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Bordetella pertussis Adenylate Cyclase: Isolation and Purification by Calmodulin-Sepharose 4B Chromatography

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Purified preparations of adenylate cyclase were obtained from crude urea extracts of *Bordetella pertussis* by a one-step calmodulin affinity chromatography technique. Diluted extract was loaded onto the column and washed, and adenylate cyclase was eluted with 10mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid]. A 104-fold purification was accomplished in one step. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the affinity-purified adenylate cyclase was dissociated into one major protein band with an apparent molecular weight of 60,000 and a minor band at 200,000. The affinity-purified adenylate cyclase was observed (i) to have adenylate cyclase enzymatic activity which was activated by calmodulin, (ii) to bind ^{125}I-calmodulin, and (iii) to be free of pertussis toxin as determined by in vivo and in vitro assays.

The human pathogen *Bordetella pertussis* causes an acute and chronic respiratory disease, particularly in young children, known as whooping cough (24). Although effective immunization programs against whooping cough occur in the United States and other industrialized countries, pertussis has reemerged recently as a major health problem. This has been due to a decrease in public confidence in the whole-cell vaccine because of reported severe side effects in young children, including neurological effects and death (5, 25). The decrease in vaccination rate has led to an increased incidence of the disease and, for example, in the United Kingdom, to increases of epidemic proportions (4). Whooping cough is still a leading cause of childhood mortality in developing countries (41).

B. pertussis produces several potential virulence factors, which include pertussis toxin (PT; 27, 29, 32, 38), filamentous hemagglutinin (8, 32), hemolysin (19, 42, 43), endotoxin (7), adenylate cyclase (AC; 6, 7, 17, 43), dermonecrotic toxin (26), and tracheal cytotoxin (12). Development of a more effective and safer vaccine (30, 33) has become increasingly difficult because of an incomplete knowledge of the role(s) these bacterial products play in the pathogenesis of the disease and in immunity to *B. pertussis* infection.

There are several features of *B. pertussis* infection which are unusual. These include absence of fever, lack of neutrophilia, persistence of infection for 3 to 8 weeks, and high frequency of secondary bacterial pneumonias (2, 28). These observations suggest that *B. pertussis* may impair host defenses locally and systemically, and this impairment may be due to one or more of the exoproducts of the bacteria. It has been suggested that the AC may be involved (6, 40).

Studies on *B. pertussis* AC have been done mainly with crude urea extracts or culture supernatants which contain a complex mixture of different proteins from the bacteria, including PT, filamentous hemagglutinin, other surface proteins, and AC (6, 16, 17, 39). Crude AC extracts were found to have enzymatic activity which was activated 10- to 1,000-fold (13, 46) by the eucaryotic regulatory protein calmodulin (CaM). The extracts were found to inhibit human polymorphonuclear leukocyte (PMN) and macrophage biological functions and bactericidal activity (6, 39). The crude AC also induces increased intracellular levels of cAMP in various mammalian cell types, including PMN, lymphocytes, monocytes, CHO cells, mouse S49 lymphoma cells, and isolated rat oocytes (6, 16). Because of the complex nature of the urea extract, it is difficult to determine whether these reported effects are due solely to the AC or to other proteins present in the extract, such as PT.

For critical studies on the role of AC in *B. pertussis* pathogenesis (as a potential virulence factor), purified preparations of the AC are required that have enzymatic and biological activities.

In this paper, a one-step CaM affinity chromatography technique is described for the isolation of AC from crude urea extracts of *B. pertussis*. The affinity-purified AC had enzymatic activity which is stimulated by CaM and had biological effects on human PMN (11).

MATERIALS AND METHODS

Bacteria. *B. pertussis* BP165 was used for the production of AC. Stock cultures were stored as a thick suspension in Stainer-Scholte medium (17) containing 50% glycerol at −70°C. Bordet Gengou agar plates were inoculated with 2 to 3 drops of a thawed cell suspension and grown for 3 to 4 days at 37°C. Purity of cultures were monitored by Gram-stain morphology, growth characteristics, and agglutination with *B. pertussis* agglutinating serum.

