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Secreted Adenylate Cyclase of *Bordetella pertussis*: Calmodulin Requirements and Partial Purification of Two Forms

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The extracellular adenylate cyclase of *Bordetella pertussis* was partially purified and found to contain high- and low-molecular-weight species. The high-molecular-weight form had a variable molecular weight with a peak at about 700,000. The smaller species had a molecular weight of 60 to 70,000 as determined by gel filtration. The low-molecular-weight form could be derived from the high-molecular-weight species. The high-molecular-weight complex purified from the cellular supernatant was highly stimulated by calmodulin, while the low-molecular-weight enzyme was much less stimulated. Active enzyme could be recovered from sodium dodecyl sulfate (SDS) gels at positions corresponding to molecular weights of about 50,000 and 65,000. Active low-molecular-weight enzyme recovered from SDS gels migrated with a molecular weight of about 50,000, which coincides with a coomassie blue-stained band. However, when both high- and low-molecular weight preparations were analyzed in 8 M urea isoelectrofocusing gels, the enzyme activity recovered did not comigrate with stained protein bands. The enzyme recovered from denaturing isoelectrofocusing or SDS gels was activated by calmodulin, indicating a direct interaction of calmodulin and enzyme. The high-molecular-weight form of the enzyme showed increasing activity with calmodulin concentrations ranging from 0.1 to 500 nM, while the low-molecular-weight form was fully activated by calmodulin at 20 nM. Adenylate cyclase on the surface of living cells was activated by calmodulin in a manner which resembled that found for the high-molecular-weight form.

Adenylate cyclase is normally a mediator between extracellular signals and the internal biochemical and genetic apparatus of procaryotic and eucaryotic cells. Two species of pathogenic bacteria secrete adenylate cyclases which may disrupt the cyclic AMP (cAMP)-dependent information processing of eucaryotic cells. These species are *Bordetella pertussis*, the organism which causes whooping cough (1, 5), and *Bacillus anthracis*, the agent of anthrax (12). The enzyme from *Bacillus anthracis* is a toxin; that of *B. pertussis* is likely to be one, but its biochemistry has not been sufficiently studied for a complete understanding of its function to emerge. Genetic evidence based on transposon-generated mutations suggests a role for the *Bordetella pertussis* adenylate cyclase in pathogenesis (15).

The *Bordetella pertussis* enzyme was discovered in commercial whooping cough vaccine (16). One property which makes the enzyme extraordinary is that its active site faces outward; another is that it is activated several hundredfold by calmodulin (18). The majority of the enzyme is bound to the cells; most of the rest is extracellular (7). For adenylate cyclase, in contrast to most calmodulin-stimulated enzymes, the effect of calmodulin does not require calcium (4, 8). The membrane-associated enzyme, extracted from the cells with 4 M urea, has been reported to enter eucaryotic cells and to elevate their intracellular cAMP concentrations (1, 5).

The interesting properties of the enzyme, its stability, secretion, invasiveness, and interaction with calmodulin, have been investigated with crude enzyme preparations. In the course of purifying both forms of the enzyme, we observed that much of the extracellular enzyme and the membrane-associated enzyme had a high molecular weight. Calmodulin is required for obtaining the high-molecular-weight activity, and, therefore, this form of the enzyme would be cryptic under assay conditions which do not include calmodulin.

To examine the forms of the enzyme, we separated partially purified adenylate cyclase on sodium dodecyl sulfate (SDS) gels and on isoelectrofocusing gels containing 8 M urea. By reactivating the enzyme recovered from the gels, we were able to demonstrate that the activities do not comigrate with Coomassie brilliant blue-stained bands during isoelectrofocusing. This indicates a much higher specific activity than was observed by Hewlett and Wolff, who reported a procedure for the purification of the extracellular enzyme (6).

MATERIALS AND METHODS

Cell culture. The World Health Organization reference strain 18323, supplied by J.-M. Alonso and C. Brezin of the Pasteur Institute, was used in these studies. Certain results were confirmed with strains 165 and Tohama I, as indicated below. Bordet-Gengou agar plates (Scott Laboratories, Fiskeville, R.I.) were inoculated from stocks maintained frozen in 15% glycerol at −80°C. After three days of growth at 37°C, the resulting lawns were recovered from the plates and used to inoculate 60-ml seed cultures of modified Stainer-Scholte medium (14). After 2 days of growth at 36°C, these cultures were used to inoculate liter cultures which were grown overnight. The inoculum was 2.5 to 3% of the final volume. Final optical densities were 0.6 to 1.0, measured at a wavelength of 625 nm in a semi-microcuvette with a double-beam spectrophotometer (model 25; Beckman Instruments, Inc., Fullerton, Calif.). Because of hospital safety regulations, cultures were treated with 0.02% thimerosal and 0.02% sodium azide before being harvested by centrifugation at 7,000 rpm in a GS3 rotor. Thimerosal did not affect the activity of the enzyme. Cultures which did not receive thimerosal were used to confirm the results.

