Accelerated Publications

Identification of a Common Domain in Calmodulin-Activated Eukaryotic and Bacterial Adenylate Cyclases†

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Received November 17, 1988; Revised Manuscript Received December 16, 1988

ABSTRACT: *Bordetella pertussis* and *Bacillus anthracis*, two taxonomically distinct bacteria, secrete adenylate cyclase toxins that are activated by the eukaryotic protein calmodulin. The two enzymes contain a well-conserved stretch of 24 amino acid residues [Escuyer et al. (1988) *Gene 71*, 293–298]. Antibodies have been obtained against two synthetic heptadecapeptides, covering part of the conserved sequences. The anti-peptide antibodies specifically reacted in Western blots with the rat brain adenylate cyclase as well as with the two bacterial enzymes. Anti-rat brain adenylate cyclase serum contained antibodies that were retained by the immobilized peptides, and the affinity-purified antibodies yielded the same recognition pattern of the eukaryotic enzyme as did the unfractionated serum. These results indicate that the eukaryotic adenylate cyclase contains an epitope closely related to that specified by the conserved bacterial sequence. The synthetic peptides and the bacterial adenylate cyclases appeared to compete for ATP (K_D of the ATP–peptide complex ca. 0.2 mM), suggesting that the conserved sequence may be part of the substrate binding site in these two enzymes.

Two pathogens, the Gram-negative bacterium *Bordetella pertussis* and the Gram-positive bacterium *Bacillus anthracis*, are known to secrete toxic adenylate cyclases that are activated by an eukaryotic protein, calmodulin, not known to occur in bacteria (Wolff et al., 1980; Leppla, 1982).

It has recently been shown that the calmodulin-stimulated rat brain adenylate cyclase catalytic subunit displays immunological cross-reactivity with the adenylate cyclase toxin of *B. pertussis* (Monneron et al., 1988). It has also been reported that the adenylate cyclase of *B. anthracis* is immunologically related to the *B. pertussis* enzyme (Mock et al., 1988). The sequence of the rat brain adenylate cyclase catalytic subunit is not yet known. The nucleotide sequence determination of the two bacterial adenylate cyclases has revealed that although the overall sequences are very different, they contain a particularly well-conserved stretch of 24 amino acids: GVATKGLGVHAKSSDWGLQAGYIP in *B. pertussis* (Glaser et al., 1988) and GVATKGLNVHGKSSDWGPVAGYIP in *B. anthracis* (Escuyer et al., 1988).

Making use of antibodies raised against synthetic peptides corresponding to the first 17 amino acids of these sequences, we show that these antibodies recognized both bacterial adenylate cyclases and the eukaryotic catalytic subunit. Furthermore, our data suggest that this peptide may be part of the catalytic domain of the bacterial enzymes.

Experimental Procedures

Purification of Enzymes. Adenylate cyclase from *B. pertussis* culture supernatants was purified according to the method of Ladant (1988); *B. anthracis* adenylate cyclase was prepared as described by Leppla (1984). They were assayed as described by Ladant (1988). Preparations of purified synaptosomal rat brain adenylate cyclase catalytic subunits were obtained according to Monneron et al. (1988). Briefly, synaptosomes were solubilized with Lubrol PX. The soluble extract was incubated with forskolin–agarose for 2 h on a roller. The resin was washed. Bound protein was eluted with 100 μM forskolin. It contained 30% of the adenylate cyclase present in the soluble extract, and it was purified 5000-fold. Rat brain adenylate cyclase activity was assayed as described by Salomon (1979). One unit of enzyme activity corresponds to 1 μmol of cAMP formed per minute at 30 °C.

Chemical Synthesis of Peptides. Synthesis of peptides A (GVATKGLGVHAKSSDWG) and B (GVATKGLNVHGKSSDWG) was performed on an Applied Biosystems peptide synthesizer (Model 430 A) by the solid-phase method developed by Barany and Merrifield (1979). *N*-Butyloxycarbonyl (Boc) amino acids with benzyl-based side-chain protecting groups [except Trp(For)] were used in combination with a PAM resin as solid support. The elongation protocol was based on a single coupling step in dimethylformamide with either Boc-amino acid symmetrical anhydride (1 mmol; i.e., 2-fold excess over free amines) or hydroxybenzotriazole active esters (2 mmol; i.e., 4-fold excess over free amines) related to Asn, Gln, and Arg couplings. After peptide-chain assembling an anhydrous hydrogen fluoride treatment (1 h, 0 °C) yielded crude peptides. Purification was achieved by filtration

† This work was supported by grants from the Centre National de la Recherche Scientifique (URA 150 and URA 37) and a grant from the Institut National de la Santé et de la Recherche Médicale (INSERM U.172).

