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Identification of residues essential for catalysis and binding of calmodulin in *Bordetella pertussis* adenylate cyclase by site-directed mutagenesis

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In order to identify molecular features of the calmodulin (CaM) activated adenylate cyclase of *Bordetella pertussis*, a truncated *cya* gene was fused after the 459th codon in frame with the α-*lacZ'* gene fragment and expressed in *Escherichia coli*. The recombinant, 604 residue long protein was purified to homogeneity by ion-exchange and affinity chromatography. The kinetic parameters of the recombinant protein are very similar to that of adenylate cyclase purified from *B.pertussis* culture supernatants, i.e. a specific activity >2000 μmol/min mg of protein at 30°C and pH 8, a K_m^{ATP} of 0.6 mM and a K_d for its activator, CaM, of 0.2 nM. Proteolysis with trypsin in the presence of CaM converted the recombinant protein to a 43 kd protein with no loss of activity; the latter corresponds to the secreted form of *B.pertussis* adenylate cyclase. Site-directed mutagenesis of residue Trp-242 in the recombinant protein yielded mutants expressing full catalytic activity but having altered affinity for CaM. Thus, substitution of an aspartic acid residue for Trp-242 reduced the affinity of adenylate cyclase for CaM >1000-fold. Substitution of a Gln residue for Lys-58 or Lys-65 yielded mutants with a drastically reduced catalytic activity (~0.1% of that of wild-type protein) but with little alteration of CaM-binding. These results substantiated, at the molecular level, our previous genetic and biochemical studies according to which the N-terminal tryptic fragment of secreted *B.pertussis* adenylate cyclase (residues 1-235/237) harbours the catalytic site, whereas the C-terminal tryptic fragment (residues 235/237-399) corresponds to the main CaM-binding domain of the enzyme.

Key words: ATP-binding site/*Bacillus anthracis*/calmodulin-binding site/cyclolysin

Introduction

Bordetella pertussis adenylate cyclase is an intensely studied enzyme because of its potential role in the pathogenesis of whooping cough (Weiss and Hewlett, 1986). Two properties of adenylate cyclase from this organism make it an attractive model for structure-function relationship studies: (i) its activation by calmodulin (CaM) which is not known to occur in bacteria (Wolff *et al.*, 1980; Greenlee *et al.*, 1982); and (ii) its ability to enter eukaryotic cells causing unregulated synthesis of adenosine 3'-5'-monophosphate (cAMP) and impairment of normal cellular function (Confer and Eaton, 1982; Hanski and Farfel, 1985). Delineation of the mechanism of activation and catalysis in *B.pertussis* adenylate cyclase would provide the first instance where the active site of this heterogeneous group of enzymes could be characterized.

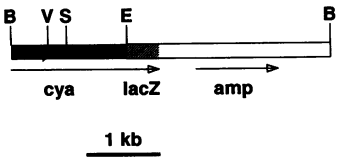
Using the interaction between adenylate cyclase and CaM as a tool we have recently cloned the corresponding gene from *B.pertussis*, determined its nucleotide sequence and expressed it in *Escherichia coli* (Glaser *et al.*, 1988a). We have found that the protein is synthesized as a large bifunctional precursor form of 1706 amino acid residues, endowed with adenylate cyclase and haemolytic activity. The protein has been named cyclolysin to indicate this fact. Its secretion mechanism involves the haemolytic carboxy-terminal end (Glaser *et al.*, 1988b) in conjunction with the product of three genes located downstream from the adenylate cyclase gene. In *B.pertussis*, the large precursor form is processed to low molecular mass forms of 43, 45 and 50 kd present in *B.pertussis* culture supernatant (Shattuck *et al.*, 1985; Ladant *et al.*, 1986). The secreted adenylate cyclase(s) corresponds to the amino-terminal domain of the large precursor form. By combining genetic and biochemical information (Glaser *et al.*, 1988a; Ladant, 1988; Ladant *et al.*, 1989), we showed that the N-terminal tryptic fragment of the 43 kd form of adenylate cyclase (T25, residues 1-235/237) harbours the active site, whereas the C-terminal tryptic fragment (T18, residues 235/237-399) corresponds to the main CaM-binding domain of the enzyme. Furthermore, a synthetic peptide corresponding to residues 235-254 of adenylate cyclase was shown to bind to CaM in a Ca^{2+}-dependent manner (Ladant *et al.*, 1989).

