide A and CaM was confirmed using the intrinsic fluorescence of the peptide. The apparent K_d for the interaction between peptide A and CaM was 3 μM, whereas the K_d for the interaction between the intact catalytic subunit and CaM is approximately 1 nM. These differences in affinity suggest that there may be other domains of the enzyme required for high-affinity CaM binding or that this interaction is sensitive to the overall conformation of the intact catalytic subunit. Domain A was chosen for mutagenesis studies because these data indicated that it contributes to CaM binding.

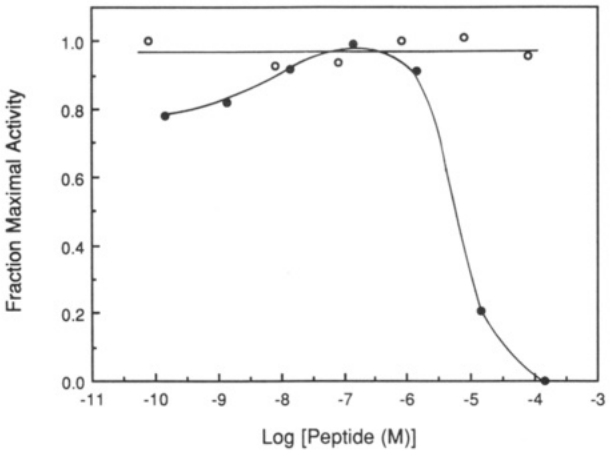

FIGURE 2: Peptide inhibition of CaM-stimulated adenylyl cyclase activity. Adenylyl cyclase activity was measured in the absence or presence of 1.0 nM CaM and 180 μM $CaCl_2$, with or without various concentrations of peptide A (●) or peptide B (○). Adenylyl cyclase enzyme activity was measured as described under Experimental Procedures. In samples containing CaM plus peptide, assays were initiated by the addition of the adenylyl cyclase to a reaction mixture containing CaM and peptide A or peptide B. Fraction (frac) maximal (max) activity was calculated using the formula frac max = $(V - V_0)/(V_{max} - V_0)$, where V_0 is the enzyme activity in the absence of CaM and peptide, V_{max} is the activity in the presence of 1.0 nM CaM and in the absence of peptide, and V is the enzyme activity in the presence of 1.0 nM CaM and a given peptide concentration. Values for V_{max} were 80 and 186 units/mL for the assay performed in the presence of peptides A and B, respectively. Values for V_0 were 8 and 7 units/mL for the assay performed with peptides A and B, respectively. The wild-type adenylyl cyclase, partially purified from *B. pertussis* culture supernatant by QAE-Sephadex anion-exchange chromatography, was used for the assays with peptides A and B.

Oligonucleotide-Directed Mutagenesis of the CaM Binding Domain of B. pertussis Adenylyl Cyclase. In order to provide additional evidence that domain A is important for CaM binding, oligonucleotide-directed mutagenesis of domain A of *cya*A was performed, with the goal of producing a mutant adenylyl cyclase with a lower affinity for CaM. Because aromatic amino acids have been implicated as critical residues in CaM binding domains (O'Neil et al., 1987), we targeted Trp-242 within domain A for site-directed mutagenesis.

We replaced the wild-type Trp-242 residue with either Leu or Glu. The wild-type and mutant *cya*A genes were expressed

in *E. coli* strain SG21173. The *cya*A constructs used for the expression of the wild-type or mutant adenylyl cyclases contained a stop codon at amino acid 436. Therefore, the recombinant wild-type and mutant adenylyl cyclase proteins were approximately 48 kDa, which is similar to the size of the catalytic subunit that we routinely isolate from *B. pertussis* conditioned media (Shattuck et al., 1985; Masure & Storm, 1988). The recombinant wild-type, Leu-242, and Glu-242 mutant adenylyl cyclases were purified to homogeneity as described under Experimental Procedures. The final step in the purification was elution of the proteins from SDS–PAGE with recovery of enzyme activity. The activity/migration profile of the Glu-242 mutant adenylyl cyclase purified by SDS–PAGE is shown in Figure 3A. The recombinant Glu-242 mutant protein had an apparent molecular weight of 47 000 ± 3000 on SDS gels. The wild-type and Leu-242 mutant adenylyl cyclases were purified by the same procedure, and the apparent molecular weights of these proteins were similar to that observed for the Glu-242 mutant.

