Purification and Characterization of a Calmodulin-Sensitive Adenylate Cyclase from *Bordetella pertussis*†

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ABSTRACT: *Bordetella pertussis*, the bacterium responsible for whooping cough, releases a soluble, calmodulin-sensitive adenylate cyclase into its culture medium. *B. pertussis* mutants deficient in this enzyme are avirulent, indicating that the adenylate cyclase contributes to the pathogenesis of the disease. It has been proposed that *B. pertussis* adenylate cyclase may enter animal cells and increase intracellular adenosine cyclic 3′,5′-phosphate (cAMP) levels. We have purified the enzyme extensively from culture medium using anion-exchange chromatography in the presence and absence of calmodulin and gel filtration chromatography. The enzyme was purified 1600-fold to a specific activity of 608 μmol of cAMP min^{-1} mg^{-1} and was free of islet activating protein. The molecular weight of the enzyme was 43 400 in the absence of calmodulin and 54 200 in the presence of calmodulin. The K_m of the bacterial enzyme for adenosine 5′-triphosphate was 2.0 mM, whereas the K_m of the calmodulin-sensitive adenylate cyclase from bovine brain was 0.07 mM. Although the enzyme was not purified to homogeneity, its turnover number of 27 000 min^{-1} is the highest documented for any adenylate cyclase preparation.

Bordetella pertussis is a small, Gram-negative bacillus that is the pathogen responsible for whooping cough (Olson, 1975; Jawetz et al., 1978). Although the molecular basis for *B. pertussis* pathology is undefined, culture medium of growing *B. pertussis* contains a number of biologically active components which are thought to play a role in the pathogenesis. One of the these, islet activating protein (IAP),[1] has been purified from the culture medium of the bacteria (Yajima et al., 1978a,b; Sekura et al., 1983). IAP attenuates receptor-mediated inhibition of adenylate cyclase in a variety of mammalian cell types by catalyzing the ADP-ribosylation of the inhibitory guanyl nucleotide component of the adenylate cyclase system (Katada et al., 1981, 1982a,b).

Adenylate cyclase activity also has been detected in the culture medium of *B. pertussis* (Hewlett et al., 1976; Hewlett & Wolff, 1976), and this enzyme is stimulated by calmodulin (CaM) even though the bacteria do not contain CaM (Wolff et al., 1980). Recently, it has been determined that *B. pertussis* mutants deficient in adenylate cyclase are avirulent (Weiss et al., 1983, 1984). Furthermore, Leppla (1982) has discovered that the *Bacillus anthracis* edema factor is also a CaM-stimulated adenylate cyclase that apparently enters animal cells and elevates intracellular cAMP levels. These observations have stimulated interest in the mechanisms for CaM stimulation of *B. pertussis* adenylate cyclase and cellular entry of the enzyme. Both of these objectives require purification of the enzyme with removal of contaminating IAP. In this study, we describe purification of *B. pertussis* adenylate cyclase to a specific activity of 608 μM cAMP min^{-1} mg^{-1} with removal of IAP. This preparation allows determination of some of the important properties of the enzyme.

Materials and Methods

Materials

QAE-Sephadex and cyanogen bromide activated Sepharose 4B were purchased from Pharmacia. Ultrogel AcA 44 was obtained from Bio-Rad. Fetuin, ATP, cAMP, and protein standards for native molecular weight determination were from Sigma. [^{32}P]ATP and [^{32}P]NAD were purchased from New England Nuclear, and [^{3}H]cAMP was purchased from International Chemical Nuclear. High and low molecular weight standards for SDS gel electrophoresis were from Bio-Rad. All other reagents were of the finest available grade from commercial sources.

Methods

Adenylate Cyclase Assay. Adenylate cyclase was assayed at 30 °C by the general method of Salomon et al. (1974), using [^{32}P]ATP as a substrate and [^{3}H]cAMP to monitor product recovery. Assays contained in a final volume of 250 μL 20 mM Tris-HCl, pH 7.5, 1 mM [^{32}P]ATP (10 cpm/pmol), 5 mM $MgCl_2$, and 0.1% bovine serum albumin. The concentrations of ATP were varied from 0.01 to 10 mM for the determination of ATP K_m values. CaM (2.4 μM) was included in some assays as indicated. Adenylate cyclase assays did not contain phosphodiesterase inhibitors since there was no phosphodiesterase activity in the preparations (data not shown). All results are presented as the mean of triplicate assays with standard errors of less than 5%. Protein concentrations were determined by the method of Peterson (1977).

Preparation of CaM. CaM was prepared from bovine brain by the procedure of Dedman et al. (1977) as modified by Olwin et al. (1985).

Fetuin–Sepharose. The fetuin affinity resin was prepared by coupling 200 mg of fetuin with 25 g of cyanogen bromide activated Sepharose 4B according to the manufacturer's recommended procedure.

Partial Purification of the CaM-Sensitive Adenylate Cyclase from Bordetella pertussis. B. pertussis (Tohama phase

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[1] Abbreviations: CaM, calmodulin; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; IAP, islet activating protein; cAMP, adenosine cyclic 3′,5′-phosphate; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; NAD, nicotinamide adenine dinucleotide; QAE, diethyl(2-hydroxypropyl)aminoethyl; BSA, bovine serum albumin.