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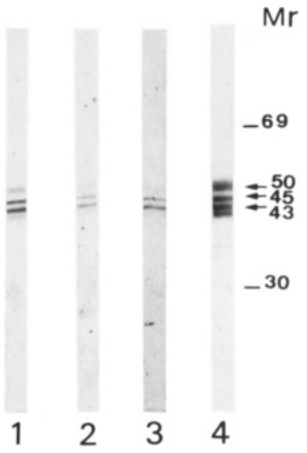

FIGURE 1: Immunochemical detection of *B. pertussis* adenylate cyclase by affinity-purified anti-peptide immunoglobulins. The purified enzyme preparation (0.2 unit/well) was separated by SDS–PAGE (10%), transferred to a nitrocellulose sheet, and reacted with affinity-purified IgGs (6 μg/mL) or homologous anti-adenylate cyclase immune serum diluted 1:1000. Lane 1, IgGs from anti-peptide A immune serum 2; lane 2, IgGs from anti-peptide B immune serum 4; lane 3, IgGs from anti-peptide A immune serum 41; lane 4, anti *B. pertussis* adenylate cyclase immune serum; M_r, molecular weight markers ($\times 10^3$).

on Sephadex G-15 equilibrated with 0.1 N acetic acid. The peptides were treated with 0.04 N NaOH (pH 11.5) for 3 min in order to ensure complete deformylation of Trp-containing peptides (Li et al., 1978) and then highly purified by C18 reverse-phase medium-pressure liquid chromatography. Homogeneity of the peptides was checked by HPLC and amino acid analysis after 6 N HCl hydrolysis (110 °C, 48 h). The uncorrected amino acid content was as follows: peptide A, Asp 0.8, Thr 0.9, Ser 1.6, Gly 3.9, Ala 2.0, Val 2.0, Leu 1.1, His 1.0, Lys 2.0; peptide B; Asp 1.7, Thr 0.8, Ser 1.6, Gly 4.1, Ala 1.0, Val 1.9, Leu 1.0, His 1.0, Lys 2.0.

Immunization Procedures. Rabbits immunized with free peptides received an injection of about 0.5 mg of peptide in Freund's complete adjuvant in each poplitean lymph node and were boosted in the foot pads followed by intramuscular injections every 3 weeks (immune sera 33 and 41 against peptide A and 35 and 42 against peptide B, respectively). Peptides were also conjugated with keyhole limpet hemocyanin, with either glutaraldehyde (immune serum 4) or 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (immune serum 2) as coupling agents (Avrameas et al., 1978). Rabbits were injected intramuscularly with 500 μg of synthetic peptide coupled to the carrier in Freund's complete adjuvant and boosted every 3 weeks with 250 μg of conjugated peptide in Freund's incomplete adjuvant. Rabbits (30 and 31) were immunized with purified preparations of rat brain adenylate cyclase, precipitated with acetone, and were immunized according to the protocol described for free peptides, with an amount of antigen corresponding to an adenylate cyclase activity of 600 nmol of cAMP/min per injection. The other anti-rat brain adenylate cyclase sera used here have been described (Monneron et al., 1988).

Immunoblots. Proteins separated by SDS–PAGE were transferred to nitrocellulose sheets and incubated as previously described (Monneron et al., 1988). The immunochemical detection was performed with alkaline phosphatase labeled anti-rabbit immunoglobulins from Promega-Biotec (Madison, WI).

Affinity Purification of Anti-Peptide Immunoglobulins. The synthetic peptides were coupled to Sepharose CL-4B (Pharmacia Fine Chemicals), 0.5 mg of peptide/1 mL of gel.

