We developed an improved method of linker insertion mutagenesis for introducing 2 or 16 codons into the *Bordetella pertussis* cyaA gene which encodes a calmodulin-dependent cyaA cyclase. A recombinant kanamycin resistance cassette, containing oligonucleotide linkers, was cloned in plasmids which carried a truncated cyaA gene, fused at its 3' end to the 5' end of the *Escherichia coli* lacZ gene, specifying the α-peptide. This construction permitted a double selection for in-frame insertions by using screening for kanamycin resistance and for lactose-positive phenotype, resulting from α-complementation. We showed that most of the two-amino acid insertions within the N-terminal moiety of the catalytic domain of adenylate cyclase abolished enzymatic activity and/or altered the stability of the protein. All two-amino acid insertions within the C-terminal part of adenylate cyclase resulted in fully stable and active enzymes. These results confirm the modular structure of the catalytic domain of adenylate cyclase, previously proposed on the basis of proteolytic studies. Two-amino acid insertions between residues 247–248 and 335–336 were shown to affect the calmodulin responsiveness of adenylate cyclase, suggesting that the corresponding region in the enzyme is involved in the binding of calmodulin or in the process of calmodulin activation. In addition, we have identified within the primary structure of adenylate cyclase several permissive sites which tolerate 16-amino acid insertions without interfering with the catalytic activity or calmodulin binding. By inserting foreign antigenic determinants into these permissive sites the resulting recombinant adenylate cyclase toxin could be used to deliver specific epitopes into antigen-presenting cells.

With the advent of recombinant DNA techniques for modifying protein sequences, considerable work has been directed toward specifically altering or improving existing proteins. Modification of specific amino acids of enzymes of known structure to alter specificity has been achieved by site-directed mutagenesis. Although knowledge of the three-dimensional structure is restricted to a limited number of enzymes, sequence comparisons between members of families of homologous proteins, as well as increasingly accurate predictions of secondary and tertiary structures, offer a useful basis for redesigning enzyme function.

Another approach of great potential interest is the insertion of new peptide sequences within a defined protein. Insertional mutagenesis has been efficiently used to study the topology of membrane proteins (Charbit et al., 1986, 1991) and, in the case of solubilizable proteins, to determine regions of natural flexibility (Barany, 1985a, 1985b; Freimuth and Ginseberg, 1986; Starzyk et al., 1989; Freimuth et al., 1990; Kumar and Black, 1991). Besides, insertion of a specific exogenous peptide within an enzyme could alter its catalytic or regulatory properties, thus providing a rational basis for protein engineering.

We describe here an improved method of insertional mutagenesis that we have applied to analyze the structure-function relationship of the catalytic domain of *Bordetella pertussis* adenylate cyclase (AC). A secreted enzyme is one of the major toxins of *B. pertussis*, the causative agent of whooping cough (Weiss and Hewlett, 1986). Adenylate cyclase exhibits two unusual properties: it is activated by calmodulin (CaM), a eukaryotic protein not known to occur in bacteria, and it can invade eukaryotic cells eliciting unregulated synthesis of cyclic AMP and impairment of cellular functions (Hanski, 1989). *B. pertussis* AC, the product of the cyaA gene, is synthesized and secreted as a 200-kDa protein (Glaser et al., 1988; Bellalou et al., 1990a). The CaM-dependent enzymatic activity is located within the first 400 amino acids (Glaser et al., 1988; Ladant et al., 1989) whereas the 1300 C-terminal residues are involved in the binding to eukaryotic target cells and in the translocation of the N-terminal enzymatic domain through the cell membrane into the intracellular compartment (Gordon et al., 1989; Hewlett et al., 1989; Rogel et al., 1989; Bellalou et al., 1990b; Gentile et al., 1990).

A truncated polypeptide of about 45 kDa, which displays all the CaM-dependent AC activity (Ladant et al., 1989), could be derived from the whole toxin by limited proteolysis (Bellalou et al., 1990a) or by expression in *Escherichia coli* of the first 400 codons of the cyaA gene (Glaser et al., 1989; Gilles et al., 1990). Using this truncated gene as a target for insertional mutagenesis, we show that most of the two-amino acid insertions within the N-terminal part of the truncated AC (residues 1–224) abolished the catalytic activity and altered the stability of the mutated proteins, whereas most insertions within the C-terminal part of the molecule (residues 225–399) did not alter its biological properties. Furthermore, we show that up to 16 amino acids could be inserted in several permissive sites without a noticeable change in the AC molecule.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**

The *E. coli* strain XL1 (endA1, hsdR17, supE44, thi1, λ−, recA1, gyrA96, relA1, Δ(lac-proB)/F′, proAB, lacF2ΔM15, Thr10(tetR))

The abbreviations used are: AC, adenylate cyclase; AP, ampicillin; CaM, calmodulin; IPTG, isopropyl-β-d-thiogalactopyranoside; Km, kanamycin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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(Stratagene) was used throughout this work for DNA manipulation and for expression of AC. The E. coli strain TP610 (Hedegaard and Danchin, 1985) was used for complementation and functional assays of the wild-type and modified ACs.