Production of *B. pertussis* AC. Growth from one Bordet Gengou plate of strain BP165 was used to inoculate 40 ml of Stainer-Scholte medium in a 125-ml flask. Cultures were incubated at 37°C with shaking at 260 rpm in an incubator shaker (model G2; New Brunswick Scientific Co., Inc.) for 24 h. These 40-ml cultures were used to inoculate 400 ml of Stainer-Scholte medium in 1-liter flasks with 5% inoculum; the flasks were incubated as described above for 24 h. Cells were harvested by centrifugation and washed two times with sterile phosphate-buffered saline.

Extraction of AC. Urea extracts were prepared by the method of Confer and Eaton (6) with modification. *B. pertussis* cells were suspended in phosphate-buffered saline containing 4.0 M urea (30 ml of 4.0 M urea solution per 3 g [wet weight] of cells) by using a Tissumizer (Tekmar Co.). The slurry was frozen at −70°C for several days. The urea extract was thawed and dialyzed exhaustively with 50 mM

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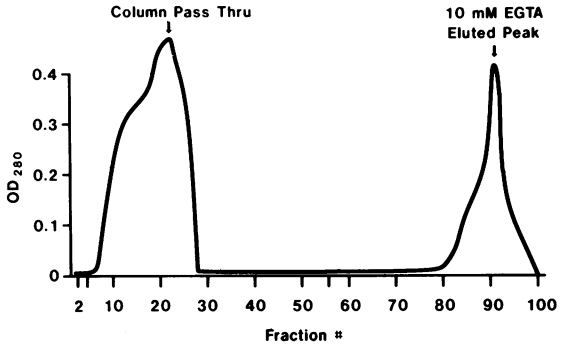

FIG. 1. CaM-Sepharose 4B chromatography of *B. pertussis* urea extracts. Urea extract was diluted 1:20 with TNT buffer and applied to the column at a flow rate of 10 ml/h. The column was washed with TNT until the optical density of the eluate at 280 nm reached 0. AC was eluted by using 50 mM Tris hydrochloride–50 mM NaCl (pH 7.6) buffer containing 10mM EGTA without Ca^{2+}.

Tris hydrochloride–50 mM NaCl buffer (pH 7.6) containing 0.05% Tween 20 and 1 mM $CaCl_2$ (TNT buffer) at 4°C. After dialysis, insoluble material was removed by centrifugation at 20,000 × *g* for 30 min at 4°C. The supernatant, which contained the AC activity (crude urea extract), was recovered and stored at −70°C.

AC enzyme assay. AC enzymatic activity of various preparations was measured by the method of Hewlett and Wolff (17) by using the neutral alumina column method of White (45). Duplicate assays were done without or with bovine CaM (Pharmacia, Inc.) at a 100 mM final concentration per assay. Samples from the alumina columns were eluted into scintillation vials containing 7 ml of H_2O, and Cerenkov radiation was monitored by using a Tracor Analytic model 689L scintillation counter (Tracor Analytic, Elk Grove Village, Ill.). Enzyme activity was given as nanomoles of cAMP formed per milligram per minute.

Binding of ^{125}I-CaM by AC. Bovine CaM was radioiodinated by using the lactoperoxidase method of Carlin et al. (3). The ability of crude and purified AC to bind ^{125}I-CaM was tested by the use of a dot blot technique by the method of Flanagan and Yost (10). Dot blots of AC were made on nitrocellulose paper (BA 85; Schleicher & Schuell), air dried, and blocked with TNT buffer. ^{125}I-CaM was added to the blots in TNT containing either 1 mM $CaCl_2$ or 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. Blots were incubated, washed, and autoradiographed.

PAGE. Cyclase preparations were subjected to polyacrylamide gel electrophoresis (PAGE) on pH 7.0 discontinuous nondenaturing 5% polyacrylamide cylindrical gels (15). Cyclase preparations were also analyzed by sodium dodecyl sulfate (SDS)-PAGE on 10 to 20% gradient gels by the method of Laemmli (22). Gels were stained with Coomassie blue to detect protein bands.