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Table I: Purification of Calmodulin-Activated Adenylate Cyclase from *Bordetella pertussis*

purification step[a]	total act. ($\times 10^4$ nmol/min)	% yield of act.	total protein (mg)	sp act. (μmol min^{-1} mg^{-1})	x-fold purification
bacteria + culture media[b]	318	100	8.4×10^3	0.38	
culture media	202	63.5	8.3×10^2	2.4	6.3
QAE-Sephadex peak I	19.1	5.9	8.5	22.8	60.0
calmodulin shift[c]	7.9	2.5			
fetuin–Sepharose[c]	6.4	2.0			
Ultrogel AcA 44	2.4	0.75	6.6×10^{-2}	360.6	948.9
peak fraction, Ultrogel AcA 44	0.608	0.2	9.9×10^{-3}	608.1	1600.3

[a] Adenylate cyclase was assayed in the presence of 5 mM $MgCl_2$ and 2.4 μM calmodulin. [b] Cultures were assayed prior to centrifugation. [c] Protein was not assayed due to contaminating free CaM.

I strain) was grown from a 5% inoculum in supplemented Stainer–Scholte medium (Stainer & Scholte, 1971) at 35.5 °C with shaking until $OD_{650} = 0.5$. Bacterial suspensions were centrifuged in a Beckman J6-3B for 1 h at 48 000 rpm, and the culture medium containing adenylate cyclase activity was removed. Five hundred milliliters of QAE-Sephadex, equilibrated in 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 2 mM $MgCl_2$, was added to 18 L of *B. pertussis* culture medium and stirred for 1 h. The resin was washed on a sintered glass funnel with 3 L of 20 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 2 mM $MgCl_2$, poured into a 5 × 20 cm column, and eluted with a linear 40 mM–1 M NaCl gradient in 20 mM Tris-HCl (pH 7.5) and 2 mM $MgCl_2$. The enzyme was pooled on the basis of adenylate cyclase activity in two separate pools, peak I and peak II (Figure 1). Peak I (or peak II, as indicated) was diluted with 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 2 mM $MgCl_2$ to a conductivity of 4.0 mΩ^{-1}. $CaCl_2$ and CaM were added to concentrations of 0.2 mM and 1 μM, respectively, and the diluted enzyme was loaded onto a 2.5 × 15 cm QAE-Sephadex column. The resin was washed with 200 mL of 20 mM Tris (pH 7.5), 40 mM NaCl, and 2 mM $MgCl_2$, and eluted with a linear 40 mM–1.2 M NaCl gradient in 20 mM Tris-HCl (pH 7.5) and 2 mM $MgCl_2$. The elution position of peak I adenylate cyclase activity shifted to higher salt concentrations when the enzyme was rerun on QAE-Sephadex in the presence of CaM. This preparation was pooled on the basis of adenylate cyclase activity and stored at −80 °C.

For further purification, five CaM-shifted peak I pools were applied to a 10-mL fetuin–Sepharose column (1.5 × 5 cm), preequilibrated in 20 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 2 mM $MgCl_2$. The protein that flowed through the fetuin–Sepharose column contained the majority of adenylate cyclase activity, and it was concentrated 25-fold by Amicon ultrafiltration using a PM-10 membrane. Four milliliters of concentrated material was applied to an Ultrogel AcA 44 column (1.75 × 90 cm) equilibrated in 20 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 2 mM $MgCl_2$. Fractions were assayed for adenylate cyclase activity and protein. All steps of the purification were carried out at 4 °C.

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) and stained with Coomassie Brilliant Blue or silver stained by the method of Garoff & Ansorge (1981).

Detection of Contaminating Islet Activating Protein. The presence of IAP was detected by the ability of this protein to catalyze the ADP-ribosylation of GTP binding proteins in cerebral cortex membranes (Neer et al., 1984; Sternweis & Robishaw, 1984). Samples of IAP, used as a standard, and CaM-shifted peak I, which had been run through a fetuin–Sepharose column, were incubated with 20 mM dithiothreitol for 30 min at 30 °C. Bovine brain membranes prepared by the method of Andreasen et al. (1983) were then incubated for 1 h at 30 °C with the IAP sample in a buffer containing 10 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP, 10 μM NAD, 40 mM Tris-HCl (pH 8), and 20 μCi of [^{32}P]NAD. The reaction was stopped by the addition of cold 100 mM Tris-HCl (pH 8). Membranes were washed, applied to a 10% polyacrylamide slab gel in SDS, and autoradiographed to detect labeling of IAP substrates.

Sucrose Density Gradient Centrifugation. Adenylate cyclase preparations were subjected to centrifugation through a 5–20% sucrose gradient in 20 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 2 mM $MgCl_2$ for 7 h at 50 000 rpm in a Beckman VTi 50 rotor at 0 °C. Protein standards included alkaline phosphatase ($s_{20,w} = 6.3$ S) and bovine cytochrome *c* ($s_{20,w} = 1.8$ S). Alkaline phosphatase was assayed by the method of Schlesinger & Barret (1965). Linearity of gradients was confirmed by refractometry.