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TABLE 1. Purification of extracellular adenylate cyclase[a]

Purification step	Total units recovered (%)	Activity (U/mg of protein)	Purification (fold)
Initial supernatant	21,000 (100)	27	1
Phenyl-Sepharose	10,700 (51)	580	21
DE52	4,800 (23)	660	24
AcA34 (HMW)	1,600 (7.6)	2,970	110
AcA34 (LMW)	1,200 (5.7)	2,280	84

[a] Values are the averages obtained with two consecutive runs of 25 to 30 liters of culture supernatant. HMW, High-molecular-weight form; LMW, low-molecular weight form.

Purification. Six liters of culture supernatant containing 0.3 M NaCl was passed through a 10-ml phenyl-Sepharose column. The column was washed with 40 ml of 50 mM Tricine-sodium hydroxide (pH 7.6) containing 0.3 M NaCl and then with 40 ml of 10 mM Tricine–10% ethylene glycol. The enzyme was eluted from the column in 10 mM Tricine–90% ethylene glycol and stored at −20°C. The enzyme contained in four or five such preparations was further purified by ion-exchange chromatography on DE52. The ethylene glycol eluates were collected directly on a DE52 column (1.5 by 3 cm) equilibrated with 50 mM Tris hydrochloride (pH 8). After loading, the column was washed with 50 mM Tris hydrochloride (pH 8) and with 50 mM Tris containing 0.1 M NaCl. The enzyme was eluted with 50 mM Tris plus 0.4 M NaCl. The enzyme eluted from the column was concentrated by ultrafiltration with a membrane filter (model PM10; Amicon Corp., Lexington, Mass.) and applied to an AcA34 gel filtration column (73 by 1.1 cm). The column was equilibrated and eluted with 50 mM Tricine containing 0.1 M NaCl and 0.02% sodium azide. The column was calibrated with the following (M_r): thyroglobulin (670,000), ferritin (440,000), immunoglobulin G (155,000), bovine serum albumin (67,000), ovalbumin (45,000), and chymotrypsinogen A (25,000). The void volume of the column was measured with dextran blue. An indication of the purifications achieved is given in Table 1. Protein assays were performed as described previously (13) with bovine serum albumin as the standard. In some experiments (see below and figure legends) a hydroxylapatite column was included. Enzyme eluted from the DE52 column was diluted fourfold with cold distilled water and applied to a hydroxylapatite column (1.5 by 4 cm) equilibrated with 50 mM Tris. The column was eluted with 50 mM sodium phosphate (pH 7), which removed the low-molecular-weight enzyme. The high-molecular-weight enzyme was eluted with 1 M sodium phosphate.

The membrane-associated enzyme was extracted from the centrifuged cells with 4 M urea as described by Confer and Eaton (1). The preparations were dialyzed, and a sample was applied to an AcA34 column.

SDS and isoelectrofocusing gels. SDS–10% polyacrylamide gel electrophoresis was performed by the method of Laemmli (11). Isoelectrofocusing gels were used as described previously (2). For analysis of the enzyme recovered from either type of gel, the gels were cut by hand into 2-mm slices. The SDS was removed from each slice by three washes for 30 min each in 0.5 ml of buffer (50 mM Tris, 0.02% sodium azide, 0.01% bovine serum albumin) at room temperature. Enzyme was eluted for 16 h at 4°C in 0.2 ml of the same buffer. The isoelectrofocusing gel slices were washed and eluted in the same way.

Assay conditions. The assay contained 0.005 to 0.05 U of enzyme activity, 50 mM Tris hydrochloride (pH 7.6), 0.01% bovine serum albumin, 1.2 mM [2,8-^{3}H]ATP, 10 mM $MgCl_2$, 1 mM cAMP, 10 mM creatine phosphate, and 0.8 U of creatine phosphokinase (type 1; Sigma Chemical Co., St. Louis, Mo.). In some experiments the creatine phosphate and creatine phosphokinase were omitted or replaced with known amounts of purified calmodulin. The specific radioactivity of the [^{3}H]ATP was 6 cpm/pmol. The reactions were carried out in a total volume of 70 μl at 37°C. Reactions were stopped by the addition of 100 μl of 10 mM ATP–2 mM cAMP containing 1.5 nCi of [8-^{14}C]cAMP. After boiling, the ATP was separated from cAMP as described by Krishna et al. (9, 10). The cAMP recovered was counted in Monofluor. One unit of enzyme activity converted 1 nmol of ATP to cAMP per min.