Chemical analysis. Protein concentration was measured by the Bradford method (Bio-Rad Laboratories) with bovine serum albumin used as the standard. AC (2 mg) was used for chemical analysis. Carbohydrate content was determined by converting carbohydrates to alditol acetates, and samples were analyzed by gas-liquid chromatography on a gas chromatograph (model 5700 A; Hewlett-Packard Co.) by the method of Grimes and Greegor (14). Fatty acid content was determined by the method of Fernando-Warnakulasuriya et al. (9).

Biological tests. Leukocytosis-promoting and histamine-sensitizing activities of purified AC were tested by the method of Arai and Sato (1). Mice were injected intravenously with various concentrations of affinity-purified AC. Mice were bled 3 days later for determination of leukocytosis and then were challenged intraperitoneally with histamine (1% solution given at 0.01 ml/g of body weight). Deaths were recorded from 2 to 24 h after injection. These studies were done in collaboration with James Cowell and C. Manclark, U.S. Food and Drug Administration, Pertussis Branch.

The CHO cell-clustering assay of Hewlett et al. (18) was used with modification. Serial dilutions (10-fold) of purified AC and PT were made directly in the wells of a flat-bottom microtiter plate (Becton Dickinson Labware) in F-12 medium containing 1% fetal calf serum (GIBCO Laboratories). CHO cells (10^4/ml) were added to each well in a 200-μl volume of tissue culture medium, and plates were incubated at 37°C in a CO_2 incubator for 24 h. After incubation, the medium was removed and the cells were stained with 2% Giemsa stain (18). PT was produced and purified by the method of Sekura et al. (34).

Western blot analysis. Western blot analysis was done by the method of Reiser and Stark (31) by using affinity-purified rabbit anti-PT antibody.

RESULTS

Purification of AC. All steps of the purification were conducted at 4°C unless otherwise indicated. Crude urea extract was diluted 1 to 20 with TNT buffer containing 1 mM $CaCl_2$ (10 ml of extract diluted with 190 ml of TNT buffer). The diluted crude urea extract was applied to a column (2.5 by 6.5 cm) of CaM-Sepharose 4B (Pharmacia) (CaM column) equilibrated with TNT buffer at a flow rate of 10 ml/h. The column was washed (Fig. 1) until the optical density of the eluate at 280 nm reached 0. The buffer was changed to TNT containing 10 mM EGTA without $CaCl_2$ to elute any bound proteins. A distinct protein peak was eluted with EGTA (Fig. 1), which was collected and dialyzed exhaustively against 50 mM Tris hydrochloride–50 mM NaCl–10 mM $CaCl_2$ (pH 7.6) buffer to remove the EGTA and Tween 20. After dialysis, glycerol was added to 2.5% and AC was

TABLE 1. Affinity purification of AC

Purification step	Vol (ml)	Total protein (mg)	AC activity (nmol of cAMP/mg per min)		Total activity (nmol of cAMP/min)[a]	Yield (%)[a]
			Without CaM	With 100 nM CaM		
Crude extract	200	104	0.24	57.3	5,959	100
CaM column EGTA eluted peak	23	9.2	25.0	52.6	484	8.1

[a] As related to AC activity in the presence of CaM.

stored at −70°C. A summary of the results of a typical AC purification is shown in Table 1.

In other experiments, a salt gradient from 50 mM to 200 mM NaCl in TNT buffer was used to wash the column before EGTA elution to remove nonspecifically bound proteins from the column. No proteins were found to be eluted under these conditions.

The peak eluted from the CaM column was found to have AC enzymatic activity (Table 1). The initial crude AC enzymatic activity (assays done without CaM added) was 0.24 nmol of cAMP formed per mg per min, whereas affinity-purified AC enzymatic activity was 25.0 nmol of cAMP formed per mg per min, suggesting a 104-fold purification. The affinity-purified AC was activated by CaM 2.1-fold, whereas crude AC was activated 238-fold when enzyme assays were done in the presence of CaM (100 nM CaM) (Table 1). By the parameter of enzymatic activity in the presence of CaM, 8.1% of the initial enzymatic activity was recovered from the crude extract.