In the present study, we examined the catalytic and CaM-binding properties of *B.pertussis* adenylate cyclase expressed in *E.coli* by a plasmid carrying a truncated *cya* gene which comprises the entire cyclase domain of cyclolysin fused to the α domain of β-galactosidase. Site-directed mutagenesis of Lys-58, Lys-65 and Trp-242 revealed the role of these residues in catalysis or in binding of CaM.

Results

Expression and purification of a truncated B.pertussis cya gene product in E.coli

Several clones deleted at the 3' end of the *cyaA* gene were obtained as side products of the sequence analysis of the *B.pertussis* adenylate cyclase gene using the cyclone strategy. Clones encoding enzyme with a M_r close to that of adenylate cyclase purified from *B.pertussis* culture supernatants were transferred into the expression vector pTZ19 (Pharmacia, Uppsala, Sweden). One such clone, plasmid pDIA5202, gave the highest level of adenylate cyclase activity. As indicated in Figure 1, it fused sequences corresponding to the whole adenylate cyclase domain of

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MQQSHQAGYANAADRESGIPAAVLDGIKAVAKEKNATLMFRLVNPHSTSLIAEGVATKGL   60
GVHAKSSDWGLQAGYIPVNPNLSKLFGRAPEVIARADNDVNSSLAHGHTAVDLTLSKERL  120
DYLRQAGLVTGMADGVVASNHAGYEQFEFRVKETSDGRYAVQYRRKGGDDFEAVKVIGNA  180
AGIPLTADIDMFAIMPHLSNFRDSARSSVTSGDSVTDYLARTRRAASEATGGLDRERIDL  240
LAKIARAGARSAVGTEARRQFRYDGDMNIGVITDFELEVRNALNRRAHAVGAQDVVQHGT  300
EQNNPFPEADEKIFVVSATGESQMLTRGQLKEYIGQQRGEGYVFYENRAYGVAGKSLFDD  360
GLGAAPGVPSGRSKFSPDVLETVPASPGLRRPSLGAVERQDSGYDSLDGVGSRSFSLGEV  420
SDMAAVEAAELEMTRQVLHAGARQDDAEPGVSGASAHWGnslavvlqrrdtenpgvtqln  480
rlaahppfastrnseeartdrpsqqlrslngetrfatfpapeavpestlecdlpeadtvv  540
vpsntqmhgydapiytnvtypitvnppfvptenptgcysltfnvdestlqegptriifdg  600
vpig*
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Fig. 1. Restriction map of plasmid pDIA5202 and deduced amino acid sequence of the recombinant adenylate cyclase. The 5′-terminal end of the cyclolysin gene has been fused in phase with a truncated *lacZ* gene containing a sequence specifying the α domain of β-galactosidase. This resulted in a chimeric protein containing the adenylate cyclase domain of cyclolysin (residues 1−399), the start of the haemolysin domain (residues 400−459) and the α domain of β-galactosidase (residues 460−604, in lower case letters). The Arg pair (399/400) is particularly sensitive to trypsin action resulting in formation of a 43 kd protein, identical to the small adenylate cyclase form present in *B.pertussis* supernatants. The black box corresponds to the inserted *B.pertussis* chromosomal DNA. The truncated *lacZ* gene is shaded. Restriction sites are represented as follows: B, *Bam*HI; E, *Eco*RI; V, *Eco*RV; S, *Sal*I. Amino acid residues are represented by the standard one-letter code. Residues submitted to site-directed mutagenesis (K58, K65 and W242) are boxed.