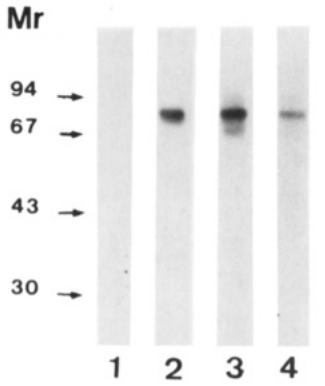

FIGURE 2: Immunoblots of the *B. anthracis* adenylate cyclase. Experimental conditions were the same as described in the legend of Figure 1 (0.15 unit/well). Lane 1, preimmune serum corresponding to immune serum 4 diluted 1:1000; lane 2, anti-peptide B immune serum 4 diluted 1:1000; lane 3, affinity-purified IgGs (6 μg/mL) from immune serum 4; lane 4, affinity-purified IgGs (6 μg/mL) from anti-peptide A immune serum 41.

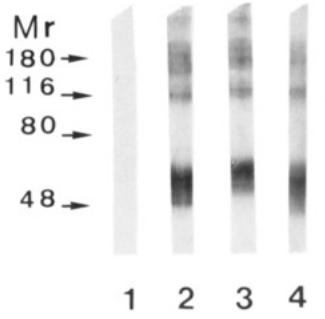

FIGURE 3: Immunochemical detection of rat brain adenylate cyclase by anti-peptide sera. The purified enzyme preparation was precipitated with acetone and separated by SDS–PAGE (7–10%) (0.015 unit/well). Proteins were transferred to a nitrocellulose sheet and reacted with different sera. Lane 1, preimmune serum 41 diluted 1:25; lane 2, anti-peptide A serum 41 diluted 1:25; lane 3, homologous anti-rat brain adenylate cyclase serum 25 diluted 1:500; lane 4, anti-peptide A immune serum 33 diluted 1:25. The corresponding preimmune sera 25 and 33 gave no reaction (not shown).

One milliliter of immune serum was applied to 2 mL of the peptide–Sepharose resin. After 30-min incubation at room temperature, the column was washed with 300 mL of PBS buffer (50 mM K_2HPO_4, 150 mM NaCl, pH 7.5). The immunoglobulins were eluted with 1 mL of buffer (glycine hydrochloride, pH 2.8, NaCl, 150 mM), neutralized with 2 M Tris, pH 10, and dialyzed against PBS–0.1% NaN_3 at 4 °C for 18 h.

Results and Discussion

Bacterial and Eukaryotic Adenylate Cyclases. B. pertussis adenylate cyclase, purified from culture supernatants, consists of three structurally related peptides of 43, 45, and 50 kDa, respectively (Ladant, 1988, and Figure 1). The gene product is synthesized as a large precursor of 177 kDa (Glaser et al., 1988), and the catalytic domain resides in the 400 amino-terminal residues. *B. anthracis* adenylate cyclase is a 89-kDa secreted protein (Leppla, 1984, and Figure 2) synthesized as a 92-kDa precursor (Escuyer et al., 1988).

Purified preparations of rat brain adenylate cyclase catalytic subunits contain four major polypeptides of 155, 116, 63, and 52 kDa, respectively (Monneron et al., 1988). Polyclonal antisera were raised against these preparations. In Western blots of purified enzyme preparations they detected the four major polypeptides. They also revealed additional molecular species in the range of 180–120 and 63–52 kDa, not detected

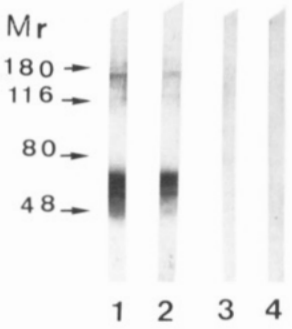

FIGURE 4: Detection of anti-peptide antibodies in immune sera raised against rat brain adenylate cyclase. Anti-rat brain adenylate cyclase serum 31 and the corresponding preimmune serum were incubated with resin coupled to peptide B. The retained IgGs were eluted. The immunochemical detection of rat brain adenylate cyclase by the immune serum (lane 1) or preimmune serum (lane 3) diluted 1:500 and by the retained IgGs (35 μg/mL) from the immune serum (lane 2) or preimmune serum (lane 4) is shown.