Determination of Stokes Radius. The Stokes radius of peak I or peak II adenylate cyclase was determined by Ultrogel AcA 44 chromatography. Cytochrome *c* ($R_s = 17.4$ Å), ovalbumin ($R_s = 27.6$ Å), and BSA ($R_s = 37.0$ Å) were used as standards. Ferritin was used as the void volume marker.

RESULTS

Purification of CaM-Activated Adenylate Cyclase from B. pertussis. Preliminary studies indicated that the levels of adenylate cyclase activity in the culture medium from *B. pertussis* (Tohama phase I) reach a maximum when the bacteria grow to an OD_{650} of 0.4–0.6. Therefore, *B. pertussis* was grown from a 5% inoculum in supplemented Stainer–Scholte medium (Stainer & Scholte, 1971) to an OD_{650} of 0.5 and used as starting material for purification of the CaM-sensitive adenylate cyclase. Since the adenylate cyclase is released by the bacteria into the culture medium, removal of the cells by centrifugation resulted in approximately a 6-fold increase in the specific activity of adenylate cyclase (Table I). Application of the soluble CaM-sensitive adenylate cyclase to QAE-Sephadex and elution with a linear 40 mM–1 M NaCl gradient resulted in two peaks of adenylate cyclase activity, peak I and peak II (Figure 1). Peak I typically eluted at a conductivity of 7.0 mΩ^{-1} and peak II eluted at a higher conductivity, 11.0 mΩ^{-1}. For the profile shown in Figure 1, peak I had a specific activity of 54.7 μmol min^{-1} mg^{-1} whereas peak II had a higher specific activity of 216.7 μmol min^{-1} mg^{-1}. Both peaks of adenylate cyclase activity were stimulated 10–30-fold by CaM, with the degree of CaM stimulation varying from one preparation to another. The percent yields of enzyme activity for peak I and peak II were 9.4% and 24.2%, respectively. The ratio of adenylate cyclase activity in peak I to peak II was typically 1:2.5. The behavior of the enzyme on QAE-Sephadex was highly reproducible, and the elution profile reported in Figure 1 was repeated in 75 different preparations. When adenylate cyclase, from either peak I or peak II, was diluted to lower the NaCl concentration and

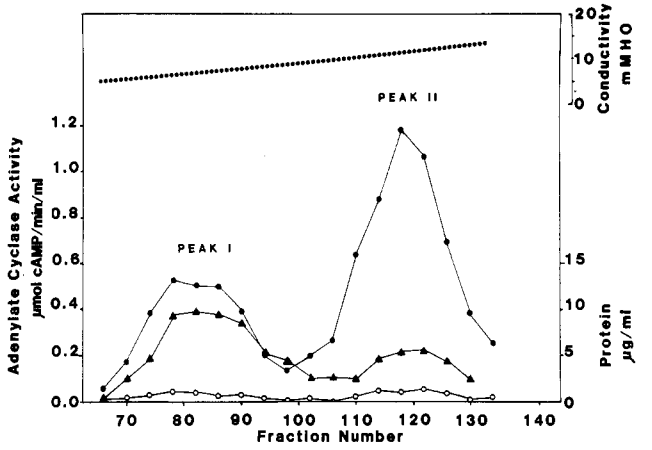

FIGURE 1: QAE-Sephadex chromatography of *Bordetella pertussis* culture medium. Culture medium from *B. pertussis* was applied to QAE-Sephadex as described under Methods. The adenylate cyclase was eluted with a 40 mM–1 M NaCl gradient in 20 mM Tris-HCl (pH 7.5) and 2 mM $MgCl_2$. Fractions (10 mL) were collected and assayed for adenylate cyclase activity in the presence (●) and absence (○) of CaM and assayed for protein (▲). The conductivity of each fraction was also measured (top curve consisting of only closed circles).

reapplied to QAE-Sephadex, only one peak of activity was eluted, indicating that the two forms are not interconvertible.

Since both peak I and peak II adenylate cyclase activities were CaM sensitive, the influence of added CaM on their elution positions from QAE-Sephadex was examined. It was anticipated that complex formation between CaM and the enzyme might change the elution position of the enzyme upon reapplication to QAE-Sephadex, yielding further purification of the enzyme. CaM is a relatively acidic protein with an isoelectric point of 3.9 (Keller et al., 1982). The addition of CaM to peak II adenylate cyclase activity, however, did not change the elution position of the enzyme when reapplied to QAE-Sephadex (Figure 2A). In contrast, the elution position of peak I adenylate cyclase was significantly shifted upon the addition of CaM (Figure 2B). Peak I adenylate cyclase, which originally eluted at a conductivity of 7.0 $m\Omega^{-1}$, was eluted at 12.5 $m\Omega^{-1}$ when CaM was added to the enzyme, and it was rerun on QAE-Sephadex. Typically, all of the activity absorbed to the resin, and 60–70% of the activity was recovered upon elution. A shift of this magnitude should separate the CaM-sensitive adenylate cyclase from the main peak of protein. However, free calmodulin eluted at approximately the same conductivity. As a result, the specific activity of the CaM-shifted peak I adenylate cyclase could not be determined due to contaminating levels of free CaM. Contaminating CaM was subsequently removed at a later stage in the purification by using gel filtration.