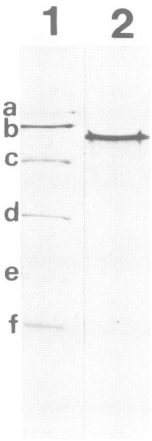

Fig. 2. SDS−PAGE (12.5%) of purified recombinant adenylate cyclase expressed in *E.coli*. **Lane 1**: M_r markers: a, phosphorylase a, (94 000); b, bovine serum albumin (67 000); c, ovalbumin (43 000); d, carbonic anhydrase (30 000); e, soybean trypsin inhibitor (20 300); f, lysozyme (14 400). **Lane 2**: purified adenylate cyclase (2 μg of proteins). Gels were stained with Coomassie Blue.

cyclolysin and the α domain of β-galactosidase. Since the transcription and translation signals were identical in different plasmids, this higher activity was probably due to a better stability of the protein. This clone was therefore kept for further studies.

The maximum level of adenylate cyclase activity was reached when cells entered the early stationary phase, after which a decrease of activity was noted, probably due to the degradation of the protein. During the exponential phase of growth, the ratio of β-galactosidase to adenylate cyclase activity was constant, as expected for α-complementation (interaction between peptide α carried by the recombinant adenylate cyclase and the partially deleted β-galactosidase coded by the *lacZ*M15 gene). The two enzyme activities

Table I. Purification of recombinant *B.pertussis* adenylate cyclase expressed in *E.coli*

Step	Protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min mg of protein)	Yield (%)
Whole cell extract	889	2410	2.7	100
8000 *g* sediment in 8 M urea	138	1968	14.2	82
DEAE−Sephacel chromatography	17.8	1032	60.0	43
CaM-agarose chromatography	0.35	738	2104	31

Bacteria from 2 l of culture were suspended in 50 ml of 20 mM K-phosphate (pH 7.4) then disrupted by ultrasound. The extract was centrifuged at 8000 *g* for 30 min and the sediment resuspended in 20 ml of 8 M urea in Tris−Triton buffer [50 mM Tris−HCl, pH 8 plus 0.1% (w/w) Triton X-100]. After removal of insoluble material by centrifugation at 13 500 *g* for 30 min, the 'urea extract' was loaded onto a DEAE−Sephacel column (20 mg of protein/ml of swollen gel) at a flow rate of 30 ml/h. The column was washed with 150 ml of Tris−Triton buffer for 1.5 h, then adenylate cyclase was eluted with 0.5 M NaCl in the same buffer. Fractions containing adenylate cyclase were supplemented with 5 mM $CaCl_2$ then purified on CaM−agarose as described by Monneron *et al.* (1988).