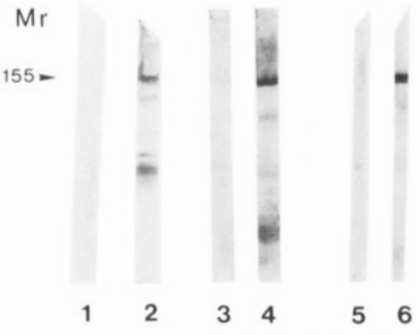

FIGURE 5: Immunochemical detection of adenylate cyclase in Western blots of synaptosomal membrane proteins. Lubrol-soluble synaptosomal proteins were precipitated with acetone, separated by SDS–PAGE (7.5–10%) (120 μg of proteins per well), and transferred to nitrocellulose. Lane 2, anti-rat brain adenylate cyclase serum 31 diluted 1:500; lane 4, anti-peptide B serum 42 diluted 1:100; lane 6, anti-peptide serum B 35 diluted 1:200; lanes 1, 3, and 5, preimmune sera corresponding to lanes 2, 4, and 6. Different Western blots are being shown in the figure; only the 155-kDa proteins are lined up, as indicated. In lane 2, the components 63 and 52 kDa are revealed.

in stained gels [Monneron et al., 1988, and Figures 3 (lane 3) and 4 (lane 1)]. When tested on Western blots of detergent-soluble synaptosomal proteins, some of these sera produced the same immunostaining pattern as that observed on blots of purified adenylate cyclase preparations (Figure 5, lane 2).

Immunological Cross-Reactions between B. pertussis, B. anthracis, and Rat Brain Adenylate Cyclase. Immune sera raised against the sequences GVATKGLGVHAKSSDWG (peptide A, corresponding to *B. pertussis*, residues 54–70) and GVATKGLNVHGKSSDWG (peptide B, corresponding to *B. anthracis*, residues 341–357) were tested in Western blots against the bacterial and eukaryotic enzymes.

Figure 1 shows that immunoglobulins, obtained by affinity purification of different anti-peptide immune sera, reacted with purified preparations of the *B. pertussis* enzyme. The recognition patterns were comparable to that obtained with the homologous anti-adenylate cyclase immune serum. Figure 2 shows an immunoblot of a partially purified preparation of *B. anthracis* adenylate cyclase. The anti-peptide immune sera as well as the corresponding affinity-purified immunoglobulins recognized material in the 89-kDa band, corresponding to the adenylate cyclase. Since no anti *B. anthracis* adenylate cyclase immune serum was available, the specificity of recognition was assessed by the absence of a signal in toxinless strains (data not shown).

Figures 3 and 4 represent Western blots of purified rat brain adenylate cyclase. The four immune sera obtained with the uncoupled, free peptides all recognized the 52- and 63-kDa components. Two of them (41 and 33, Figure 3, lanes 2 and 4) and the corresponding affinity-purified immunoglobulins (not shown) reacted with all the molecular forms of the enzyme, presenting the same recognition pattern as that obtained with sera raised against the purified eukaryotic adenylate cyclase (Figure 3, lane 3). When tested on Western blots of synaptosomal proteins, the anti-peptide immune sera recognized a 155-kDa protein (Figure 5, lanes 4 and 6). The 52- and 63-kDa proteins were, however, not detected by all sera. Finally, when an anti-rat brain adenylate cyclase polyclonal serum was incubated with the resin-coupled peptides, immunoglobulins were retained on the resins and, upon elution, reacted with all the components of the purified enzyme preparations (Figure 4, lane 2).

These data indicate that the rat brain adenylate cyclase contains an epitope specified by the peptide which is common to both bacterial enzymes or is closely related to it. This particular epitope accounts, at least in part, for the immunological relatedness between the eukaryotic and the calmodulin-dependent bacterial enzyme (Monneron et al., 1988). It was detected in a 155-kDa protein present both in membrane fractions and in purified adenylate cyclase preparations. The other components of the latter system also contained this epitope, but such proteins were generally not detected in the membrane preparations. These observations tend to suggest that the 155-kDa protein could be an undegraded form of the catalytic subunit and that the lower molecular weight components of the purified enzyme preparation, i.e., the 116-, 63-, and 52-kDa components, might represent contaminant or degraded forms of the enzyme produced in the course of purification, or both.

Function of the Conserved Peptide Sequence. The highly conserved peptide sequence in *B. pertussis* and *B. anthracis* adenylate cyclases is represented by the stretch of amino acid residues 54–77 in the *B. pertussis* enzyme and 342–358 in the *B. anthracis*. Since it has been shown that the calmodulin-activated catalytic domain of the protein resides in the 400 amino-terminal residues of the *B. pertussis* enzyme, the 54–77 peptide might be involved in the binding of the substrate. Therefore, we performed competition experiments with the synthetic peptides and ATP.