To ensure complete removal of IAP from the preparation, the CaM-shifted peak I pool was applied to a fetuin–Sepharose column. Fetuin is a serum sialoglycoprotein which binds IAP and has been used in its purification (Sekura et al., 1983). Adenylate cyclase activity did not absorb to fetuin–Sepharose. As shown in Table I, when CaM-shifted peak I adenylate cyclase was applied to fetuin–Sepharose, 81% of the adenylate cyclase activity flowed through the column. To determine if IAP had actually been removed from the preparation, bovine brain membranes were labeled with various sources of IAP and [^{32}P]NAD (Figure 3). When either pure IAP or concentrated culture medium containing IAP was incubated with [^{32}P]NAD and bovine brain membranes, two labeled polypeptides with molecular weights of 39 000 and 41 000 were readily detected on SDS gels. The partially purified adenylate cyclase preparation that had been submitted to fetuin–Se-

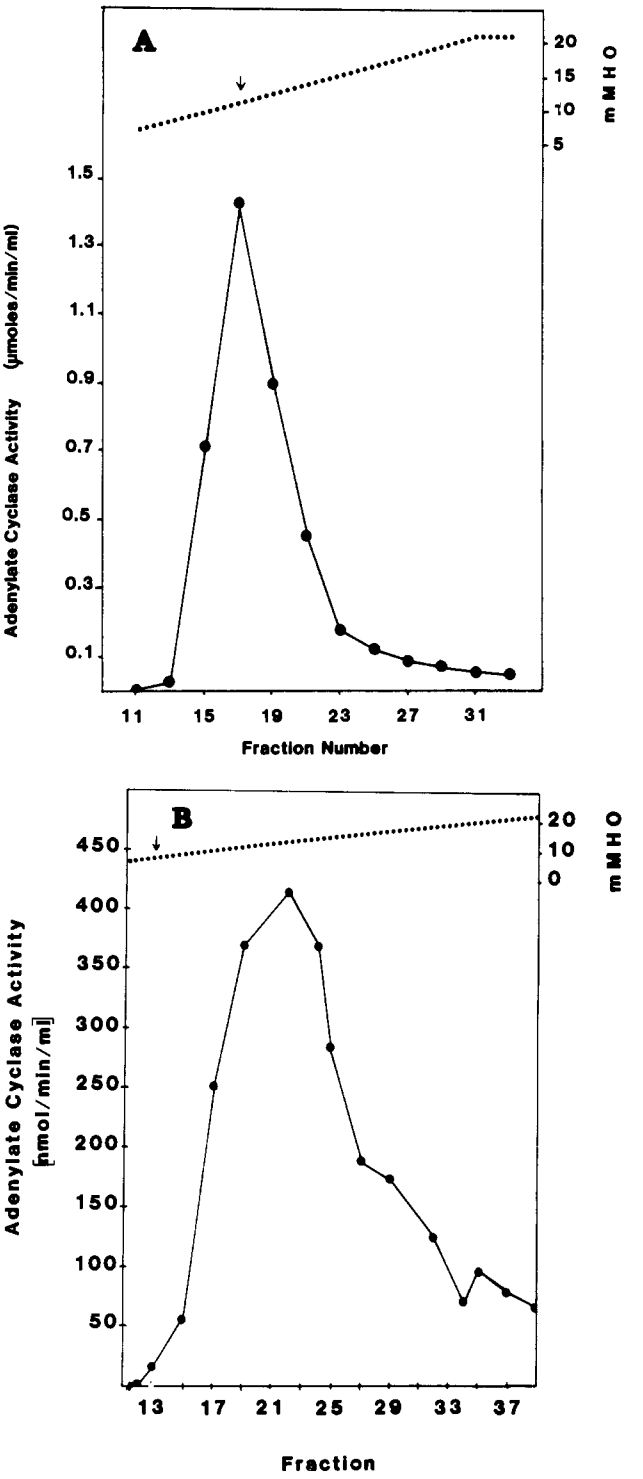

FIGURE 2: (A) Reapplication of peak II with CaM added to QAE-Sephadex. Peak II, pooled from QAE-Sephadex, was diluted to 4.0 $m\Omega^{-1}$ with 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 2 mM $MgCl_2$. $CaCl_2$ and CaM were added to final concentrations of 0.2 mM and 1 μM, respectively. This material was applied to a column of QAE-Sephadex. The column (2.5 × 15 cm) was washed with 20 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 2 mM $MgCl_2$ and eluted with a 40 mM–1.2 M NaCl gradient in 20 mM Tris-HCl (pH 7.5) and 2 mM $MgCl_2$. Fractions (4 mL) were assayed for adenylate cyclase activity (●), and conductivity was measured (top curve consisting only of closed circles). The arrow (↓) indicates the conductivity at which peak II was eluted in the absence of CaM. (B) Reapplication of peak I with CaM to QAE-Sephadex. Peak I, pooled from QAE-Sephadex, was reapplied to QAE-Sephadex and eluted as described in (A). Fractions (4 mL) were assayed for adenylate cyclase activity (●), and conductivity was measured (top curve consisting only of closed circles). The arrow (↓) indicates the conductivity at which peak I was originally eluted in the absence of CaM.