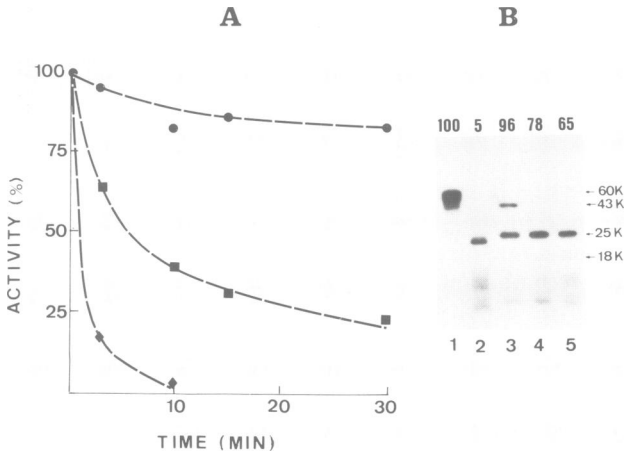

Fig. 3. Trypsin digestion of recombinant adenylate cyclase in the presence and absence of CaM. (**A**) Seven units of purified recombinant adenylate cyclase in 100 μl of Tris−Triton buffer (50 mM Tris−HCl, pH 8 plus 0.1% Triton X-100) supplemented with 1 mM $CaCl_2$ and 50 ng TPCK-trypsin were incubated at 4°C in the presence of 2 μM CaM (●, ■) or 0.5 mg/ml human serum albumin (◆). At different time intervals 5 μl aliquots were withdrawn and diluted in 200 μl of Tris−Triton buffer containing 2 μg of soybean trypsin inhibitor (●, ◆) or in 35 μl of 8 M urea in the same buffer (■). After 10 min at 4°C, samples in urea were diluted to 200 μl with Tris−Triton buffer, then residual adenylate cyclase activity (expressed as percentage of initial activity) was determined. Controls run in the absence of trypsin and diluted with Tris−Triton buffer or 8 M urea Tris−Triton showed no decrease in enzyme activity. (**B**) 0.1 units of pure [^{125}I]adenylate cyclase (7×10^5 c.p.m.) in 50 μl of Tris−Triton buffer containing 1 mM $CaCl_2$ were submitted to TPCK-trypsin (25 ng) proteolysis for 15 s (**lane 3**), 3 min (**lane 4**), 5 min (**lane 2**) or 7 min (**lane 5**), in the absence (**lane 2**) or in the presence of 0.15 μM CaM (**lanes 3, 4** and 5). Proteolysis was stopped with an excess of soybean trypsin inhibitor (2 μg/sample). Residual adenylate cyclase activity (expressed as percentage of initial activity on the top of the gel lanes) was determined for each sample. The remaining samples were run onto 12.5% (w/v) SDS−PAGE and autoradiographed. The arrows to the right indicate the positions of the undigested protein (**lane 1**, 100% activity), the 43 kd form of adenylate cyclase, and the two complementary peptides resulted from trypsin digestion of *B.pertussis* adenylate cyclase (25 and 18 kd, respectively).

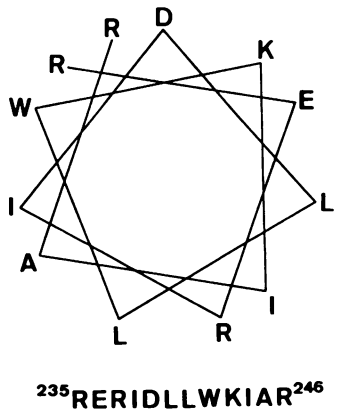

Fig. 4. A helix wheel representation of the putative CaM-binding sequence situated between residues 235 and 246 in *B.pertussis* adenylate cyclase.

Table II. Adenylate cyclase activity of crude extracts

Strain	Activity (nmol/min mg of protein)	
	10 nM CaM	2 μM CaM
WT (5202)	2610	2540
W242D	87	1330
W242G	480	2220
W242R	1465	2430
W242V	2530	2820
K65Q	1.51	1.40
K58Q	0.55	0.59

Bacteria was disrupted by ultrasound and the extract was diluted with 8 M urea in Tris–Triton buffer to 'unmask' cryptic adenylate cyclase activity. K65Q and K58Q mutant extracts were analysed for enzyme activity using incubation times of 1 h instead of 5 or 10 min.

were recovered in the pellet fraction after cell breakage and centrifugation.

Formation of adenylate cyclase–β-galactosidase aggregates was found to be helpful in enzyme purification. Insoluble proteins once treated with urea in buffer solution contained >80% of the original adenylate cyclase activity. Chromatography on DEAE–Sephacel allowed removal of the chaotropic agent and recovery of a fully active enzyme by elution with salt. Finally, affinity chromatography on CaM-agarose yielded pure adenylate cyclase (Table I and Figure 2). The mol. wt of the protein calculated from a calibration curve is at 60 kd, somewhat lower than that expected from the deduced sequence of the protein (M_r = 64 995). Several faint bands, visible between 60 and 43 kd correspond most probably to products of cleavage of the recombinant protein by endogenous proteases. Antibodies directed against adenylate cyclase of *B.pertussis* (Monneron *et al.*, 1988) recognized these polypeptides in Western blots (data not shown).