Nondenaturing PAGE. Crude and affinity-purified AC were run on pH 7.0 discontinuous nondenaturing 5% polyacrylamide cylindrical gels. In the crude urea extract (Fig. 2, lane 1), numerous Coomassie blue staining protein bands were observed, whereas the affinity-purified AC migrated as a single band that just entered the gel (Fig. 2, lane 2) when as much as 50 μg of protein was analyzed.

To determine which band(s) had enzymatic activity, duplicate unstained gels were run and sliced, and fractions were macerated and eluted by diffusion into buffer. AC enzymatic activity was found associated with the high-molecular-weight band that just entered the gel in both the crude and affinity-purified AC preparations. By Sepharose CL-6B gel filtration chromatography, affinity-purified AC was found to have a molecular weight of 680,000. This high-molecular-weight peak had both enzyme and biological activity (data not shown). When acidic or alkaline polyacrylamide gels were run (15), affinity-purified AC did not enter the separating gel, but remained in the stacking gel.

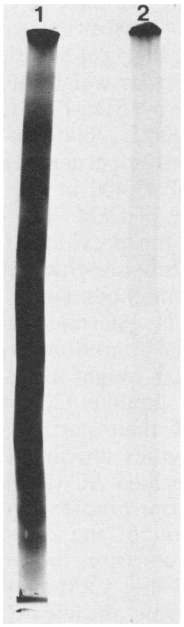

FIG. 2. Nondenaturing PAGE of AC. Crude (lane 1) and affinity-purified (lane 2) AC (50 μg) were run on pH 7.0 discontinuous nondenaturing 5% polyacrylamide cylindrical gels. Gels were stained with Coomassie blue to detect protein bands.

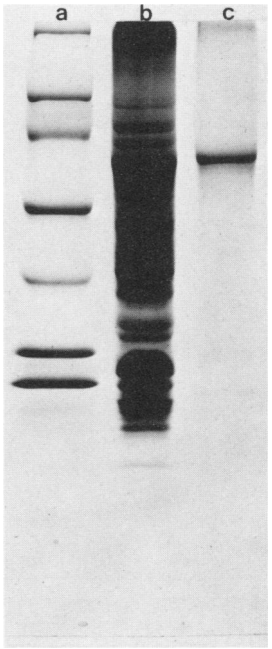

FIG. 3. SDS-PAGE of CaM affinity-purified AC. Samples were mixed 1:1 with sample buffer without 2-mercaptoethanol, boiled for 5 min, and subjected to SDS-PAGE on a 10 to 20% gradient gel. Lanes: a, molecular weight standards (from the top of the gel) of 200,000, 97,400, 68,000, 43,000, 25,700, and 18,400; b, crude AC; c, 25 μg of affinity-purified AC.

SDS-PAGE. Affinity-purified and crude AC preparations were run on a 10 to 20% SDS-PAGE gradient gel (Fig. 3). Numerous protein bands were observed in the crude extract (lane b). Affinity-purified AC was dissociated into at least two protein bands, i.e., a minor band with a molecular weight of 200,000 and a major band with a molecular weight of 60,000, respectively (lane c). The molecular weights of these bands were estimated based on a calibration curve obtained from molecular weight standards (lane a).

Binding of CaM by purified AC. Dot blots of crude cyclase, CaM column-passaged, and affinity-purified AC were made and probed with ^{125}I-CaM. It was found that all three preparations bound ^{125}I-CaM in the presence of 1 mM $CaCl_2$ (Fig. 4A) but not in the presence of 1 mM EGTA (Fig. 4B). From the autoradiograph it can be observed that affinity-purified AC bound more ^{125}I-CaM than did the crude cyclase or column-passaged AC, suggesting significant purification of AC by this parameter.