Construction of plasmid pDIA5240 has already been described (Sebo et al., 1991). Plasmid pCa13 contains a synthetic CaM gene (restricted) cloned in the BamH1 site of plasmid pTZ19R (Pharmacia LKB Biotechnology Inc.). Then, the KmR gene of pUC4K (Pharmacia) was excised by cleavage with PstI, purified by gel electrophoresis, and cloned into the PstI site of pKMII, yielding pKMII.

**Linker Insertion Mutagenesis**

Two-codon Insertion Mutagenesis—Plasmid pMKII was digested by PstI, and the KmR gene cartridge KmO was purified by gel electrophoresis. Plasmid pDIA5240 was linearized by partial digestion with one of the following restriction enzymes which generate blunt ends: EcoRV, HaeIII, HincII, NdeI, NruI, and PvuII. The linearized plasmid was purified by gel electrophoresis and ligated with the purified KmO cartridge. The E. coli strain XL1 containing the linearized pMKII was transformed with the ligation mixture and plated on LB plates containing 100 mg/liter ampicillin, 50 mg/liter kanamycin, and 40 mg/liter X-gal. Plasmid DNA was isolated from white colonies using the alkaline lysis procedure. The sites of insertion were localized by restriction mapping. The DNA of interest were digested with PstI, religated, transformed in E. coli XL1, and plated on LB/Ap/X-gal. Two blue colonies were chosen to be kanamycin-sensitive indicating that the KmR gene cartridge had been excised. One blue colony for each mutant was selected for further analysis. Correct insertion of the hexameric PstI linker was confirmed by local DNA sequencing (Sanger et al., 1977).

Sixteen-codon Insertion Mutagenesis—Mutated derivatives of pDIA5240 were digested with PstI and incubated, in the presence of dNTPs, with T4 DNA polymerase which digested the 3′ overhang extremities to yield blunt ends. Plasmid pUC4K was digested with EcoRI, and the KmR gene cartridge was purified by gel electrophoresis. The EcoRI ends were filled in by incubation with T4 DNA polymerase in the presence of dNTPs. The blunt-ended KmR gene cartridge was then ligated with the blunt-ended mutated plasmids, and the ligation mixtures were transformed in E. coli XL1, selecting for kanamycin and ampicillin resistance. Ligation of the filled-in EcoRI site with the KmR gene cartridge but which now contained an inverted repeat of 45 nucleotides was performed by ligation into the PstI site of pUC4K. The correct insertion of this sequence was checked by local DNA sequencing.

**In Vivo Characterization of Modified ACs**

The parental plasmid pDIA5240 and its mutated derivatives were transformed into competent TP610 or TP610/pCa13 cells and plated on MacConkey agar medium containing 1% maltose, 100 mg/liter ampicillin, and, in the case of TP610/pCa13, 30 mg/liter chloramphenicol. The plates were incubated overnight at 37°C.

**Purification of AC and Its Modified Forms**

The E. coli strain XL1 harboring pDIA5240 was grown at 37°C in 2YT medium (Sambrook et al., 1989) containing 100 mg/liter ampicillin, to an optical density (600 nm) of 0.5–0.7. Then, IPTG (1 mM final concentration) was added to induce synthesis of AC cloned downstream from the E. coli lac regulatory region. After 4 additional h of growth at 37°C, the cells were collected by centrifugation, resuspended in 50 mM Tris-HCl, pH 8 (buffer A: 15 mM Tris-HCl of original CaM sequence, and dithiothreitol 10 mM, The sonicated extract was centrifuged 10 min at 13,000 rpm at 4°C. The supernatant, which contained about 70% of total AC activity, was resuspended in 8 M urea in buffer B, (10 ml/liter of culture) and shaken 1 h at 4°C. After 10 min of centrifugation at 13,000 rpm, the supernatant (“urea extract”) was collected. Adenylate cyclase was further purified by CaM-sagrose chromatography as previously described (Muñier et al., 1991) or by chromatography through DEAE-Sepharose in denaturing conditions (8 M urea in buffer A, 1.5 volumes of packed gel/volume of urea extract); the flow-through fractions consisted of an almost pure preparation of the 45-kDa AC species.