Characterization of the B.pertussis adenylate cyclase expressed in E.coli

E.coli carrying the truncated gene of *B.pertussis* adenylate cyclase expressed not only an insoluble, but also a cryptic, form of the enzyme after growth at 37°C, most probably due to the fact that adenosine 5′-triphosphate (ATP) and/or

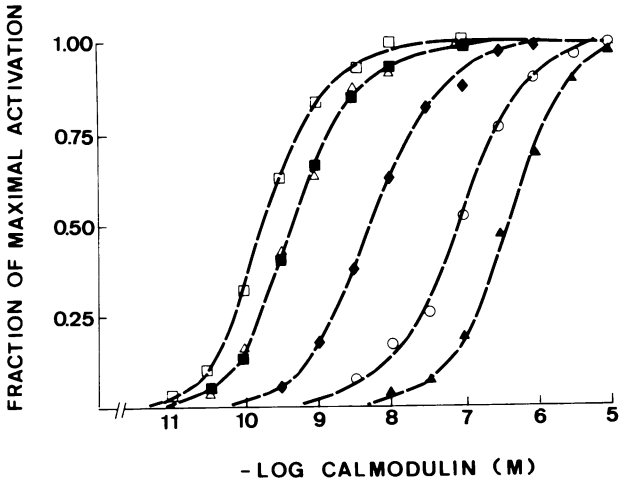

Fig. 5. Activation by calmodulin of wild-type (□) and different mutant forms (W242V, ■; W242R, ◆; W242D, ▲; W242G, ○; and K65Q, △) of recombinant adenylate cyclase expressed in *E.coli*. Bovine brain calmodulin (100 μM stock solution) was diluted to the desired concentrations with Tris–Triton buffer containing 1 mg/ml human serum albumin and then added in 5 μl-aliquots to the reaction medium described in Materials and methods. $CaCl_2$ was present at 0.1 mM. The reaction was initiated by the addition of adenylate cyclase followed by 10–60 min incubation at 30°C. The fraction of maximal activation was calculated as $(V-V_o)/(V_{max}-V_o)$ where V_o and V_{max} are the reaction rates in the absence of CaM or in the presence of saturating concentrations of CaM, respectively; V is the reaction rate at a given concentration of CaM. The wild-type and K65Q mutant enzymes were used as purified preparations; the Trp mutants were derived from crude bacterial extracts.

CaM were less accessible to the cyclase moiety of the complemented protein aggregates. Dilution of crude extracts with 8 M urea instead of buffer prior to activity assays unmasked this cryptic activity. Trypsin rapidly inactivated the recombinant adenylate cyclase in the absence of CaM as was the case with enzyme secreted from *B.pertussis* (Figure 3A). Proteolysis performed in the presence of CaM converted the 60 kd protein to a 43 kd form with no loss of activity. Further proteolysis of the 43 kd form yielded the complementary fragments T25 and T18 (Ladant, 1988), still maintained in an active native-like structure by CaM (Figure 3B). It is therefore clear that the α peptide of β-galactosidase, as well as residues between 400 and 458 of recombinant adenylate cyclase, could easily be removed from the protein by tryptic digestion with no significant loss of activity. The kinetic parameters of the recombinant protein are very similar to those of adenylate cyclase purified from *B. pertussis* culture supernatants, i.e. a specific activity >2000 μmol/min mg of protein at 30°C and pH 8, a K_m for ATP of ~0.6 mM and a K_d for CaM of 0.2 nM in the presence of Ca^{2+} ions. Ethylene *bis*(oxyethylenenitrilo) tetraacetic acid (EGTA) decreased the affinity of recombinant adenylate cyclase for CaM by about two orders of magnitude, as was the case with enzyme secreted from *B.pertussis* (Ladant, 1988), while the maximum catalytic activity was increased by a factor of 1.7 (data not shown).

Site-directed mutagenesis of Trp-242 in recombinant adenylate cyclase

Attempts to identify the CaM-binding site of *B.pertussis* adenylate cyclase by sequence comparison with well-characterized CaM-activated enzymes was rather inconclusive.