Chemical analysis. Affinity-purified AC was assayed for carbohydrate and fatty acid content. No detectable levels of carbohydrate were observed at a limit of less than 0.1%. Only trace levels of lipid were detected at 1 to 2%, with detection limits of less than 0.1%.

Biological tests. Studies were performed to determine the effect of affinity-purified AC on mice. Affinity-purified AC was injected intravenously into mice to monitor leukocytosis-promoting and histamine-sensitizing activities, tests used routinely to monitor for the presence of PT (1, 8). It was observed that 50 μg of purified AC caused no overt mouse toxicity and did not induce leukocytosis in 10 mice tested. In contrast, as little as 20 ng of PT induced leukocytosis. This dose of affinity-purified AC induced some histamine sensi-

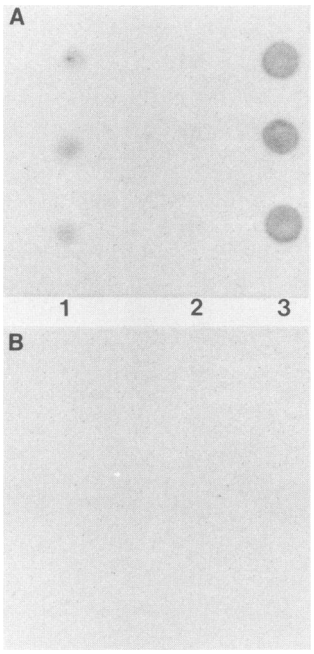

FIG. 4. Binding of ^{125}I-CaM to crude and affinity-purified AC. Dot blots (5 μg per dot blot) of crude AC (lane 1), CaM column-passaged AC (lane 2), and affinity-purified AC (lane 3) were made, dried, blocked, and probed with ^{125}I-CaM in the presence of $CaCl_2$ (A) or EGTA (B). Dot blots were washed and autoradiographed.

tivity, killing 4 of 10 mice. From control tests done with purified PT, 1 to 2 ng of PT caused similar results.

Affinity-purified AC was also tested for the presence of PT by using the CHO cell-clustering assay of Hewlett et al. (18) (three different preparations were tested). This is a highly sensitive in vitro assay that detects PT at picogram levels. Affinity-purified AC and PT were tested at concentrations of 10 μg/ml to 10 pg/ml. Affinity-purified AC did not cause CHO cell clustering or any toxic effects at any of the concentrations tested, whereas PT caused CHO cell clustering down to a 100-pg/ml concentration. Western blot analysis of 50 μg of affinity-purified AC was done by using affinity-purified rabbit anti-PT antibody. No PT was detected in the AC preparations by this assay (data not shown), which can detect PT down to 30 pg/ml.

DISCUSSION

The procaryote enzyme AC of *B. pertussis* has the unusual property of being stimulated by CaM, a eucaryotic regulatory protein. This activity was first described by Wolff et al. (46). More recently, Leppla (23) reported that the edema factor toxin of *Bacillus anthracis* is also an AC which is stimulated by CaM.

It was hypothesized that since CaM activates and binds to crude preparations of *B. pertussis* AC (Fig. 4), one could use CaM as an affinity ligand to isolate and purify the AC. CaM affinity chromatography has been previously used to purify various CaM-modulated enzymes and other CaM-binding proteins (35, 36, 44). This is the first reported attempt to isolate and purify *B. pertussis* AC by this method. It was also hoped that by this method an enzymatically and biologically active AC could be recovered whereas previously only enzymatic activity was recovered (17).

B. pertussis AC was significantly purified (104-fold) by the one-step CaM affinity chromatography technique described in this report (Fig. 1 and Table 1). The addition of the detergent Tween 20 (at 0.05%) was found to be critical for the binding of AC to the affinity column (data not shown). Flanagan and Yost (10) observed that Tween 20 enhances CaM binding to proteins bound to nitrocellulose paper and decreases nonspecific protein interactions. The Tween 20 may allow the AC to be in a correct or optimal conformation to bind to CaM on the column.