The identity of the product was confirmed by ascending paper chromatography on filter paper (no. 1; Whatman, Inc., Clifton, N.J.). The solvent system was 70 parts 95% ethanol and 30 parts 1 M ammonium acetate. The product was more than 95% sensitive to degradation by cyclic nucleotide phosphodiesterase whether synthesized in the presence or absence of calmodulin. After digestion with phosphodiesterase, the product comigrated with 5′-AMP. Calmodulin was iodinated with Bolton-Hunter reagent as described by the manufacturers (New England Nuclear Corp., Boston, Mass.). The presence of protease was tested with a kit produced by Bio-Rad Laboratories, Richmond, Calif., and used as described in the instructions of the manufacturers.

Materials. [2,8-^{3}H]ATP and [8-^{14}C]cAMP were purchased from ICN Pharmaceuticals, Inc., Irvine, Calif., or Amersham Corp., Arlington Heights, Ill. Monofluor was obtained from National Diagnostics. Calmodulin (from bovine brain) and phenyl-Sepharose CL4B were obtained from Sigma. Ultragel AcA34 and ampholytes were obtained from LKB Instruments, Inc., Rockville, Md., and DE52 was obtained from Whatman. Pentex bovine serum albumin was from Miles Laboratories, Inc., Elkhart, Ind. All other reagents were of the highest quality available.

RESULTS

Two size classes of adenylate cyclase. The extracellular enzyme was concentrated and purified by passing 6 liters of culture supernatants through a 10-ml phenyl-Sepharose column. Two to four percent of the protein was recovered after elution of hydrophobic molecules with 90% ethylene glycol. The enzyme was further purified by ion exchange on DE52, as described in Materials and Methods. The concentrated DE52 eluate was applied to an AcA34 gel filtration column (Fig. 1). Two peaks were detected: one with a broad range of molecular weights with a peak at 700,000, and a second with a sharp peak corresponding to a molecular weight of 60,000 to 70,000. When the A_{280}s were measured, no peaks corresponding to the peaks of activity were found, indicating that we were not yet dealing with a single protein species. Identical results were obtained when strain 165 was used or when thimerosal was not used. The Tohama I strain also produced a high-molecular-weight enzyme. An indication of the degree of purification is given in Table 1. Crude preparations of the membrane-associated enzyme also produced 2 peaks (data not shown). Hanski and Farfel have also observed high- and low-molecular-weight components in enzyme extracted from the bacterial surface with 4 M urea (5).

Conversion of the high-molecular-weight form to the smaller form. The fractions of the AcA34 column containing the high-molecular-weight form of the extracellular enzyme were pooled and concentrated. The high-molecular-weight species broke down spontaneously but incompletely to pro-

duce the low-molecular-weight form of the enzyme (see Fig. 3). Treatment with 0.5 M NaCl or 4 M LiCl did not reduce the size of the complex. There was increased conversion of the high-molecular-weight enzyme to the low-molecular-weight form with 1% Triton or acetone-ethanol precipitation (data not shown). We infer that a hydrophobic interaction is partially responsible for holding the enzyme in its high-molecular-weight form.

Differential reactions with calmodulin. In the results shown in Fig. 1, the assay mixture contained an ATP-regenerating system which includes phosphocreatine and phosphocreatine kinase. Leaving out the creatine phosphate does not reduce the activity of the enzyme, which indicates that no regenerating system is necessary and that the phosphocreatine kinase is contaminated with calmodulin (3). Subsequent experiments showed that the activation caused by the phosphocreatine kinase was comparable to the activation by 5 nM calmodulin (data not shown). When the fractions of the AcA34 columns described above were assayed without the regenerating system, that is, without the calmodulin which contaminates the phosphocreatine kinase, very little activity was found in the high-molecular-weight fractions. There was activity in the low-molecular-weight fractions (Fig. 2). Addition of 240 nM calmodulin revealed adenylate cyclase activity in the high-molecular-weight fractions. The activation was greater than 200-fold, the largest activation of extracellular enzyme yet reported. In a number of preparations, we observed a 5- to 20-fold stimulation of the low-molecular-weight form of the enzyme by calmodulin. In unseparated preparations containing high- and low-molecular-weight enzyme, the degree of calmodulin activation depended on the fraction of the preparation which was in the monomeric form.