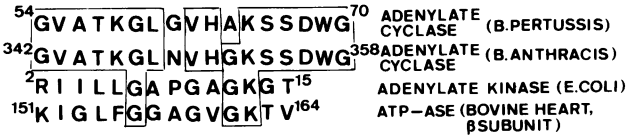

Fig. 6. Alignment of the putative polyphosphate-binding sites of *B.pertussis* adenylate cyclase with sequences belonging to well-characterized ATP-binding enzymes.

However, the sequence situated around Trp-242 in the bacterial enzyme (Figure 4) had some features characteristic of CaM-binding peptides such as presence of basic and hydrophobic residues forming amphiphilic α-helix structures (Blumenthal *et al.*, 1985; Buschmeier *et al.*, 1987; Kennelly *et al.*, 1987; O'Neil *et al.*, 1987; Glaser *et al.*, 1988a; Hanley *et al.*, 1988; James *et al.*, 1988). Since Trp is frequently present in CaM-binding sequences, Trp-242 was chosen as the first target for analysis of *B.pertussis* adenylate cyclase by site-directed mutagenesis.

As shown in Table II replacement of Trp-242 in wild-type adenylate cyclase with Val, Arg, Gly or Asp (W242V, W242R, W242G and W242D mutants) had little effect on enzyme activity in crude extracts, as long as CaM concentration was well above saturation. However, at 10 nM CaM (concentration at which the activator is almost saturating for the wild-type enzyme) the four mutants displayed considerable differences in activity: mutant W242V was the least affected (90% of maximal activity at 10 nM CaM), whereas mutant W242D was the most affected (only 6.6% of maximal activity at 10 nM CaM). Analysis of CaM activation curves for the four Trp mutants showed that the half-maximal concentration required for activation was of 0.5 (W242V), 6.3 (W242R), 95 (W242G) and 350 nM (W242D), respectively, as compared to a value of 0.20 nM for the wild-type protein (Figure 5). As expected, the K_m for ATP of all mutants showed no significant difference when compared with that of the wild-type enzyme.

Two modified forms of adenylate cyclase (W242V and W242D) were purified to homogeneity to determine whether mutation had affected the maximum catalytic activity. The specific activity of the adenylate cyclase W242V was very close to that of the wild-type protein (2200 μmol/min mg of protein), whereas mutant W242D had a V_m 30% lower than the wild-type enzyme. It is possible that under experimental conditions the latter mutant would not be saturated by the activator. Determination of CaM activation curves of purified enzymes gave the same K_d values as those obtained with enzyme from crude extracts.

Site-directed mutagenesis of Lys-58 and Lys-65 in recombinant adenylate cyclase

Previous experiments showed that isolated T25 fragment (residues 1–235/237) of *B.pertussis* adenylate cyclase exhibited a low but measurable enzyme activity, which indicated that it harboured the catalytic site (Ladant *et al.*, 1989). In addition, it was observed, by comparison with another CaM-activated enzyme, *Bacillus anthracis* adenylate cyclase (Escuyer *et al.*, 1988), that a 17 amino acid polypeptide was similar in both proteins and contained the sequence G----GKS (AKS in *B.pertussis*) (Figure 6) which is known to be part of many ATP-binding proteins (Walker *et al.*, 1982; Miller and Amons, 1985; Fry *et al.*, 1986). This suggested that the nucleotide-binding site might be located near Lys-65. In order to substantiate this hypothesis we substituted Gln for Lys-65. Another basic residue, Lys-58, situated in the vicinity of the former Lys residue was also submitted to the same substitution. Both mutants (K65Q and K58Q) showed drastically reduced catalytic activity in crude extracts as compared to wild-type protein at saturating or near-saturating concentrations of CaM (Table II). As a first approximation we can assume that both mutants were much less, or not at all, affected in their CaM-binding properties. Pure K65Q mutant adenylate cyclase exhibited a specific activity of 2.2 μmol/min mg of protein when assayed with 2 mM ATP and 100 nM CaM. Determination of enzyme activity as a function of ATP and CaM concentration indicated a K_m for ATP of 1.5 mM and half-maximum concentration required for activation by CaM of 0.5 nM. The latter value is most probably an overestimate since enzyme concentration in the assay mixture was of the same order of magnitude to ensure accurate determination of its activity. As in the case of wild-type or W242 mutants, the adenylate cyclase K65Q was converted by proteolysis with trypsin into complementary T25 and T18 peptides in the presence of CaM (data not shown).