Figure 6 depicts an experiment in which adenylate cyclase and synthetic peptide A competed for the binding of ATP (similar results were obtained with peptide B). With both bacterial enzymes, the peptide inhibited the initial rate of reaction when ATP concentrations were below the K_m values for the substrate, namely, 0.6 and 0.2 mM for the *B. pertussis* and the *B. anthracis* enzymes, respectively. High concentrations of ATP reversed the inhibition displayed by the peptide. At constant ATP concentration, and increasing concentrations of peptide from 0.1 to 0.6 mM, a dose-dependent inhibition of the adenylate cyclase activity was observed; this allowed for an estimation of the apparent dissociation constant of the peptide–ATP complex of ca. 0.2 mM. The inhibition exerted by peptide A or peptide B appeared to be specific: another peptide, corresponding to residues 235–254 of the *B. pertussis* enzyme (RERIDLLWRIARAGARSAVG) and shown to be part of the calmodulin-binding domain (Glaser et al., 1988; Ladant et al., 1989), was without effect on enzymatic activity.

Our results suggest that peptides A and B bind ATP with a dissociation constant of the same order of magnitude as the

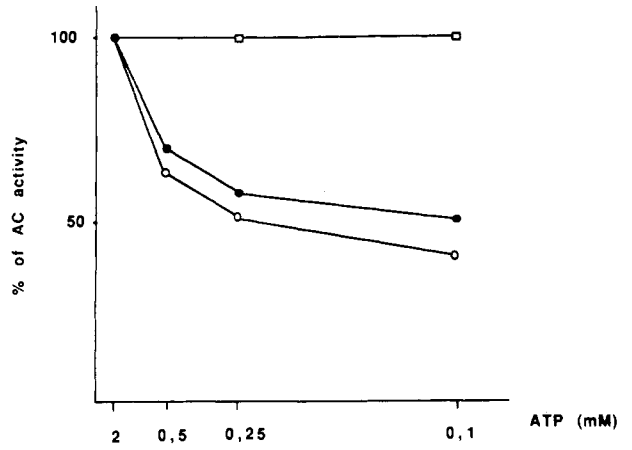

FIGURE 6: Effect of peptide A and of an unrelated peptide on the activity of *B. pertussis* and *B. anthracis* adenylate cyclases. Purified preparations of enzymes (5×10^{-4} unit) were incubated in the presence of 0.6 mM peptide A (residues 54–70 of *B. pertussis* enzyme) (○, ●) or of 0.6 mM synthetic peptide corresponding to residues 239–254 of *B. pertussis* enzyme (□). *B. pertussis* enzyme (open symbols) and *B. anthracis* enzyme (closed symbols) were assayed in the presence of 0.1 μM calmodulin; AC represents adenylate cyclase.

K_m of both bacterial adenylate cyclases for ATP. They are, therefore, compatible with the hypothesis that the common sequence could be part of the ATP-binding site in the two bacterial enzymes. Further support for this hypothesis is provided by the conserved sequence G----GKS (AKS in *B. pertussis*), which is a characteristic motif found in nucleotide-binding proteins (Ahnn et al., 1986). It is thus tempting to suppose that, in the brain adenylate cyclase too, the epitope recognized by the anti-peptide antibodies corresponds to a portion of the substrate-binding site. Attempts to validate experimentally this assumption were unsuccessful: the peptides failed to inhibit activity of the eukaryotic enzyme, probably due to the relatively low affinity of the peptide for ATP (K_D ca. 0.2 mM) as compared to the much higher value found for the brain adenylate cyclase (K_m = 18 μM).

In conclusion, we have shown that antibodies raised against a peptide corresponding to a sequence common to two bacterial calmodulin-activated adenylate cyclases reacted specifically with the SDS-denatured rat brain adenylate cyclase catalytic subunit. The data suggest that the bacterial and eukaryotic enzymes might have a common origin. They also may provide, in the absence of published sequences of the eukaryotic enzyme, a basis for the use of such antibodies as potential probes for cloning the cDNA of the rat brain enzyme.

ACKNOWLEDGMENTS

We are grateful to O. Barzu, M. Goldberg, and D. Ladant for helpful discussions and to M. Ferrand for secretarial help.

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