Binding of ^{125}I-labeled calmodulin. Reaction with an excess of iodinated calmodulin followed by chromatography on ultragel AcA34 showed comigration of calmodulin with the high-molecular-weight extracellular enzyme (Fig. 3A). Calmodulin fractionated anomolously on Ultragel AcA34, behaving as if it were a globular protein with a molecular weight of about 40,000. Since calmodulin was in excess, the shoulder of the calmodulin peak in Fig. 3A overlaps the low-molecular-weight adenylate cyclase peak. The fractions between the brackets were pooled, concentrated, and rechromatographed. Iodinated calmodulin comigrated with low-molecular-weight adenylate cyclase (Fig. 3B). Because calmodulin association does not require calcium (4, 8), the standard control experiment, with calcium removed, could not be done. By assuming a 1:1 stoichiometry of calmodulin and adenylate cyclase and knowing the specific activity of the iodinated calmodulin, one can estimate the specific activity of the activated enzyme. We calculate that the activity of the fully activated enzyme approaches 1,000 μmol/min per mg of protein.

The results in Fig. 3A also illustrate that the high-molecular-weight material broke down to the smaller form of the enzyme because the enzyme used in this experiment was initially all high molecular weight.

Renaturation of the enzyme from SDS and isoelectrofocusing gels. Before gel analysis, both high- and low-molecular-weight forms of the enzyme were further purified by binding to and elution from hydroxylapatite. The low-molecular-weight form was eluted with 50 mM phosphate buffer, and the high-molecular-weight form was eluted with 1 M phos-

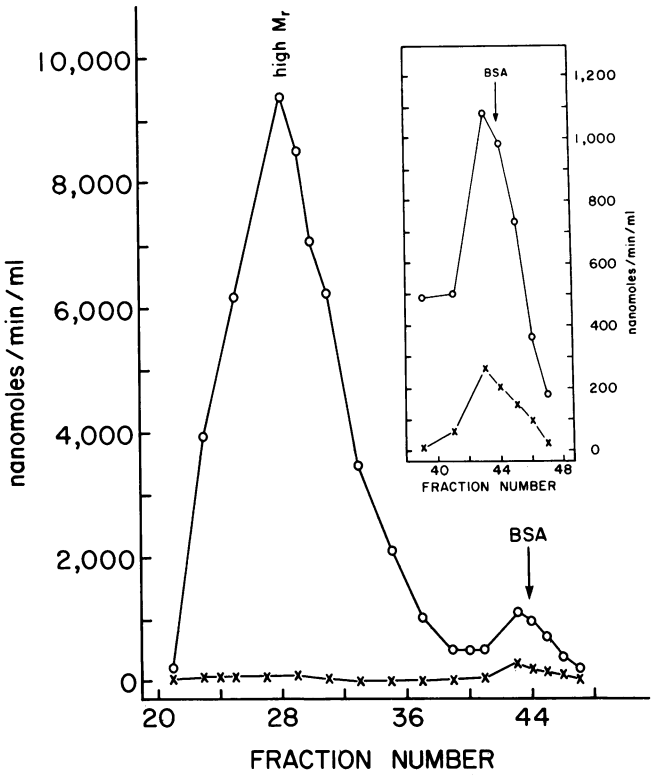

FIG. 2. Differential effect of calmodulin. Partially purified enzyme was applied to the same AcA34 column as described in the legend to Fig. 1 and in Materials and Methods. Fractions were assayed in the total absence of calmodulin (×) or in the presence of 240 nM calmodulin (○). All assays included 10 μM $CaCl_2$. The inset shows the activity of the low-molecular-weight enzyme plotted on an expanded scale.