Glaser et al. (1989) have reported that mutation of the Trp-242 residue to Asp-242 in *B. pertussis* adenylyl cyclase gave an enzyme with lowered affinity for CaM. However, these investigators used unpurified *E. coli* extracts containing adenylyl cyclase–β-galactosidase fusion proteins. The recombinant adenylyl cyclases used in this study were purified to homogeneity and assayed for CaM stimulation (Figure 3B). CaM-stimulated enzyme activity was observed for the recombinant wild-type and mutant adenylyl cyclases. The Leu-242 mutant had a slightly lower affinity for CaM ($K_{act.}$ for CaM = 8 nM) compared to the wild-type enzyme ($K_{act.}$ for CaM = 1 nM). The apparent affinity of the Glu-242 mutant for CaM ($K_{act.}$ for CaM = 250 nM) was 2 orders of magnitude lower than the wild-type enzyme.

Construction of a B. pertussis Strain Containing the Glu-242 Adenylyl Cyclase Mutant. The Glu-242 mutation described above was returned to *B. pertussis* by homologous recombination as described under Experimental Procedures. The *cya*A gene and the mutations introduced into the gene are depicted in Figure 4. Parental strain, Nalr11, produces wild-type adenylyl cyclase and hemolytic activity. Mutant S7c2 has a 1.6-k Kan^r cassette insertion within *cya*A that abolishes both adenylyl cyclase and hemolytic activity. S7c2 was used as the acceptor strain for the replacement of the 1.6-kb Kan^r cassette and flanking wild-type *cya*A sequences with the Glu-242 mutation.

Adenylyl cyclase from *B. pertussis* strains were partially purified from the culture media using QAE-Sephadex as described under Experimental Procedures. The partially purified wild-type enzyme prepared by this method invades animal cells (Shattuck & Storm, 1985; Masure et al., 1988). Therefore, this purification method was used to evaluate the ability of the mutant adenylyl cyclase to invade neuroblastoma cells.

The adenylyl cyclases that were partially purified from Nalr11 or BP8-8 were maximally stimulated by micromolar concentrations of CaM, but the relative CaM affinities of the enzymes from these strains were quite different (Figure 5). The $K_{act.}$ for CaM stimulation of the Glu-242 mutant was 240 nM, whereas the $K_{act.}$ of the wild type as 1 nM. The CaM activation curves for the adenylyl cyclase preparations from Nalr11 and BP8-8 were very similar to those observed for the recombinant wild-type and Glu-242 mutant enzymes purified from *E. coli*, respectively.

Activation of Adenylyl Cyclase Activity by Neuroblastoma Cell Extracts. The only assay available to detect the entry