B. pertussis AC was eluted from the CaM column by buffer containing 10 mM EGTA, which chelates Ca^{2+}. These results and the dot blot binding of ^{125}I-CaM only in the presence of Ca^{2+} (Fig. 4) suggest that AC binding to the CaM column requires Ca^{2+} at least under the described conditions. Others have reported that *B. pertussis* AC activation by CaM is calcium independent (13, 21). These results suggests that CaM binding and activation of AC may occur at different sites on the AC molecule or by different mechanisms.

The results of PAGE suggest that under the conditions used for affinity purification, the AC in its native form is a high-molecular-weight molecule (Fig. 2). By SDS-PAGE, affinity-purified AC was separated into a minor band of 200,000 molecular weight and a major protein band at 60,000 molecular weight (Fig. 3). These results together with the PAGE and Sepharose CL-6B chromatography data suggest that (i) affinity-purified AC is a high-molecular-weight protein that is composed of subunits or (ii) that AC may be forming aggregates with itself. Kessin and Franke (20) recently reported the partial purification of AC from culture supernatant and found a high-molecular-weight species of 700,000 and a low-molecular-weight species of 60,000 to 70,000. Both the high- and low-molecular-weight fractions have enzyme activity, but no studies were reported for biological activity (20). They also observed that the low-molecular-weight species is less stimulated by CaM, whereas the high-molecular-weight species is highly stimulated by CaM (20). Hanski and Farfel (16) using crude urea extracts reported that the invasive AC has a high molecular weight of 190,000 in the presence of Ca^{2+} and in its absence the MW increases to 340,000 as determined by Ultrogel AcA 34 (LKB Instruments, Inc.) gel filtration. Hewlett and Wolff (17) reported the molecular weight of *B. pertussis* AC to be 70,000 as determined by SDS-PAGE and sucrose density gradient methods. Recently, Shattuck et al. (37) isolated AC from *B. pertussis* culture supernatant and reported it to have a molecular weight of 43,400 in the absence of CaM and 54,200 in the presence of CaM. The difference in reported molecular weights in the previous studies may be due to isolating AC from culture supernatant or crude extracts by different biochemical methods (16, 17, 20, 37). Before release from the bacteria, AC may be in a high-molecular-weight form and after secretion it may be degraded to various lower-molecular-weight forms. Both Shattuck et al. (37) and Kessin and Franke (20) reported high specific enzymatic activity of their partially purified AC. These reported specific activities are much higher than that observed with affinity-purified AC (Table 1). The low specific enzymatic activity of our preparation may be due to denaturation or inactivation of the enzymatic activity during purification or to effects from the presence of Tween 20. Another possibility is that CaM may have been liberated from the affinity column during AC elution, binding to purified AC and maximally activating it. Affinity-purified AC was screened for the presence of CaM after column elution by dot blot assays with anti-CaM antiserum (Calbiochem-Behring), and none was detected (data not shown). Although

the specific enzymatic activity was low, affinity-purified AC was found to have biological activity (11). Affinity-purified AC induced increases in PMN intracellular cAMP levels and inhibited various PMN functions, including chemiluminescence, chemotaxis, and superoxide generation (11). What effects the partially purified AC preparations of Kessin and Franke (20) and Shattuck et al. (37) have on human PMN have not been reported.

Affinity-purified AC was tested in various biological assays for the presence of PT contamination. The AC preparation was found to have no leukocytosis-promoting activity, to be negative by the CHO cell-clustering assay (18), and to be negative by Western blot analysis of 50 μg of AC with affinity-purified anti-PT antibody (data not shown). Yet affinity purified AC was found to induce some histamine sensitivity in mice. Further detailed studies need to be done to confirm these observations.

In summary, *B. pertussis* AC was isolated from crude urea extracts by CaM affinity chromatography. Affinity-purified AC was found to have enzyme activity which was activated by CaM and was not contaminated by PT. Affinity-purified AC was also found to have biological effects on human PMN. These studies will be presented in another paper (11).

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