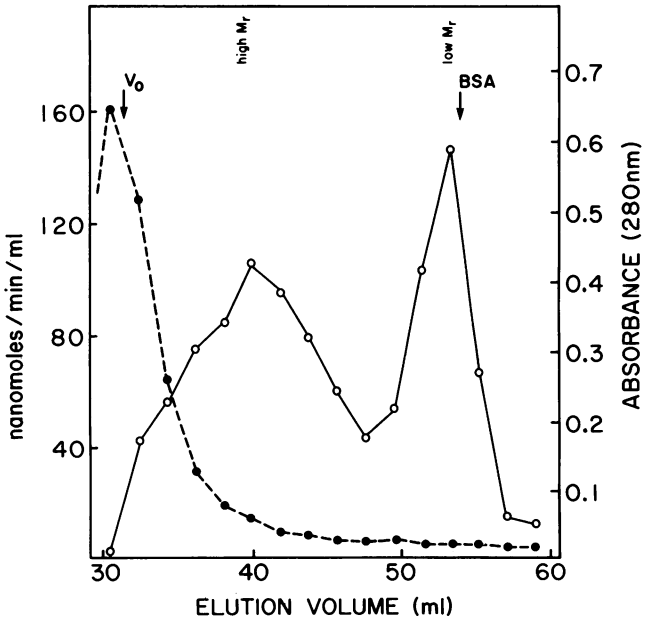

FIG. 1. Size classes of adenylate cyclase. Partially purified enzyme was applied to an ultragel AcA34 column and eluted with 50 mM Tricine-NaOH (pH 7.6)–0.1 M NaCl–0.02% sodium azide. The flow rate was 4.8 ml/h, and the fraction size was 1.6 ml. Adenylate cyclase activity was measured in the presence of 5 nM calmodulin. Symbols: ○, enzyme activity; ●, A_{280}.

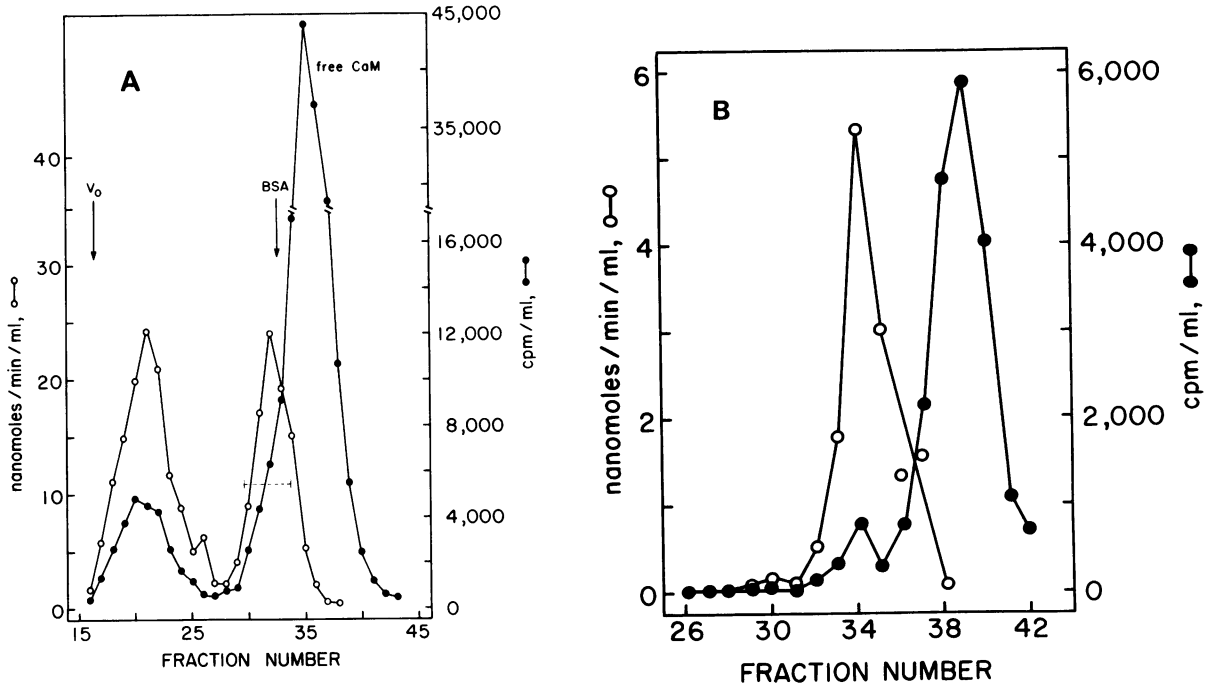

FIG. 3. Binding of iodinated calmodulin to adenylate cyclase. (A) ^{125}I-labeled calmodulin (10^6 cpm; specific activity, 44 mCi/nmol) was reacted for 10 min at 37°C with 150 U of high-molecular-weight enzyme. The complex was applied to an AcA34 column. Fractions were collected, and samples were counted or assayed for adenylate cyclase activity. (B) The fractions indicated by the brackets in panel A were pooled, concentrated, and reapplied to the same AcA34 column.

phate. These preparations were concentrated and analyzed by SDS-polyacrylamide gel electrophoresis and isoelectrofocusing in 8 M urea.