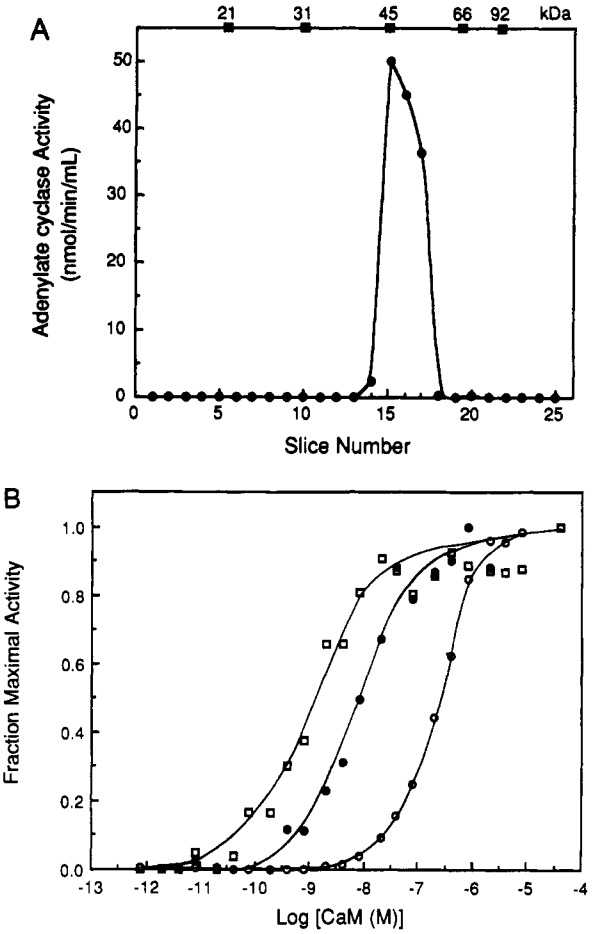

FIGURE 3: Calmodulin activation of recombinant wild-type and mutant adenylyl cyclases purified from *E. coli*. (A) Recombinant wild-type and mutant adenylyl cyclases were expressed in *E. coli*, and the proteins were purified by SDS–PAGE as described under Experimental Procedures. Recombinant Glu-242 mutant adenylyl cyclase was subjected to SDS–PAGE, and the gel slices were analyzed for adenylyl cyclase activity in the presence of CaM as described under Experimental Procedures. A single peak of enzyme activity was observed with the recombinant Glu-242 mutant, corresponding to an apparent molecular weight of 47 000 ± 3000. The SDS–PAGE elution profiles of the recombinant wild-type and Leu-242 mutant adenylyl cyclases were indistinguishable from that shown for the Glu-242 mutant. The migration of the molecular weights standards is shown at the top of the graph. (B) Recombinant wild-type and mutant adenylyl cyclases purified by SDS–PAGE as described under Experimental Procedures were assayed for CaM-stimulated enzyme activity. The purified wild-type (□), Leu-242 mutant (●), and Glu-242 (○) adenylyl cyclases were assayed for enzyme activity at CaM concentrations ranging from 0.8 pM to 40 μM in the presence of 80 μM $CaCl_2$. Fraction maximal activity was calculated using the formula frac max = $(V - V_0)/(V_{max} - V_0)$, where V_0 and V_{max} are the enzyme activity in the absence of CaM and at saturating levels of CaM, respectively, and V is the enzyme activity at a given CaM concentration. The enzyme activity values for the recombinant adenylyl cyclases were determined for the wild type (V_{max} = 12 units/mL, V_0 = 2 units/mL), Leu-242 mutant (V_{max} = 43 units/mL, V_0 = 12 units/mL), and Glu-242 (V_{max} = 80 units/mL, V_0 = 0.1 unit/mL).

of the *B. pertussis* adenylyl cyclase into animal cells is to measure increases in intracellular [cAMP] that occur when animal cells are exposed to the enzyme. This increase in intracellular [cAMP] requires that the bacterial adenylyl cyclase be fully stimulated by CaM present in the target cell. Therefore, it was crucial to determine whether endogenous CaM levels in neuroblastoma cells were sufficient to maximally activate the mutant enzyme.

The Glu-242 mutant adenylyl cyclase had decreased affinity for CaM compared to the wild-type enzyme, but it was fully