Discussion

Although adenylate cyclases from various organisms have been the subject of intense study for the past 15 years not much is known about the detailed molecular structure of their catalytic or regulatory centres. The genes coding for adenylate cyclase from two very different bacteria, *B. pertussis* (a Gram-negative organism) and *B.anthracis* (a Gram-positive bacillus), were cloned and sequenced (Escuyer *et al.*, 1988; Glaser *et al.*, 1988a; Mock *et al.*, 1988). We observed that these enzymes although having similar properties, displayed only limited sequence similarity, except in three regions. The first region (situated between residues 54–70 in *B.pertussis* enzyme and between residues 342–358 in *B.anthracis* adenylate cyclase) contained a sequence that resembled the generally accepted binding site for ATP (Walker *et al.*, 1982; Miller and Amons, 1985; Fry *et al.*, 1986) (Figure 6). We have demonstrated here in the case of *B.pertussis* adenylate cyclase that, indeed, this region must be closely involved in the enzyme active centre. From amino acid sequence comparison we can therefore confidently infer that the cognate site is also involved in the catalytic site of *B.anthracis*. The data presented are also in line with biochemical experiments using the pure enzyme fragments and suggesting that the catalytic centre was located in the first half of the protein (residues 1–235/237) secreted in *B.pertussis* culture supernatants (Ladant *et al.*, 1989).

The case of the CaM regulator site is more complex. When analysing the amino acid sequence of the protein, as predicted from the nucleotide sequence, we were prompted to suggest a tryptophan-containing peptide (Trp-242) might be involved in CaM-binding as it is in some known cases (Glaser *et al.*, 1988a). However, comparison with the *B.anthracis* enzyme did not display any significant primary structure similarity in the corresponding region. It was observed, however, that the region was bracketed by two regions of strong similarity (Escuyer *et al.*, 1988). This suggested either that we are in the presence of regions flanking a polypeptide fold where CaM is accommodated, and/or that one of these regions

contacts CaM while the other one would be involved in the information transfer between the CaM-binding site and the catalytic centre. In any case the fact that alteration of Trp-242 results in a much lower CaM-binding demonstrates that this region must be somehow involved in the association between the two proteins. The fact that there is no conservation of the corresponding primary structure indicates that it is a secondary rather than primary structure which is important for binding. It should be emphasized here that our results are the first experimental data where the putative α-helix involved in CaM-binding has been modified, and thus shown to influence binding.

In keeping with this hypothesis are the results of the specific mutation effects we observed. Aspartate, when replacing Trp diminishes drastically CaM interaction; it is known that this amino acid residue, perhaps through its specific interaction with water molecules, is often a helix breaker, whereas aromatic amino acid residues (namely Trp and Phe), are helix formers. A branched-chain amino acid such as Val should be, in this context, more like Trp and Gly more like Asp, as they indeed are. Thus we propose, in line with Cox *et al.* (1985) that an α-helix is part of the CaM-binding site of bacterial pathogen adenylate cyclase, as is the case for other CaM-binding proteins. This could correspond to a dipolar moment orienting CaM in a correct position with respect to the enzyme; the conserved sites would in such a picture be more likely to correspond to zones of contact between the enzyme and CaM.

Materials and methods

Chemicals

Adenine nucleotides, restriction enzymes and T_4 DNA ligase were from Boehringer Mannheim. L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) trypsin, soybean trypsin inhibitor, bovine brain CaM and CaM-agarose were from Sigma. Oligonucleotides were synthesized according to the phosphamidinate method using a commercial DNA synthesizer (Applied Biosystems). [α-^{32}P]ATP (3000 Ci/mmol), [^{3}H]cAMP (40 Ci/mmol) and Na^{125}I (1000 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, UK).