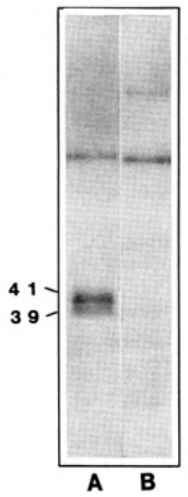

FIGURE 3: IAP labeling of bovine brain membranes. Bovine brain membranes were labeled with either 10 μg of concentrated culture medium from *B. pertussis* (A) or 10 μg of CaM-shifted peak I adenylate cyclase which had been run over a fetuin–Sepharose column (B) and [^{32}P]NAD (20 μCi/sample) in the presence of 10 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP, 10 μM NAD, and 40 mM Tris-HCl (pH 8). Labeling was done at 30 °C for 1 h. The reaction was stopped by the addition of cold 100 mM Tris-HCl (pH 8). Membranes were washed and applied to a 10% polyacrylamide slab gel in SDS, and labeled polypeptides were identified by autoradiography of the dried gel.

pharose chromatography showed no IAP activity detectable by this assay.

In the final purification step, an Ultrogel AcA 44 gel filtration column was used to separate excess CaM from adenylate cyclase (Figure 4). Use of this resin resulted in separation of adenylate cyclase activity (fractions 34–40) from the peak of free CaM (fractions 43–48), which was one of the major sources of contaminating protein at this stage of the preparation. The adenylate cyclase obtained from this column was no longer sensitive to CaM, presumably because of CaM strongly associated with the enzyme. SDS gel electrophoresis of the peak fractions containing adenylate cyclase activity revealed a number of polypeptides with two major bands, which have molecular weights of 64 000 and 45 000. The latter polypeptide is of particular interest since hydrodynamic data, presented in a later section, indicated that the native molecular weight of the enzyme is approximately 45 000 (Figure 4B). CaM was detectable in peak fractions of adenylate cyclase activity (e.g., fractions 36 and 38); however, stoichiometry between CaM and the polypeptide with a molecular weight of 45 000 was not evident on silver-stained gels. Coomassie blue stained gels of pooled adenylate cyclase activity from this column showed comparable staining of CaM and the band with a molecular weight of 45 000 (data not shown).

This purification protocol resulted in a 1600-fold purification of adenylate cyclase activity in the peak fraction, or 1000-fold purification of pooled activity (Table I). The specific activity of the most highly purified enzyme was 608 μmol of cAMP min^{-1} mg^{-1}, higher than any previously reported preparation of adenylate cyclase. The yield of total activity relative to starting activity was low; however, peak I adenylate cyclase activity comprised only a small fraction of the total starting activity.

Molecular Weights of Peak I and Peak II Adenylate Cyclases. The molecular weights of adenylate cyclases were obtained in the presence and absence of CaM by determination of sedimentation coefficients and Stokes radii. Peak I adenylate cyclase plus CaM migrated with an $s_{20,w}$ of 4.17 S and a Stokes radius of 31 Å (Figures 5 and 6). Assuming a partial

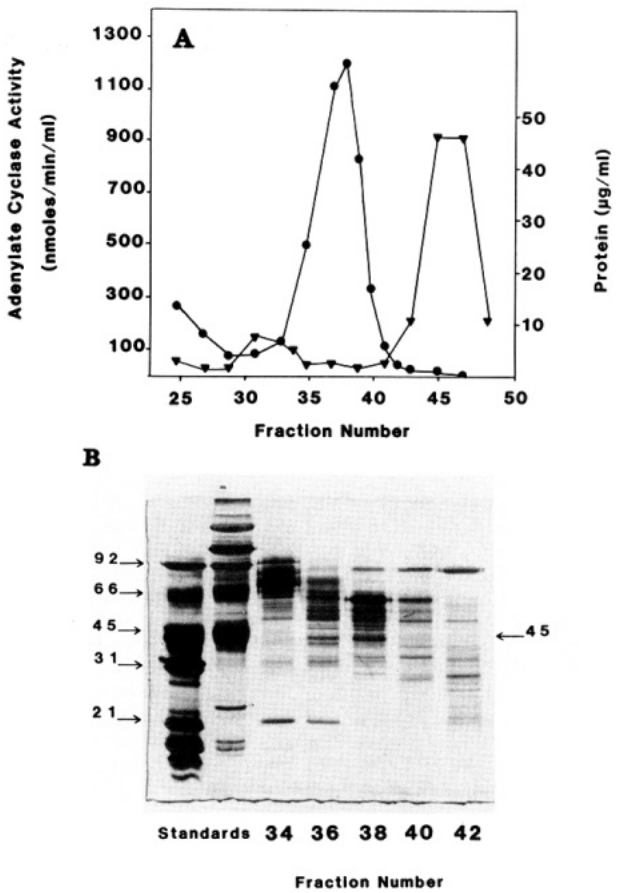

FIGURE 4: (A) Ultrogel AcA 44 chromatography. Five CaM-shifted peak I pools from QAE-Sephadex were applied to a 10-mL fetuin–Sepharose column. The adenylate cyclase activity that flowed through the column was then concentrated by ultrafiltration using an Amicon PM-10 membrane. Four milliliters of concentrated adenylate cyclase was applied to an Ultrogel AcA 44 column (1.75 × 90 cm) equilibrated in 20 mM Tris-HCl, pH 7.5, 40 mM NaCl, and 2 mM $MgCl_2$. Fractions (4.0 mL) were collected and assayed for adenylate cyclase activity (●) and for protein (▼). (B) SDS gel electrophoresis of the Ultrogel AcA 44 elution profile. Samples (1 mL) from the Ultrogel AcA 44 elution profile were precipitated with 72% trichloroacetic acid at 4 °C. The precipitates were pelleted by microcentrifugation. The pellets were then washed 2 times with 1 mL of cold acetone and recentrifuged. The acetone was removed, and the pellets were dissolved in 50 μL of Laemmli sample buffer. The samples were subjected to SDS–polyacrylamide gel electrophoresis on a 12.5%, 0.75-mm-thick slab gel. The gel was then silver stained.