A rapid purification procedure was devised for analysis of the various modified ACs. The XL1 strain harboring pDIA5240 or its mutated derivatives was grown at 37°C in 5 ml of 3YT medium containing 100 mg/liter of ampicillin until an OD600nm of 0.5–0.7 and induced with IPTG (1 mM final concentration) for 4 additional h. The cells collected by centrifugation were resuspended in 0.8 ml of 8 M urea in buffer A supplemented with 0.1% Nonidet P-40, and sonically disrupted (20 s, 18 kHz). After centrifugation (10 min, 15,000 rpm), the supernatant (“sonicated extract”) was loaded onto a DEAE-Sepharose column (1.2 ml of packed gel) equilibrated and eluted with the same buffer. The 1.5 ml flow-through fraction contained 80% of the input AC activity applied.

**Analytical Procedures**

Adenylate cyclase activity was measured as previously described (Ladant et al., 1986) in medium containing 50 mM Tris-HCl, pH 8, 6 mM MgCl2, 0.1 mM CaCl2, 0.1 μM bovine brain CaM, and 2 mM [α-32P]ATP (1–2 × 107 cpm/assay). One unit of AC activity corresponds to 1 pmol of cAMP formed in 1 min at 30°C and pH 8. Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (1970). The gels were stained with Coomassie Blue. Immunoblot analyses were performed essentially as described by Goyard et al. (1989) using a rabbit polyclonal antiserum raised against the 45-kDa AC purified from B. pertussis (Monneron et al., 1988). Single-strand DNA for nucleotide sequence analysis was prepared according to Bentley and Thillet (1991). Recombinant DNA manipulations were performed according to standard protocols (Sambrook et al., 1989).

**Results**

Adenine nucleotides were from Boehringer Mannheim. Bovine brain CaM was from Sigma. Urea was a product of Schwarz/Mann. [α-32P]ATP (400 Ci/mmol), [3H]cAMP (40 Ci/mmol), and [α-32P]dATP were obtained from Sigma Chemical Co. (United Kingdom). DNA sequencing kit was from Pharmacia LKB, and enzymes for routine DNA manipulations were obtained from Bethesda Research Labs, Pharmacia LKB, United States Biochemicals, or New England Biolabs. Anti-rabbit immunoglobulin labeled with alkaline phosphatase was obtained from Promega Biotec (Madison, WI). Oligonucleotides were synthesized by the Organic Chemistry Unit, Institut Pasteur (Paris).

**Linker Insertion Mutagenesis**—In order to examine the tolerance of B. pertussis AC to amino acid insertions, we devised an improved approach of linker insert mutation, using two positive selection procedures. Plasmid pDIA5240 (Sebo et al., 1991) was used as a target for insertional mutagenesis (Fig. 1A). It contained the first 459 codons of B. pertussis cyd gene under the control of transcriptional and transcripational signals of the E. coli lacZ gene. The truncated cydA gene was fused at its 3′ end to the beginning of lacZ gene, encoding the α-peptide (Ullmann et al., 1967). The 400th codon CAG was converted by site-directed mutagenesis into a TAG amber stop codon (Gilles et al., 1990). Hence, pDIA5240 expressed a 399-residue-long AC (molecular mass of 45 kDa) which exhibited all the catalytic properties of the full-length enzyme (Ladant et al., 1989; Gilles et al., 1990). However, in strains such as strain XL1 carrying an amber suppressor, 10–15% of AC was expressed as a AC-LacZα fusion protein (molecular mass of 60 kDa) (Fig. 1A and Fig. 5, lane 2) which was able to complement the lacZ M accumulation of the host strain, resulting in the formation of active β-galactosidase (Ullmann et al., 1967). Thus, the XL1 Blue strain harboring pDIA5240 formed blue colonies on plates containing X-gal.

We also constructed a recombinant kanamycin resistance cassette, KmO (Fig. 1B), which could be cloned in plasmids.
at restriction sites that generate blunt ends. This allowed a convenient screening (Km\(^r\)) for recombinants. In a second step, the kanamycin carboxylic acid could be excised upon cleavage with PstI and religation, leaving in the recombinant plasmids the hexanucleotide, CTGCAG, corresponding to the PstI recognition site. The prerequisite of the correct excision step was the absence on the target plasmid of other PstI sites, which was the case for plasmid pDIA5240.

Plasmid pDIA5240 was linearized at various restriction sites (see "Experimental Procedures"), ligated with the recombinant kanamycin cassette, Km\(^r\), transformed into XL1 cells, and plated on LB, Ap, Km, and X-gal plates. Insertion of Km\(^r\) into the cyaA gene prevents the synthesis of AC-LacZ\(\alpha\) fusion protein; thus, the clones harboring such recombinant plasmids could easily be identified as white colonies on X-gal plates (Fig. 1C). In contrast, insertion of the Km\(^r\) cassette outside the cyaA gene did not prevent complementation and yielded Km\(^r\) blue colonies on X-gal plates (Fig. 1C). This positive selection greatly increased the efficiency of the insertion mutagenesis; more than 90% of the white colonies were shown to harbor recombinant plasmids mutagenized within the cyaA gene.