The enzyme separated on SDS gels could be recovered from gel slices and reactivated, even after boiling in the presence of 2% mercaptoethanol. The recovery was approximately 4% of the activity applied to the gel. The high-molecular-weight enzyme was analyzed by SDS gel electrophoresis and a predominant coomassie blue staining band at a molecular weight of 65,000 and a minor one at 40,000 were detected. The migration of the stained bands and of renatured enzyme are shown in Fig. 4A. One peak of activity corresponded to the 65,000-molecular-weight band, while a peak with more activity but a lower molecular weight was not associated with a stained band. We suspect that the lower-molecular-weight peak of activity is a fragment of the larger one. Activity was nearly absent from either peak when calmodulin was not included in the assay. SDS gels of low-molecular-weight enzyme showed a 50,000-molecular-weight stained band with a comigrating activity (Fig. 4B).

We analyzed the same preparations by isoelectrofocusing in 8 M urea. Recovery of enzyme activity from denaturing isoelectrofocusing gels was approximately 25%. Neither the high-molecular-weight enzyme nor the low-molecular-weight species comigrated with a major stained band (Fig. 5A and B). We believe that the component which was separated from the adenylate cyclase activity during isoelectrofocusing was a contaminant which may have been previously confused with adenylate cyclase. All of the assays were done in the presence of calmodulin. When it was omitted, no activity was found. These results illustrate that (i) enzyme separated under denaturing conditions retains its ability to interact with calmodulin after renaturation and (ii) the specific activity of the enzyme is much higher than was previously thought (6). By comparison of faint silver-stained bands in the region of the gel containing adenylate cyclase activity with standards of known concentration, we estimated a specific activity of at least 100,000 nmol/min per mg of protein. Estimates based on calmodulin binding gave even higher values.

Kinetic properties of the high- and low-molecular-weight forms. The high- and low-molecular-weight proteins reacted differently with calmodulin (Fig. 6). The low-molecular-weight form purified under our conditions was stimulated 5- to 20-fold by 20 nM calmodulin, with half-maximal activation at 0.5 to 1.0 nM. Freshly prepared high-molecular-weight enzyme was responsive to calmodulin stimulation over a wide range of concentrations (Fig. 6). The degree of stimulation was several hundredfold, with half-maximal stimulation at 50 to 100 nM. Goldhammer and co-workers (3, 17) have observed that whole *Bordetella pertussis* cells or crude membranes respond to calmodulin in a manner nearly identical to that which we observed for the high-molecular-weight enzyme. We added increasing amounts of calmodulin to samples of whole cells and confirmed that the stimulatory effects of calmodulin extended over a wide range of calmodulin concentrations (Fig. 6). We infer from these results that the high-molecular-weight component is derived from the enzyme on the surface of the living cells. The K_m of the high-molecular-weight enzyme for ATP (in the presence of calmodulin) is 2.4 mM, while the K_m of the enzyme measured on whole cells was 1.9 mM (data not shown). This is in the same range as previously reported for the low-molecular-weight enzyme (6).

DISCUSSION

The adenylate cyclase of *Bordetella pertussis* has been used to inhibit phagocytosis and to stimulate the production of cAMP by various mammalian cells (1, 5). In both cases, crude membrane-bound enzyme was used. The evidence