Bacterial strain and growth conditions

E.coli strain BMH7118 was used in this work (Gronenbrun, 1976). This strain expresses a *lac*I^q gene and a deleted *lac*Z gene, *lac*ZM15. The product of the first gene represses the lactose promoter in the absence of the inducer (isopropyl-β-D-thiogalactoside) (IPTG), whereas the product of the second gene, M15, is an acceptor in α-complementation (Ullmann and Perrin, 1970). Cultures were performed in LB medium (Miller, 1972) supplemented with 100 μg/ml ampicillin, and when necessary 0.1 mM IPTG until the end of the exponential phase of growth. Bacteria were harvested by centrifugation for 30 min at 5000 *g*, then disrupted by sonication (2 × 3 min at 20 KHz and 100 watts) after being resuspended in 20 mM K-phosphate buffer (pH 7.4).

Plasmids

Plasmid pDIA5202 harbours a truncated *cya* gene, fused in frame after the 459th codon with the α-*lac*Z′ gene (Figure 1). The fusion gene encodes a 604 residue long protein. The fusion to the α segment of β-galactosidase increased the stability of the adenylate cyclase, allowing a simplified purification of the protein.

Site-directed mutagenesis and sequence analysis

Oligonucleotide directed mutagenesis was performed using the Amersham kit following the supplier's instructions. The Lys (AAA) codon at position 58 was modified to a Gln (CAA) codon using the oligonucleotide: GTGGCCACCCAAGGATTGG. The Lys (AAG) codon at position 65 was modified to a Gln (CAG) codon using the oligonucleotide GTGCACGCCCAGTCGTCCG. Both modifications were performed on the *Bam*HI–*Eco*RV DNA fragment cloned in phage M13tg130 (Nakamaye and Eckstein, 1986). The Trp (UUG) codon at position 242 was first modified to a Asp (GAC) codon using the oligonucleotide CTTGTTGGACAAAATCGC. This Asp codon was then modified to a Val (GTC) codon using the oligonucleotide CTTGTTGGTCAAAATCGC. Finally the Trp codon was also modified in a single step to either Gly (GGG), or Arg (CGG) codons using the ambiguous oligonucleotide GACTTGTTG(C/G)GGAAAATCGC. The modifications at position 242 were performed on the *Eco*RV–*Eco*RI DNA fragment cloned in phage M13tg130. For each mutagenesis the mutated fragment was controlled for the absence of any other mutations by the dideoxynucleotide sequencing method (Sanger *et al.*, 1977).

Analytical procedures

Adenylate cyclase activity was measured as previously described (Ladant *et al.*, 1986) in 100 μl of medium containing 50 mM Tris–HCl (pH 8), 6 mM $MgCl_2$, 0.1 mM $CaCl_2$ (or 2 mM EGTA), bovine brain CaM (between 0.03 nM and 10 μm), 0.1 mM [^{3}H]cAMP (10^4 c.p.m./assay) and 2 mM [α^{32}P]ATP (5×10^5 c.p.m./assay). One unit of adenylate cyclase activity corresponds to 1 μmol of cAMP formed in 1 min at 30°C and pH 8. β-galactosidase activity was measured according to Pardee *et al.* (1959). Iodination of adenylate cyclase (0.08–0.25 mol of ^{125}I/mol of enzyme) and cleavage of protein by trypsin were performed exactly as previously described (Ladant *et al.*, 1988). Cleaved products were analysed by SDS–PAGE as described by Laemmli (1970) and detected by exposure of dried gels at −80°C to Kodak X-O-Mat AR films for 4–48 h with intensifying screens.

Protein concentration was measured according to Bradford (1976) or by amino acid analysis on a Biotronik amino acid analyzer LC 5001 using a single column procedure. Samples containing 10–15 μg of protein were hydrolysed in vacuum in 0.1 ml of 6 M HCl for 20 h at 110°C.

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