Table II: Determination of Molecular Weights of Peak I and Peak II Adenylate Cyclases

sample[a]	R_s (Å)[b]	$s_{20,w}$[c] (S)	M_r[d]
peak I	32.5	3.25	44 300
peak I + CaM	31.0	4.17	54 200
peak II	30.5	3.52	45 100
peak II + CaM	30.2	4.29	54 500

[a] Adenylate cyclases were prepared as described under Methods. [b] Stokes radii, R_s, were determined by Ultrogel AcA 44 chromatography as described under Methods. [c] Sedimentation coefficients, $s_{20,w}$, were determined by sucrose density gradient centrifugation as described under Methods. [d] Molecular weights, M_r, were calculated from Stokes radii and sedimentation coefficients by assuming a partial specific volume of 0.725. All results are presented as the mean of at least two separate determinations with standard deviations less than 5%.

specific volume of 0.725, the molecular weight of peak I adenylate cyclase plus CaM was determined to be 54 200 (Table II). The molecular weight of peak I adenylate cyclase in the absence of CaM was 44 300 (Table II), which compares favorably with one of the major silver-stained bands in the most highly purified preparation (Figure 4). Also reported in Table

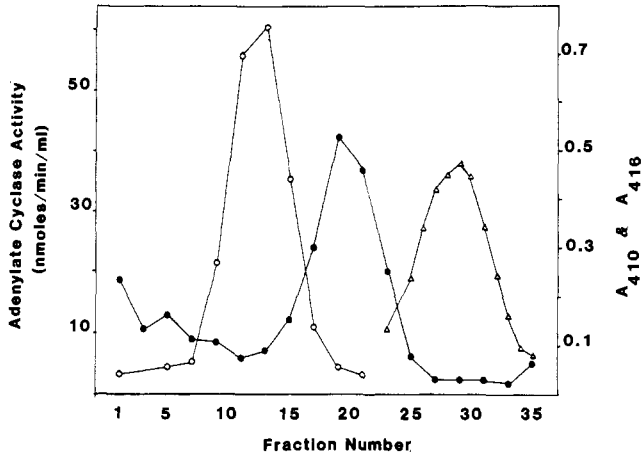

FIGURE 5: Sucrose density gradient centrifugation of peak I adenylate cyclase from QAE-Sephadex in the presence of CaM. A 1-mL sample of peak I adenylate cyclase with 2 μM added CaM was subjected to centrifugation through a 5–20% sucrose gradient in H_2O-based buffer for 9 h at 50 000 rpm at 0 °C, using a VTi 50 rotor. The sample included bovine cytochrome *c* ($s_{20,w}$ = 1.8 S) and alkaline phosphatase ($s_{20,w}$ = 6.3 S) as internal standards. Sample and gradient buffer contained 20 mM Tris, pH 7.5, 20 mM NaCl, and 2 mM $MgCl_2$. Gradient buffers were 5% and 20% in sucrose prior to gradient preparation. After centrifugation, 1-mL fractions were collected and measured for adenylate cyclase activity (●) and alkaline phosphatase activity (○) as described under Methods. Cytochrome *c* was measured by the absorbance at 416 nm (△). Sucrose density was determined for each fraction by refractometry.

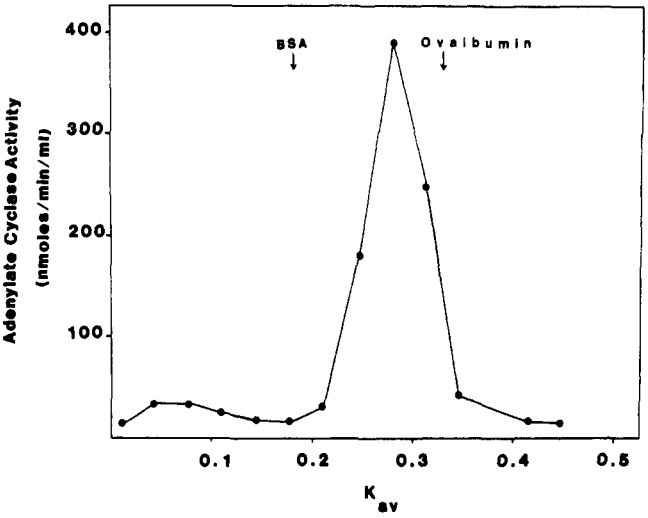

FIGURE 6: Determination of the Stokes radius of peak I adenylate cyclase in the presence of CaM. A 2.5-mL sample of pooled peak I adenylate cyclase was applied to an Ultrogel AcA 44 column in the presence of 0.2 mM $CaCl_2$ and 1 μM CaM. The column had been preequilibrated with 20 mM Tris-HCl, pH 7.5, 40 mM NaCl, and 2 mM $MgCl_2$. The column was calibrated by using bovine cytochrome *c* (R_s = 17.4 Å), ovalbumin (R_s = 27.6 Å), and BSA (R_s = 37.0 Å), and ferritin was used to determine the void volume.