The sites of insertion were mapped by restriction analysis and the Km cassette was excised from the recombinant plasmids by screening for recovery of both Km sensitivity and blue phenotype on X-gal (Fig. 1C). The mutations were further confirmed by local DNA sequencing.

The mutations resulted in the insertion of two amino acids into the AC primary structure. Fig. 2 illustrates the location of the 14 different insertions generated within the cyaA gene. The nomenclature of the mutant enzymes and their characteristics are summarized in Table 1. For example, AC188LQ corresponds to the modified enzyme having the two amino acids Leu and Gln inserted between residues 188 and 189.

In Vivo Characterization of the Modified ACs—The modified ACs were first tested in vivo for their ability to complement an adenylate cyclase-deficient mutant (TP610) which does not synthesize cAMP and is thus unable to ferment maltose; it exhibits white phenotype on MacConkey maltose plates (Hedegaard and Danchin, 1985). Complementation of this strain with a functional AC restores its ability to ferment maltose and yields red colonies on MacConkey maltose plates. As shown in Table 1, the parental plasmid pDIA5240 transformed into TP610 gave red colonies on MacConkey maltose suggesting that B. pertussis AC expressed at a high level in E. coli (~1% of total bacterial proteins) exhibited a residual activity even in the absence of its activator, CaM. We do not know yet whether this activity could be accounted for by an intrinsic CaM-independent enzymatic activity or was due to activation by a CaM-like molecule present in E. coli. Evidence for such CaM-like proteins have been reported in various bacteria (Inouye et al., 1983; Swan et al., 1987).

Concerning the modified ACs, a clear distinction could be made between modification in the N- and C-terminal moieties of the AC molecule; all the two amino acid insertions within the N-terminal part of the AC molecule, except one (AC137AA), yielded nonfunctional enzymes, whereas all in-
**Table 1**

| Plasmid    | AC allele   | Restriction site modified | Relevant mutation | In vivo complementation | In vitro analysis |
|------------|-------------|---------------------------|-------------------|------------------------|------------------|
| pTZ19      | None        | None                      | None              | W                      | -/-              |
| pDIA5240   | AC wild-type| None                      | None              | W                      | -/-              |
| pACM55     | AC55AA      | None                      | Val^{60}AlaAlaAla | R                      | 0.23             |
| pACM78     | AC78LQ      | HincII                    | Val^{60}LeuGlnAsn | W                      | 0.23             |
| pACM100    | AC100LQ     | HincII                    | Val^{60}LeuGlnAsn | W                      | 0.23             |
| pACM107    | AC107CS     | HincII                    | Gly^{52}CysSerHis | W                      | 0.23             |
| pACM111    | AC111LQ     | HincII                    | Val^{52}LeuGlnAsp | W                      | 0.23             |
| pACM137    | AC137AA     | NruI                      | Val^{52}AlaAlaAla | R                      | 0.23             |
| pACM188    | AC188LQ     | EcoRV                     | Asp^{188}LeuGlnAsn| W                      | 0.23             |
| pACM224    | AC224AA     | HaeIII                    | Arg^{224}AlaAlaAla| R                      | 0.23             |
| pACM228    | AC228AA     | HaeIII                    | Gly^{228}AlaAlaAla| R                      | 0.23             |
| pACM235    | AC235CS     | NruI                      | Arg^{235}CysSerGlu| R                      | 0.23             |
| pACM247    | AC247LQ     | Nrd1                      | Ala^{247}LeuGlnGly| R                      | 0.23             |
| pACM317    | AC317AA     | HaeIII                    | Ser^{317}AlaAlaAla| R                      | 0.23             |
| pACM335    | AC335CS     | HaeIII                    | Gly^{335}CysSerGlu| R                      | 0.23             |
| pACM384    | AC384CR     | NaeI                      | Pro^{384}CysArgAla| R                      | 0.23             |

*Bold characters indicate inserted amino acids at defined positions in wild-type AC.*

*W*, white phenotype on MacConkey maltose plates; R, red phenotype on MacConkey Maltose plates; 0, no growth.

*The level of the 45-kDa AC in different mutants was estimated by SDS-polyacrylamide gel electrophoresis of partially purified enzyme preparations: =, undetectable level; +/-, intermediate level; +, wild-type level.

*Expressed in percent of wild-type activity, 100% corresponding to 2,200 to 2,400 units per mg of protein.

*Determined from activation curves of adenylate cyclase by various concentrations of CaM.

*ND, not determined.