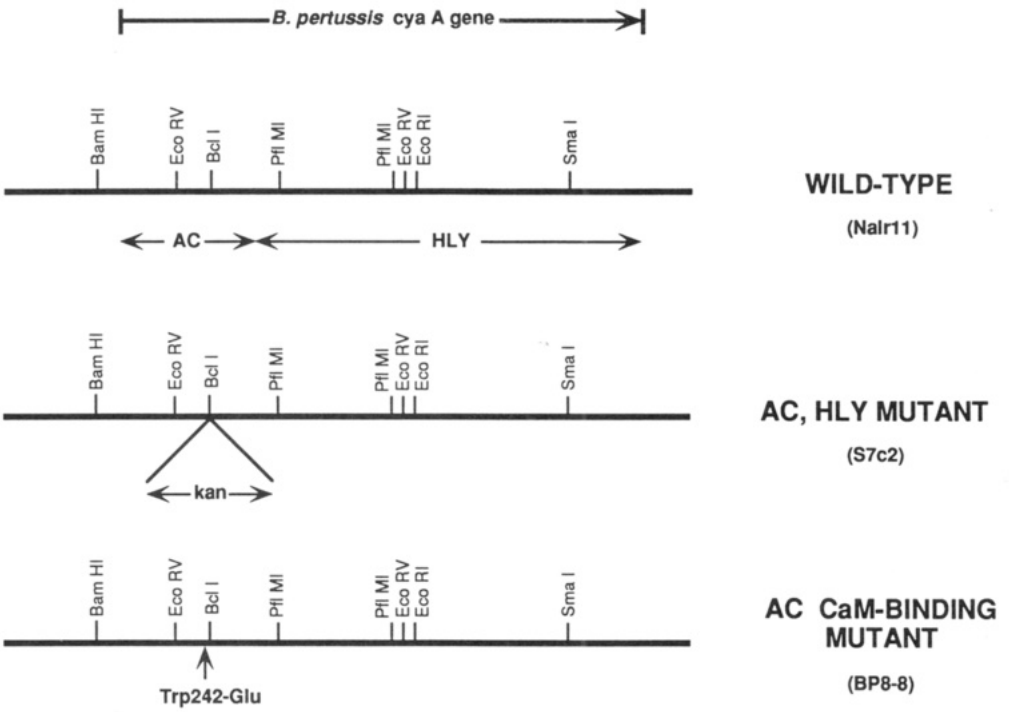

FIGURE 4: *B. pertussis* strains. Homologous recombination was used to replace the wild-type *cya*A with a single point mutation within the CaM binding region. Nalr11 is the wild-type parental strain. S7c2 contains a 1.6-kb Kan^r cassette that was introduced by homologous recombination. Biparental mating of acceptor strain S7c2 and donor strain SM10(pCO5V) was performed to produce the mutant strain BP8-8 (see Table I). BP8-8 contains a single point mutation in *cya*A that changes Trp-242 to Glu.

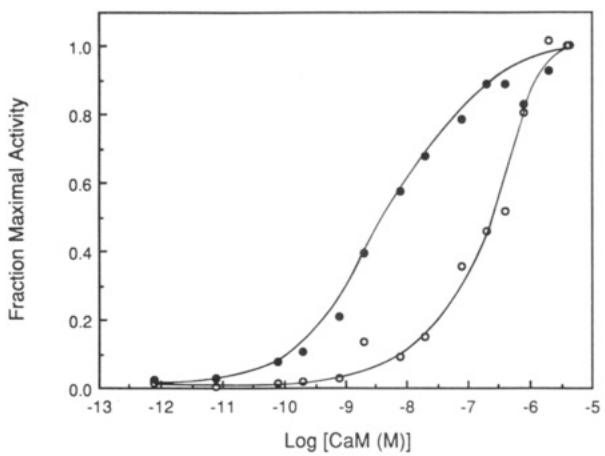

FIGURE 5: CaM sensitivity of the Glu-242 mutant adenylyl cyclase expressed in *B. pertussis*. The wild-type (●) and Glu-242 mutant (○) adenylyl cyclases were partially purified from the culture supernatant of *B. pertussis* strains Nalr11 and BP8-8 using QAE-Sephadex as described under Experimental Procedures. CaM activation of the wild-type and Glu-242 mutant adenylyl cyclases was determined at CaM concentrations ranging from 0.8 mM to 40 μM in the presence of 80 μM $CaCl_2$. Fraction maximal activity was calculated using the formula frac max = $(V - V_o)/(V_{max} - V_0)$, where V_0 and V_{max} are the enzyme activity in the absence of CaM and at saturating levels of CaM, respectively, and V is the enzyme activity at a given CaM concentration. The enzyme activity values for the adenylyl cyclases were determined for the wild type (V_{max} = 85 units/mL, V_0 = 2 units/mL) and Glu-242 (V_{max} = 13 units/mL, V_0 = 0.1 unit/mL).