II are the molecular weights for peak II adenylate cyclase in both the presence and absence of CaM. Peak I adenylate cyclase was not significantly different in size from peak II adenylate cyclase with molecular weights of 44 300 and 45 100, respectively. Furthermore, the addition of CaM to either enzyme increased its molecular weight by approximately 10 000. This increment in molecular weight is somewhat lower than would be expected for the binding of one CaM to a polypeptide with a molecular weight of 45 000. The molecular weight of CaM is 16 700 (Watterson et al., 1980). However, considering the inherent error associated with these molecular weight determinations, it is concluded that both forms of

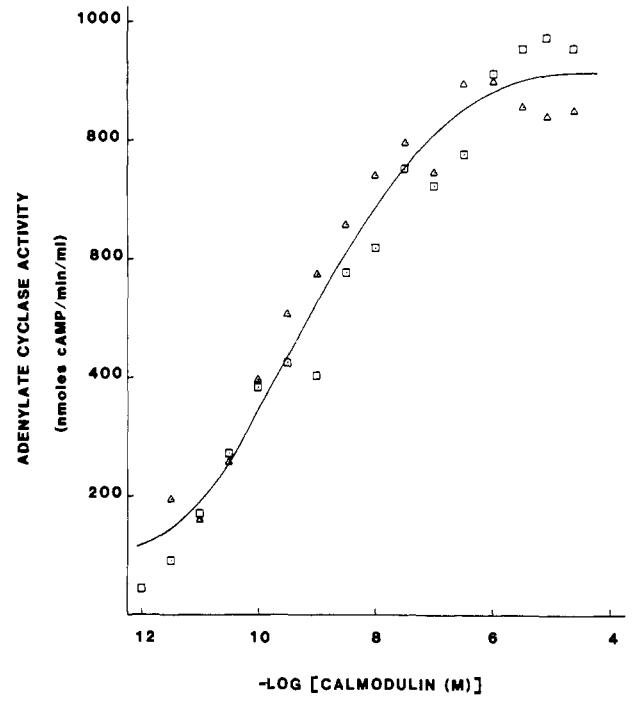

FIGURE 7: CaM activation of peak I and peak II adenylate cyclase. Adenylate cyclase activity from peak I (△) and peak II (□) was assayed at varying concentrations of CaM in the presence of 0.1 mg/mL BSA, 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, and 2 mM $MgCl_2$.

Table III: Determination of the K_m of ATP for Peak I and Peak II Adenylate Cyclases

adenylate cyclase	K_m (mM)[a]	correlation coefficient[b]
peak I	1.2 ± 0.2	0.92
peak I + CaM	2.0 ± 0.2	0.99
peak II	3.0 ± 0.3	0.99
peak II + CaM	1.9 ± 0.2	0.99

[a] Adenylate cyclase from either pooled peak I or pooled peak II was assayed as a function of ATP concentration, as described under Materials and Methods, with a $MgCl_2$ concentration of 10 mM. When present, [CaM] was 2.4 μM. [b] Determined by linear regression analysis of Lineweaver–Burk plots.

adenylate cyclase have a native molecular weight of about 45 000 and bind one CaM. This suggests that CaM interacts directly with the catalytic subunit of this enzyme as is the case with other CaM-regulated enzymes (Andreasen et al., 1981).

CaM Dose–Response Curves for Adenylate Cyclase Stimulation. Both forms of adenylate cyclase isolated by QAE-Sephadex were stimulated by CaM, and the dose–response curves were indistinguishable (Figure 7). CaM stimulated enzyme activity over a concentration range of 6 log units, indicating that interactions between CaM and the enzymes are complex and cannot be fit by a simple, single-site binding isotherm. Similar CaM dose–response curves, showing stimulation over a very broad range of CaM concentration, have been seen with the membrane-bound CaM-sensitive adenylate cyclase from *B. pertussis* (Goldhammer & Wolff, 1982). Since our hydrodynamic data indicated that both enzymes probably exist as simple monomers binding one CaM, the activation curves suggest heterogeneity of CaM binding sites rather than cooperativity between different CaM binding sites. Nevertheless, the two forms of adenylate cyclase isolated by QAE-Sephadex have identical sensitivities to CaM.

ATP Concentration Dependence. The K_m's of peak I and peak II adenylate cyclase for ATP were also determined (Table III). Both enzymes were assayed with increasing concen-

trations of ATP up to 10 mM in the presence and absence of CaM. Saturation of the enzyme was not reached until the ATP concentration was 6 mM or higher. It is apparent that the K_m of ATP was not significantly different for the two forms of the enzyme. The K_m for peak I in the presence of CaM was 2.0 mM, and that for peak II was 1.9 mM (Table III). The addition of CaM to either enzyme did not alter the K_m for ATP greatly, and in neither case was the K_m lowered. Therefore, CaM stimulation must be due to an increase in the turnover number of the enzymes. It is interesting that the K_m for ATP for both forms of the *B. pertussis* enzyme was very high relative to that determined for the CaM-sensitive adenylate cyclase from mammalian brain which is 0.07 mM (Keller et al., 1980).