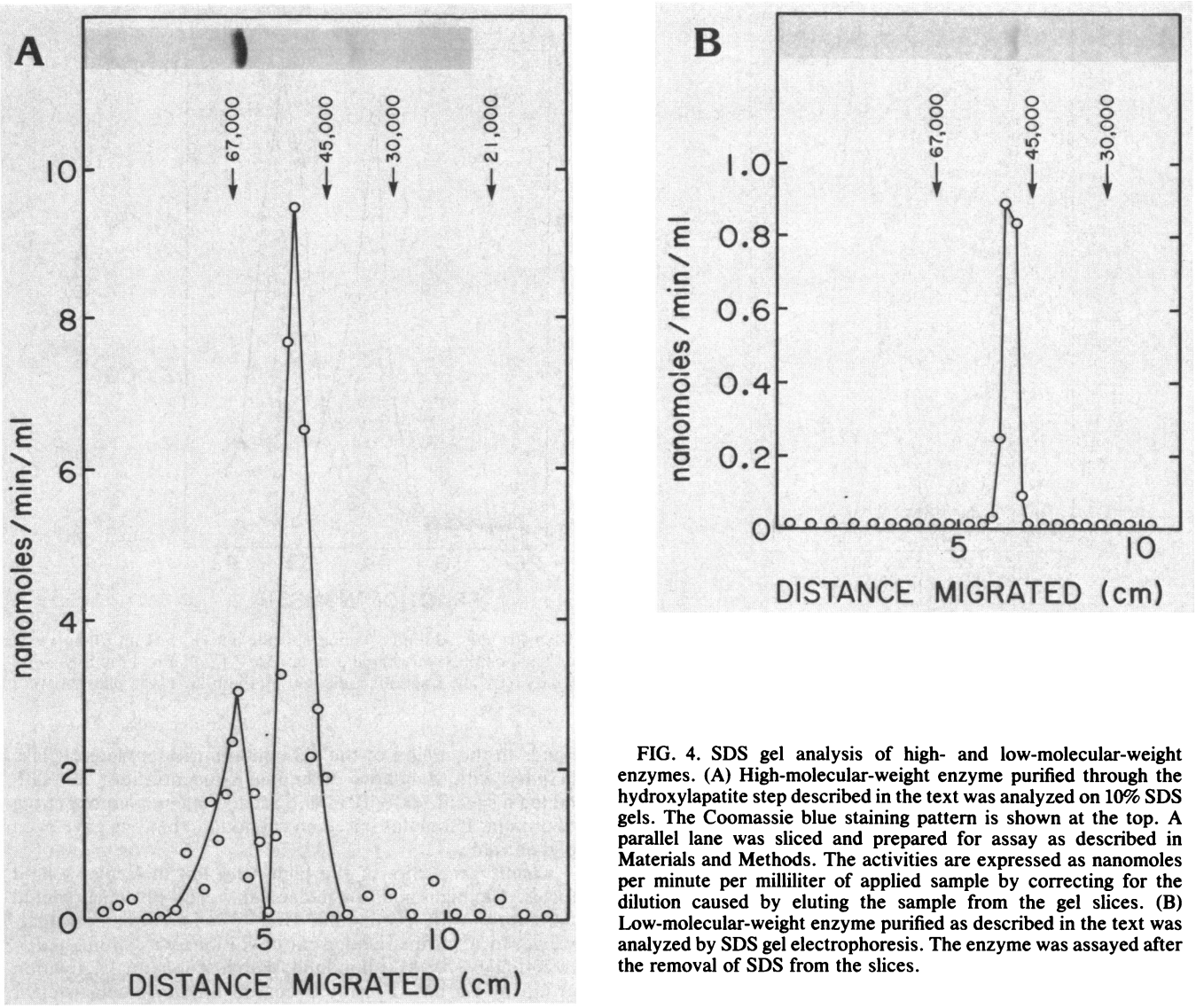

FIG. 4. SDS gel analysis of high- and low-molecular-weight enzymes. (A) High-molecular-weight enzyme purified through the hydroxylapatite step described in the text was analyzed on 10% SDS gels. The Coomassie blue staining pattern is shown at the top. A parallel lane was sliced and prepared for assay as described in Materials and Methods. The activities are expressed as nanomoles per minute per milliliter of applied sample by correcting for the dilution caused by eluting the sample from the gel slices. (B) Low-molecular-weight enzyme purified as described in the text was analyzed by SDS gel electrophoresis. The enzyme was assayed after the removal of SDS from the slices.

that it is the adenylate cyclase in this preparation which causes the synthesis of cAMP in the target cells rests on the stability of the enzyme to boiling (1, 5). To define the properties of the enzyme more precisely, we began purification of the extracellular adenylate cyclase.

We found that the majority of the extracellular enzyme activity in our preparations was in a high-molecular-weight form and that the activity in this species was cryptic unless calmodulin was present in the assay. This may account for previous failure to find the high-molecular-weight form, although it has been observed by Hanski and Farfel in the membrane-extracted enzyme (5). We also observed the high-molecular-weight species in enzyme extracted from the cell surface (results not shown).

The high- and low-molecular-weight forms differed in their interaction with calmodulin. The high-molecular-weight form of the enzyme was increasingly stimulated by calmodulin over the range of 0.1 to 500 nM, with half-maximal activation at about 50 nM. The high-molecular-weight form of the enzyme was nearly inactive without calmodulin, and the degree of stimulation was several hundredfold. The low-molecular-weight enzyme was less stimulated by calmodulin, with a maximum activation of about 20-fold and half-maximal activation at 0.5 to 1 nM. The low-molecular-weight enzyme had residual activity in the absence of calmodulin (Fig. 2).

Goldhammer and Wolff (3) presented a dose-response curve for calmodulin activation of adenylate cyclase retained on cell membranes. Their curve resembles the one we obtained for the high-molecular-weight form of the enzyme. We repeated their experiment with whole cells and increasing amounts of calmodulin. The pattern of calmodulin activation closely matched that found with the high-molecular-weight enzyme (Fig. 6). The argument might be made that the high-molecular-weight entity is an artifact of purification. This is unlikely for two reasons: (i) crude membrane-associated enzyme has a high-molecular-weight form which interacts with calmodulin, and (ii) there is a correspondence of calmodulin sensitivities between the high-molecular-weight form and the enzyme located in situ on the cells.