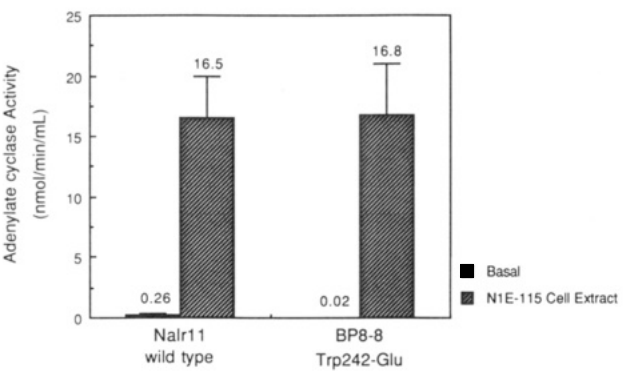

FIGURE 6: Effect of neuroblastoma cell extracts on the activity of wild-type and Glu-242 mutant adenylyl cyclases. Extracts from neuroblastoma cells were assayed for stimulation of adenylyl cyclase activity to ensure that there was sufficient endogenous CaM to fully activate both the wild-type and mutant enzymes. A cell extract from N1E-115 mouse neuroblastoma cells was prepared and assayed for stimulation of the wild-type and mutant adenylyl cyclases from *B. pertussis* strains Nalr11 and BP8-8, respectively, as described under Experimental Procedures. Basal adenylyl cyclase activity of the wild-type and Glu-242 mutant enzymes was measured in the absence of CaM and without any cell extract added. Enzyme stimulation by CaM in cell extracts was determined by adding an aliquot of the lysed cells to the wild-type and Glu-242 enzyme preparations.

stimulated by micromolar concentrations of CaM. The concentration of free CaM in neuroblastoma cells is 2 μM, which is sufficient to maximally activate the mutant enzyme. In order to confirm that there was enough free CaM to activate both the mutant and wild-type enzymes, their activities were assayed in the presence of a soluble cell extract from neuroblastoma cells, under conditions approximating those in cell invasion assays (Figure 6). In order to make this comparison valid, the ratio of total adenylyl cyclase activity/cellular protein was adjusted so that it was the same as that used during the cell invasion assays. Both the wild-type and Glu-242 mutant adenylyl cyclases were stimulated to equivalent levels by the neuroblastoma cell extract. These data indicate that the intracellular CaM concentration in N1E-115 cells is sufficient to maximally activate the Glu-242 mutant enzyme. Although it might be argued that the local concentration of CaM was not high enough to activate the mutant enzyme, the concentration of CaM at the inner surface of the cytoplasmic membranes of animal cells is generally comparable to or greater than that in the total cell extract. Since the amount of free CaM in the extract was at least 10-fold higher than