The mutagenized plasmids were also assayed for their ability to transform TP610 harboring a plasmid (pCa13) which expresses high levels of CaM. The parental plasmid pDIA5240 was unable to transform TP610 pCa13; it is likely that the high level of ACs (pACM137, pACM224 to pACM385) were unable to transform TP610 harboring a plasmid (pCal3) which directed the expression of an active AC in the presence of its activator, CaM, was lethal for the host strain as it should lead to a rapid depletion of endogenous ATP and accumulation of cAMP. However, we expected to find among the mutated enzymes, which were nonfunctional in TP610, some which could become active in presence of CaM. This appeared to be the case for AC78LQ because as pDIA5240 the plasmid pACM78 could not be transformed in TP610/pCa13 (Table I). All other mutated enzymes remained inactive even in the presence of calcium as evidenced by their white phenotype on the indicator plates.

**In Vitro Characterization of the Modified ACs**—The effects of linker insertion into the cyd gene on the expression and the stability of AC were evaluated after partial purification of the modified proteins. As shown in Fig. 3 more than 50% pure preparation of AC could easily be obtained from a sonicated total extract of induced culture of XL1/pDIA5240 by chromatography through DEAE-Sepharose under denaturing conditions. This simple procedure was applied to partially purify the various modified ACs.

As shown in Fig. 4, the modified enzymes that have been shown to be functional in vivo were synthesized at levels similar to wild-type AC. On the contrary, the modified inactive enzymes (AC55AA, AC100LQ, AC107CS, AC111LQ) could not be detected on SDS-PAGE after Coomassie Blue staining. Anti-AC immune sera failed to detect a 45-kDa polypeptide in the corresponding bacterial extracts but reacted with shorter polypeptides (data not shown), suggesting that the modified ACs became highly susceptible to proteolysis in situ. Indeed, only residual enzymatic activity could be detected in the corresponding bacterial extracts (Table I).

Two particular cases should be mentioned. AC78LQ appeared to be less unstable and exhibited a CaM-dependent enzymatic activity (~0.5% of wild type, Table I) in accord with the in vivo complementation test. The modified protein AC188LQ was expressed at high levels and appeared to be as stable as the wild-type enzyme (Fig. 4) but was devoid of enzymatic activity (Table I). This was not unexpected since in AC188LQ insertion of the two amino acids was located in one of the regions that have been recently established to play a crucial role in the catalytic process (Glaser et al., 1991).

The CaM activation of the enzymatically active, modified...
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FIG. 4. Visualization of enzymes with two-amino acid insertions. Various modified forms of AC were partially purified by DEAE-Sepharose chromatography and analyzed on 10% SDS-PAGE. The gels were stained with Coomassie Blue. Molecular mass standards are as in Fig. 3.

FIG. 5. Calmodulin activation of wild-type and different mutant forms of AC. CaM (100 μM stock solution) was diluted to the desired concentration in 50 mM Tris- HCl, pH 8, containing 1 mg/ml bovine serum albumin and then was added to the reaction mixture as described under "Experimental Procedures." The reaction was initiated by addition of adenylate cyclase followed by a 5-10-min incubation at 30 °C. Wild-type, O; AC137AA, ◆; AC224AA, □; AC228AA, ▼; AC235CS, ▽; AC247LQ, ◻; AC317AA, △; AC335CS, ▼; AC384CR, □.

ACs is depicted in Fig. 5. Six out of eight modified enzymes were similar to the wild-type AC with respect to the half-maximum concentration of CaM required for activation (0.2-0.4 nM) and to the specific activities (1800-2200 μmol/min/mg) at saturating CaM concentration (Table I). This identifies permissive sites in AC, where two amino acid insertions had no effect on CaM binding or on catalytic activity. Two other modified ACs, AC247LQ and AC335CS, exhibited a profound alteration in their CaM-binding characteristics although their specific activities at high CaM concentrations reached that of the wild-type enzyme (Table I). This suggests that the corresponding regions are either directly involved in the interaction of the enzyme with its activator or play an important structural role in the protein.

Insertion of 16 Amino Acid Residues into AC—The observation that two amino acids could be inserted in several sites of B. pertussis AC without altering its stability, its activity, or its affinity for CaM prompted us to investigate the tolerance of AC to insertions of larger peptide sequences.

Insertional mutagenesis has been performed, using the Km resistance cartridge from pUC4K, on plasmids containing the newly created PstI site and which encoded enzymatically active modified ACs. KmR transformants were selected and, subsequently, the Km cartridge was removed by digestion with PstI to yield insertions of 16 codons. The characteristics of the resulting mutant enzymes expressed in E. coli are summarized in Table II. They were stable and could be partially purified, as the wild-type AC, by chromatography through DEAE-Sepharose (Fig. 6). Inspection of CaM activation curves for all the five AC mutants (Fig. 7 and Table II), indicates that their specific activity and their affinity for CaM were similar to those of the parental enzyme except for AC317P16 which exhibited 5-fold lower affinity for CaM than the native enzyme. These results show that B. pertussis AC could accommodate 16-amino acid insertions in several sites without apparent alteration of the structural and catalytic properties.