Discussion

In this report, we describe a procedure which provides a significant purification of the *B. pertussis* adenylate cyclase and removes IAP as a contaminating activity. The purification is based on two novel properties of the enzyme: it is released extracellularly into the culture medium free from many contaminating bacterial proteins, and its affinity for CaM causes a very significant shift in its elution position on anion-exchange chromatography. Although SDS gels indicated that the enzyme was not purified to homogeneity, the most highly purified preparations were purified 1600-fold to a specific activity of 608 μmol of cAMP min^{-1} mg^{-1}, which is the most active adenylate cyclase preparation reported from any source. Hewlett & Wolff (1976) described a procedure for the purification of *B. pertussis* adenylate cyclase from culture medium to apparent homogeneity. The specific activity of their most highly purified enzyme was 0.14 μmol of cAMP min^{-1} mg^{-1}. More recently, Wolff et al. (1983) have reported a specific activity of approximately 100 μmol of cAMP min^{-1} mg^{-1} for adenylate cyclase purified from whole *B. pertussis*, and Leppla (1983) has reported a specific activity of 400 μmol of cAMP min^{-1} mg^{-1} for a CaM-sensitive adenylate cyclase from *Bacillus anthracis*. In neither of these latter two cases have details of the purification been reported. The adenylate cyclase from *Brevibacteriun liquifaciens*, which is not a CaM-sensitive enzyme, has been purified to homogeneity with a specific activity of 30 μmol of cAMP min^{-1} mg^{-1} (Takai et al., 1974).

One can estimate the turnover number of the *B. pertussis* adenylate cyclase in the presence of CaM from data reported in this study. The minimum turnover number of the pure enzyme would be 27 000 min^{-1} using the molecular weight of 44 300 and the specific activity of 608 μmol of cAMP min^{-1} mg^{-1} and assuming one catalytic site per monomer. Although the turnover number of the CaM-sensitive adenylate cyclase from brain is not known, the most highly purified preparation has a turnover number of 80 min^{-1} (Yeager et al., 1985). Levitzki has estimated that mammalian adenylate cyclases may have turnover numbers as high as 1000 min^{-1} (Levitzki et al., 1975). It appears that the *B. pertussis* cyclase may be inherently more active than mammalian adenylate cyclases, which would be consistent with its role as a toxin. If indeed the biological function of this enzyme is to enter animal cells and elevate cAMP levels, then it may have evolved with very high intrinsic activity to overcome endogenous phosphodiesterase activity. In this respect, it is interesting that crude preparations of *B. pertussis* adenylate cyclase cause elevations of cAMP in human neutrophils, implying that the adenylate cyclase can enter animal cells (Confer & Eaton, 1982). Alternatively, it could be argued that IAP or another factor present in the preparation entered neutrophils and stimulated adenylate cyclase activity. The more highly purified preparations described in this study, which lack IAP, would be more suitable to determine if the enzyme actually enters animal cells. Preliminary studies, using the enzyme purified through fetuin–Sepharose, indicate that the partially purified enzyme can elevate cAMP levels in animal cells (Shattuck, 1985).

The partially purified *B. pertussis* adenylate cyclase obtained in this study has a native molecular weight of 44 300, it apparently binds one CaM, and it has a K_m for ATP of 2 mM. Previous studies have established that the bacterial enzyme can be stimulated by CaM in the absence of free Ca^{2+} (Greenlee et al., 1982; Kilhoffer et al., 1983). In contrast, the CaM-sensitive adenylate cyclase purified from bovine cerebral cortex has a native molecular weight of 328 000, its catalytic subunit has a molecular weight of 150 000, it binds one CaM per catalytic subunit, its K_m for ATP is 0.07 mM, and the enzyme requires Ca^{2+} for CaM stimulation (Andreasen et al., 1983; Yeager et al., 1985). These differences in physical and catalytic properties most likely reflect the functional differences between the two enzymes. The mammalian enzyme has evolved to respond to fluctuations in free intracellular Ca^{2+} concentrations; the bacterial adenylate cyclase is apparently a toxin which has evolved to enter animal cells by using intercellular CaM as a positive regulator.

During the course of purifying the *B. pertussis* adenylate cyclase, we found two forms of the enzyme separable on QAE-Sephadex. These two enzyme activities had identical sensitivities to CaM, similar K_m's for ATP, and comparable hydrodynamic properties in both the presence and absence of CaM. They could be distinguished only by their behavior on anion-exchange columns, and the relationship between these two enzymes remains to be established. Although they may arise by proteolytic removal of a small peptide, making them indistinguishable by the criteria applied, these two forms of enzyme may ultimately be useful in defining the properties of the enzyme.

In summary, an adenylate cyclase from *B. pertussis* has been purified to a very high specific activity and was found to be free of IAP. The physical and catalytic properties of the enzyme are quite distinct from the mammalian enzyme, which is consistent with different biological functions of the two enzymes.

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