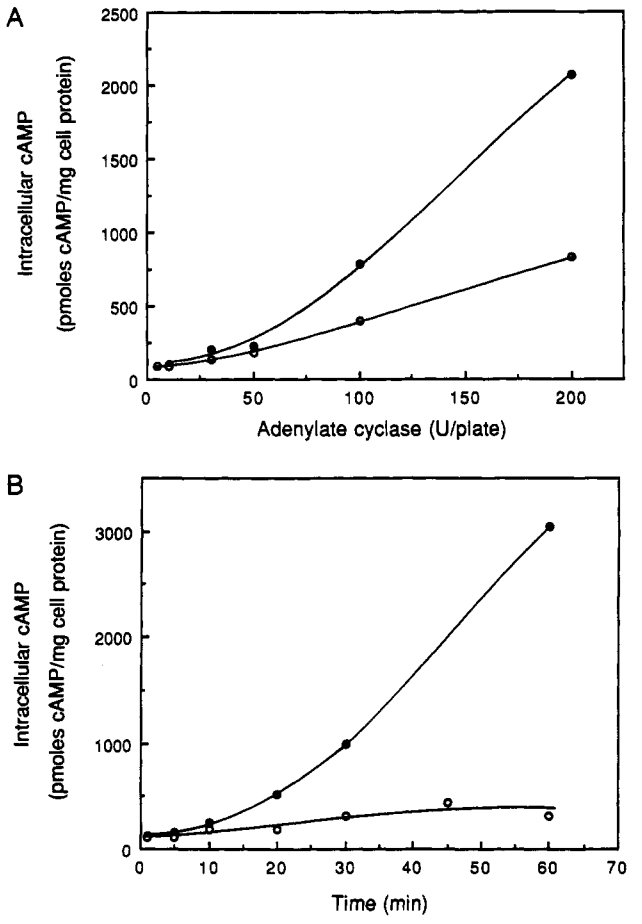

FIGURE 7: Effect of wild-type and Glu-242 mutant adenylyl cyclases on neuroblastoma cell intracellular cAMP levels. (A) Mouse neuroblastoma cells were incubated with various concentrations of wild-type (●) or Glu-242 mutant (○) adenylyl cyclases and assayed for intracellular cAMP as described under Experimental Procedures. The volume of wild-type and mutant adenylyl cyclases applied to each plate of neuroblastoma cells was adjusted in order to give equivalent amounts of enzyme activity. The activities of the enzyme preparations used in the cell entry assays were 320 units/mL for the wild-type and 330 units/mL for the Glu-242 mutant adenylyl cyclases, measured in the presence of 2.4 μM CaM (1 unit = 1 nmol of cAMP/min). The neuroblastoma cells were incubated with adenylyl cyclase preparations for 20 min at 37 °C. (B) The wild-type (●) and Glu-242 mutant (○) adenylyl cyclases were incubated with mouse neuroblastoma cells for various times and assayed for intracellular cAMP as described under Experimental Procedures. 100 units of either the wild-type or the Glu-242 mutant adenylyl cyclase was added to each plate.

the $K_{act.}$ for CaM stimulation of the Glu-242 mutant, and both enzymes were stimulated to the same level by the cell extract, we feel that these cells contained sufficient CaM to fully activate the mutant enzyme.

Invasion of Neuroblastoma Cells by the Wild-Type and Glu-242 Mutant Adenylyl Cyclases. The effect of the wild-type and Glu-242 mutant adenylyl cyclases on intracellular [cAMP] in N1E-115 neuroblastoma cells was determined as a function of the amount of adenylyl cyclase added to neuroblastoma cells (Figure 7A). Both enzyme preparations showed similar activities in the presence of CaM (320 units/mL for wild type and 330 units/mL for the Glu-242 mutant). The volume of sample applied to each plate of cells was adjusted so that equivalent amounts of wild-type or Glu-242 mutant enzyme activity were applied. A dose-dependent increase in intracellular [cAMP] was observed with cells treated with either the wild-type or the Glu-242 mutant adenylyl cyclase. However, the level of intracellular cAMP observed when cells were treated with the Glu-242 mutant was significantly lower than that observed with the wild-type enzyme. When 200 units of enzyme activity was added, the intracellular cAMP level obtained was approximately 2000 pmol of cAMP/mg of cell protein for the wild type and 800 pmol of cAMP/mg of cell protein for the Glu-242 mutant enzyme.

The difference in invasion of neuroblastoma cells between the wild-type and Glu-242 mutant enzymes was even more evident when the kinetics for these increases in intracellular [cAMP] were examined at a constant value of applied enzyme activity (Figure 7B). In this experiment, each plate of neuroblastoma cells was incubated with 100 units of either the wild-type or the Glu-242 mutant adenylyl cyclase. Both the wild-type and the Glu-242 mutant adenylyl cyclases showed a time-dependent increase in intracellular [cAMP]. However, the levels of cAMP observed for the wild-type enzyme were significantly higher than those observed for the mutant. At 60 min, the intracellular cAMP level observed for the wild-type enzyme was approximately 10-fold higher than the level observed for the Glu-242 mutant. These differences in the rate of production of intracellular [cAMP] were not due to intrinsic differences in the enzyme-catalyzed reaction rates because identical rates of cAMP production were seen for the two enzymes in vitro, in the presence of saturating CaM. These data indicate that the rate of entry of the adenylyl cyclase was lower for the mutant enzyme compared to the wild-type enzyme.