DISCUSSION

We describe here an improved method for insertional mutagenesis using phenotypic screening for insertions which map within the desired gene. This technique has been applied to analysis of the structure-function relationship of the catalytic domain of B. pertussis AC, encoded by the first 400 codons of the cyaA gene.

TABLE II
Characteristics of Bordetella pertussis AC mutants having 16-amino acid insertions

| AC alleles | Inserted residues | AC specific activity | $K_d$ for CaM |
|------------|-------------------|----------------------|--------------|
| AC wild-type None | 2,300 | 0.23 |
| AC137P16 V132A| 2,200 | 0.25 |
| AC224P16 P165R | 2,200 | 0.23 |
| AC228P16 P165R | 2,000 | 0.24 |
| AC235P16 N172S, P177R | 2,000 | 0.21 |
| AC317P16 S192A | 1,900 | 1.1 |

* Bold characters indicate inserted amino acids (one-letter code) at defined positions in wild-type AC.

b Expressed in units/mg protein.

c Determined from CaM activation curves.

FIG. 6. Visualization of AC mutants having 16-amino acid insertions. Various modified forms of AC were partially purified by DEAE-Sepharose chromatography and analyzed on 10% SDS-PAGE. The gels were stained with Coomassie Blue. Molecular mass standards are as in Fig. 3.
Our approach is an extension of the classical insertion mutagenesis method (Heffron et al., 1978; Lobil and Goff, 1984; Barany, 1985a, 1985b; Freimuth and Ginsberg, 1986) by using two different biological selections. The first one allows screening of recombinant plasmids having inserted an additional hexanucleotide, by means of a selectable cartridge. Secondly, a phenotypic screening based on α-complementation (Ullmann et al., 1967) has been devised. For this purpose, the target gene (in our case part of the peptide. Expression of the resulting AC-α fusion protein (Glaser et al., 1989) can be detected by Lac' phenotype when the plasmid has been introduced into a strain carrying a small deletion in the 5' end of the lacZ gene, which encodes the α-peptide. Expression of the resulting AC-α fusion protein (Glaser et al., 1989) can be detected by Lac' phenotype when the plasmid has been introduced into a strain carrying a small deletion in the 5' end of lacZ. Insertion of the selectable cartridge within the target gene prevents synthesis of the fusion protein and results in Lac' phenotype. Removal of the selectable cartridge should lead to the recovery of the Lac' phenotype. This double selection provides a simple test for in frame insertions and should be useful for extensive linker insertion scanning mutagenesis, particularly in the case of proteins that do not exhibit a straightforward phenotype for rapid screening.

Fusion of the foreign α-peptide to the C-terminal part of a target protein could in some instances modify its biological properties. To avoid this potential problem, an amber codon was introduced at the 3'-terminal end of the truncated cyoA gene. In a recipient strain carrying an amber suppressor, only a small fraction (10–15%) of the protein was expressed as a CyaA-α fusion protein, which was, however, sufficient for phenotypic screening by α-complementation. After identification of the insertions of interest, the relevant proteins can be expressed, for further characterization, in a strain lacking the amber suppressor. This latter step was not required in our particular case, since the 400-amino acid-long AC was resolved from the AC-LacZ fusion protein by the purification procedure that we used.

Our present results confirm the modular structure of the catalytic domain of B. pertussis AC, previously proposed on the basis of limited proteolysis studies (Ladant, 1988; Ladant et al., 1989; Munier et al., 1991). They showed that the N-terminal domain of AC (residues 1–224, a 25-kDa fragment) was resistant to trypsin proteolysis both in the presence and in the absence of CaM, whereas the C-terminal moiety (residues 225–399, a 18-kDa fragment) was highly susceptible to trypsin digestion in the absence of CaM but was largely protected by the binding of the activator (Ladant, 1988). We have suggested on the basis of these data that the N-terminal domain is able to fold in a compact structure, whereas the C-terminal part of the molecule would adopt a flexible conformation in the absence of CaM and could be wrapped around CaM upon binding of the activator, thus being less accessible to proteolysis.

The finding that most of the two-amino acid insertions within the N-terminal part of AC abolished enzymatic activity and/or generated unstable molecules is consistent with the proposed tightly folded structure. Indeed, insertion of two additional amino acid residues into such a compact domain would introduce considerable constraints, which in most cases could not be accommodated in the native three-dimensional structure (Go and Miyazawa, 1980; Barany, 1985a, 1985b; Freimuth et al., 1990). However, insertion of two amino acids into two specific sites of the N-terminal domain were found compatible with the overall stability of AC, suggesting that these permissive insertion sites could map at the surface regions of the protein. One of these sites (amino acids 137–138) allowed insertion of as much as 16 residues without altering the catalytic activity of the enzyme or its CaM-binding properties. In this case, the additional amino acids would probably adopt an omega-loop structure as defined by Leszczynski and Rose (1988). The second permissive site in the N-terminal domain, leading to inactive albeit stable protein, was found to be between Asp'ss and Ile'ss. For this latter mutant, AC188LQ, it seems more likely that the two additional amino acids are accommodated in the ATP-binding pocket rather than at the surface of the AC molecule. Indeed, it is interesting to note that insertion of 16 amino acids at the same position yielded an unstable protein, undetectable in crude extract (not shown). Asp'ss and Asp'ss have been shown to be essential for catalysis, and it was suggested that they participate in the binding of Mg²⁺-ATP (Glaser et al., 1991). Thus the polypeptide segment around Asp'ss/Asp'ss may constitute one side of the ATP-binding site. That the two amino acids introduced in AC188LQ are buried in the ATP-binding site would explain the lack of enzymatic activity of the modified enzyme.

Concerning the C-terminal domain (residues 224–399) our data support the hypothesis of a flexible polypeptide chain which should be able to accommodate various amino acid insertions, provided that they do not lie within regions which make essential contacts with CaM. The modified enzymes AC247LQ and AC353CS clearly fell into this category, as they exhibited severely altered CaM-binding properties. For AC247LQ the insertion maps in the middle of a peptide segment (amino acids 235–254) which has been shown to be an essential CaM-binding site in AC (Glaser et al., 1989; Ladant et al., 1989). The main feature of this sequence, in agreement with other CaM-binding sites, is the presence of basic and hydrophobic residues which have the potential to form an amphiphilic α-helical structure (O'Neil and DeGrado, 1990). Our observation that insertion of Leu and Gln residues between Ala'ss and Gly'ss greatly diminished the affinity of AC for its activator not only confirms the essential role of this peptide segment in the binding of CaM, but also substantiates the importance of the amphiphilicity of this putative helix for an efficient interaction with the activator. Indeed, insertion of the two helix former amino acids Leu and Gln in the middle of the CaM-binding site of AC should maintain or even strengthen the α-helical structure but will introduce a half-turn rotation of the two moieties of the helix thus disrupting the initial segregation of basic and polar residues on the two opposite faces of the α-helix.

Insertions of up to 16 additional amino acids in the vicinity of the CaM-binding site (between residues 224 and 225, 228 and 229, and 235 and 236) had no effect on the CaM-binding properties of the enzyme suggesting that sequences located.
next to the N-terminal end of the CaM-binding helix are not involved in direct contacts with the activator. Besides, a deletion extending from residues 224 to 235 does not affect the catalytic and the CaM-binding properties of AC (data not shown). Altogether, these results permit to map precisely one extremity of the CaM-binding site to residue Arg^{255}.

The drastically altered CaM responsiveness of AC35CS allowed us to identify an additional region important for activation of AC by CaM. It remains to be determined whether this region is directly involved in binding of CaM or it corresponds to a polypeptide segment which is essential for the CaM activation process itself.

The finding that foreign peptides of up to 16 residues could be inserted into various sites of B. pertussis AC without altering the stability, the catalytic, and the CaM-binding properties of the modified enzyme offers some interesting possibilities for protein engineering. It should be possible to construct hybrid ACs by inserting into the permissive sites peptides displaying defined characteristics, such as ligand binding capability. An example of a similar approach has been recently reported by Toma et al. (1991) who grafted a calcium-binding loop of thermolysin to Bacillus subtilis neutral protease. This modification conferred a Ca^{2+}-dependent modulation of the stability of the recombinant enzyme.

An even more interesting possibility would be to use the full-length, invasive AC toxin of B. pertussis as a vehicle to deliver foreign epitopes into eukaryotic cells. Adenylate cyclase toxin is especially suited for such purpose because the toxin is able to invade a large variety of eukaryotic cells including cells of the immune system (Hanski, 1989, Trends Biochem. Sci. 14, 459-463).