DISCUSSION

There is considerable evidence in the literature that *B. pertussis* adenylyl cyclase enters animal cells and catalyzes the synthesis of cAMP from intracellular ATP (Confer & Eaton, 1982; Shattuck & Storm, 1985; Hanski & Farfel, 1985; Gentile et al., 1990), but the mechanism for cell entry has not been elucidated. The major objective of this study was to determine whether high-affinity CaM binding is required for the rapid entry of *B. pertussis* adenylyl cyclase into neuroblastoma cells. We have shown that a mutant adenylyl cyclase with a lowered CaM affinity, Glu-242, entered animal cells at a significantly slower rate than the wild-type enzyme. The data reported in this study support the hypothesis that high-affinity CaM binding may be important for the rapid entry of the *B. pertussis* adenylyl cyclase into neuroblastoma cells. However, there are several other interpretations of the data that must be considered. For example, the difference in the rates of cell invasion between the wild-type and mutant enzymes may be due to a global conformational change caused by the mutation that indirectly affected other domains of the protein. Alternatively, the mutant enzyme may have decreased stability within neuroblastoma cells, or it may be more susceptible to proteolysis than the wild-type enzyme.

Whenever a mutation is introduced into an enzyme, there is the possibility that it may alter the overall conformation of the protein and indirectly affect more than one function of the protein. Without X-ray crystallographic data, it is impossible to rule out global conformational effects of mutants. We have attempted to minimize this possibility by the use of a point mutant introduced into a sequence of the protein that was strongly implicated for CaM binding. Other than the lowered affinity of the mutant enzyme for CaM, the mutant and wild-type enzymes had very similar properties. The activities of the two enzyme in the absence or presence of saturating CaM were very similar. Furthermore, both the wild-type and mutant enzymes were renatured after boiling in SDS, indicating that there were no major differences in the ability of the two proteins to refold. When the wild-type and

mutant enzyme preparations were assayed with sonicated neuroblastoma cell extracts, in the absence of protease inhibitors, similar enzyme activities were observed in the presence of saturating CaM. There was no indication that the mutant enzyme is less stable or more susceptible to proteolysis than the wild-type enzyme.

It might be argued that the Glu-242 mutation affected interactions between the catalytic subunit and invasive factor (Donovan et al., 1989). However, the apparent affinity of the mutant adenylyl cyclase for calmodulin was the same using the purified catalytic subunit or the enzyme in extracts of *B. pertussis* that contained invasive factor. If CaM and invasive factor interacted at the same site, then we might expect to see a difference in CaM binding in the presence of invasive factor. Furthermore, we have determined the site of invasive factor interaction is quite distinct from the CaM binding domain (Oldenberg and Storm, unpublished observations).

The translocation of a large polypeptide across a membrane requires energy and a mechanism for coupling this energy to the translocation of the protein. *B. pertussis* adenylyl cyclase does not enter animal cells by receptor-mediated endocytosis (Gentile et al., 1988; Gordon et al., 1988; Donovan & Storm, 1990), and the mechanism and energy source for membrane translocation are not known. Presumably, the catalytic subunit must undergo extensive reversible conformational changes in order to cross the cytoplasmic or intracellular membranes. *B. pertussis* adenylyl cyclase enters animal cells very rapidly, and its affinity for CaM is unusually high. We hypothesize that energy derived from the binding of CaM may be used for membrane translocation. The interaction of the catalytic subunit with intracellular CaM, while it is entering or subsequent to entry, may be a major source of energy for accumulation of the enzyme within animal cells. For example, if the enzyme unfolds and crosses the cytoplasmic membrane through a channel, then the interaction of the CaM binding domain with intracellular CaM may promote membrane translocation. The rate of cell entry for the wild-type and mutant enzymes may differ, even though both are saturated by intracellular CaM, because the free energy of CaM binding is greater with the wild-type enzyme. Regardless of the detailed mechanism for cell entry, data reported in this study suggest that the high affinity of this enzyme for CaM may be required for rapid entry into animal cells.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. Scott Stibitz for the use of vector pSS1129 and Dr. M. Maurizi of the *E. coli* strain SG21173. We also acknowledge Tom Serwold and Anna Widehov for their technical assistance. We thank Ed Chapman and Dr. Roy Duhe for critical reading of the manuscript.

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