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REFERENCES

Barany, F. (1985a) Proc. Natl. Acad. Sci. U.S.A. 82, 4202-4206

Barany, F. (1985b) Gene (Amst.) 37, 111-123

Bellalou, J., Ladant, D., and Sakamoto, H. (1990a) Infect. Immun. 58, 1195-1200

Bellalou, J., Sakamoto, H., Ladant, D., Geoffroy, C., and Ullmann, A. (1990b) Infect. Immun. 58, 3242-3247

Blondel, A., and Thillet, J. (1990) Nucleic Acids Res. 19, 181

Chang, A. C., and Cohen, S. N. (1978) J. Bacteriol. 134, 1141-1156

Charbit, A., Boulin, J.-M., Ryter, A., and Hofnung, M. (1986) EMBO J. 5, 3029-3037

Charbit, A., Ronco, J., Michel, V., Werts, C., and Hofnung, M. (1991) J. Bacteriol. 173, 262-275

Freimuth, P. I., and Ginsberg, H. S. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 7816-7820

Freimuth, P. I., Taylor, J. W., and Kaiser, E. T. (1990) J. Biol. Chem. 265, 896-901

Gentile, F., Knippling, L. G., Sackett, D. L., and Wolff, J. (1990) J. Biol. Chem. 265, 10686-10692

Gilles, A.-M., Munier, H., Rose, T., Glaser, P., Krin, E., Danchin, A., Pellecuer, C., and Bärzu, O. (1990) Biochemistry 29, 8126-8130

Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullmann, A., and Danchin, A. (1988) Mol. Microbiol. 2, 19-30

Glaser, P., Elmaleoglou-Lazaridou, A., Krin, E., Ladant, D., Bärzu, O., and Danchin, A. (1989) EMBO J. 8, 967-972

Glaser, P., Munier, H., Gilles, A.-M., Krin, E., Poizat, T., Bärzu, O., Sarfati, R., Pellecuer, C., and Danchin, A. (1991) EMBO J. 10, 1683-1688

Go, M., and Miyazawa, S. (1990) Int. J. Peptide Protein Res. 15, 211-224

Gordon, V. M., Young, W. W., Jr., Lechler, S. M., Gray, M. C., Leplaa, S. H., and Hewlett, E. L. (1989) J. Biol. Chem. 264, 14792-14796

Goyard, S., Orlando, C., Sabatier, J.-M., Labruyère, E., d’Alayer, J., Fontan, G., Van Rietchooten, J., Mock, M., Danchin, A., Ullmann, A., and Monneron, A. (1988) Biochemistry 28, 1964-1967

Hanski, E. (1989) Trends Biochem. Sci. 14, 459-463

Hedegaard, L., and Danchin, A. (1985) Mol. Gen. Genet. 201, 38-42

Heffron, F., So, M., and McCarthy, B. J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 6012-6016

Hewlett, E. L., Gordon, V. M., McCaffery, J. D., Sutherland, W. M., and Gray, M. C. (1989) J. Biol. Chem. 264, 13979-13984

Inouye, S., Franceschini, T., and Inouye, M. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 6823-6833

Kumar, G. B., and Black, P. N. (1991) J. Biol. Chem. 266, 1348-1353

Ladant, D. (1988) J. Biol. Chem. 263, 2612-2618

Ladant, D., Brézin, C., Alonso, J.-M., Crenon, I., and Guiso, N. (1986) J. Biol. Chem. 261, 16264-16269

Ladant, D., Michelson, S., Sarfati, R., Gilles, A.-M., Predeleanu, R., and Bärzu, O. (1989) J. Biol. Chem. 264, 4015-4020

Laemmli, U. K. (1970) Nature 227, 680-685

Leszczynski, J. F., and Rose, G. D. (1990) Science 234, 849-855

Lobel, L. K., and Goff, S. P. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 4149-4153

Monneron, A., Ladant, D., D’Alayer, J., Bellalou, J., Bärzu, O., and Ullmann, A. (1988) Biochemistry 27, 536-539

Munier, H., Gilles, A.-M., Glaser, P., Krin, E., Danchin, A., Sarfati, R., and Bärzu, O. (1991) Eur. J. Biochem. 196, 469-474

O’Neill, K. T., and DeGrado, W. F. (1990) Trends Biochem. Sci. 15, 59-64

Robert, D. M., Cree, R., Malecha, M., Alvarado-Urbina, G., Chirello, R. H., and Watterson, D. M. (1985) Biochemistry 24, 7050-7058

Roberts, J. A., McInerney, J. R., and Ironside, D. J. (1987) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467

Sebo, P., Glaser, P., Sakamoto, H., and Ullmann, A. (1991) Gene (Amst.) 104, 19-24

Starzyk, R. M., Burburn, J. J., and Schimmel, P. (1989) Biochemistry 28, 8479-8484

Swan, D. G., Hale, R. S., Dhillon, N., and Leadlay, P. F. (1987) Nature 329, 84-86

Toma, S., Campagnoli, S., Margarit, I., Gianna, R., Grandi, G., Bolognesi, M., De Filippis, V., and Fontana, A. (1991) Biochemistry 30, 97-106

Ullmann, A., Jacob, F., and Monod, J. (1967) J. Mol. Biol. 24, 339-345

Weiss, A. A., and Hewlett, E. L. (1986) Annu. Rev. Microbiol. 40, 661-686