We do not know the nature of the interaction of the enzyme with itself or other components which give it a high molecular weight. The fact that enzyme isolated in the high-molecular-weight form did not break down to the mo-

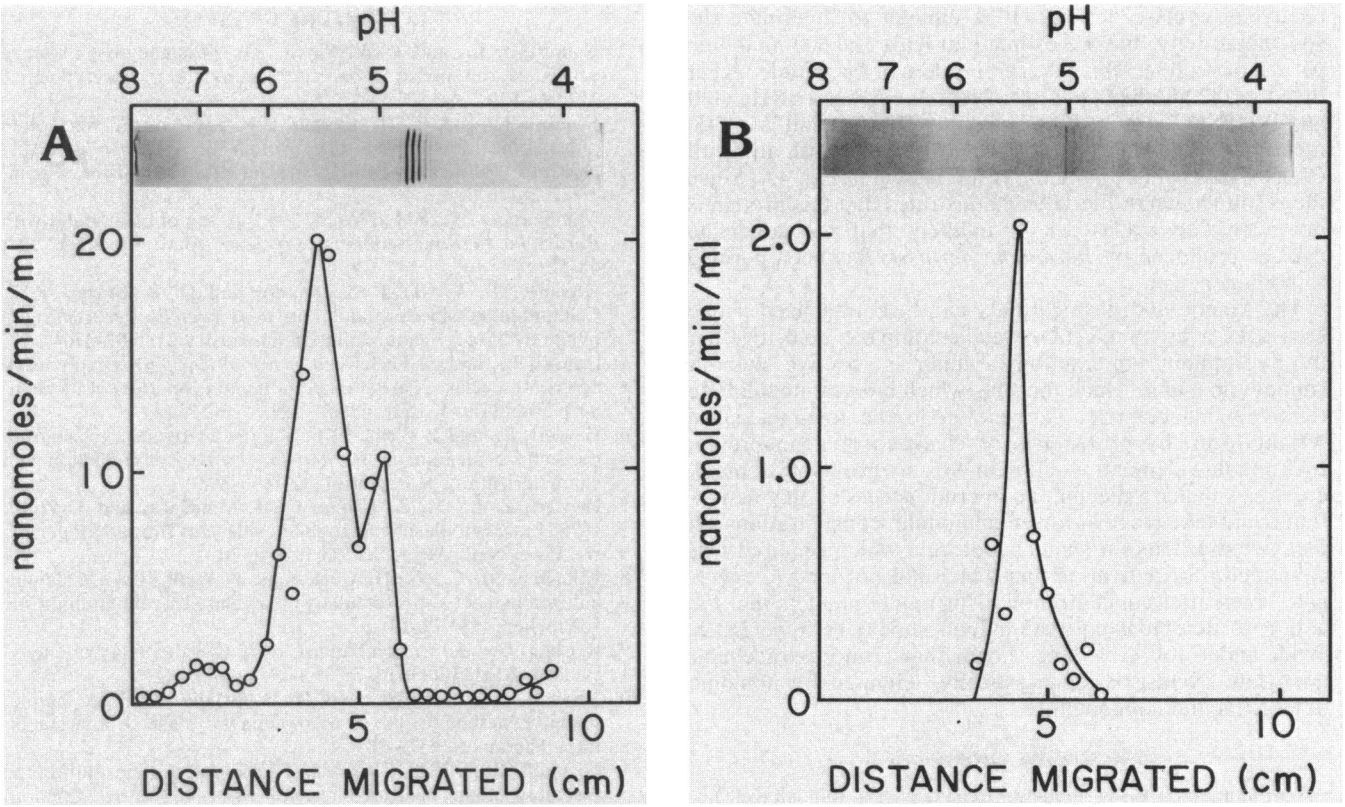

FIG. 5. Isoelectrofocusing of high- and low-molecular-weight adenylate cyclase. The enzyme preparations used in the experiments described in the legend to Fig. 4 were applied to isoelectrofocusing gels in 8 M urea. Before focusing, the enzyme in 8 M urea was heated for 1 min at 100°C. Proteins were focused to equilibrium at constant voltage (500 V for 7 h). Focusing was monitored with aniline blue, Evan's blue, fast green, and cytochrome *c*. One lane of the gel was fixed and stained with Coomassie brilliant blue while another was sliced into 3-mm segments and assayed as described in Materials and Methods. (A) High-molecular-weight enzyme; (B) low-molecular-weight enzyme.

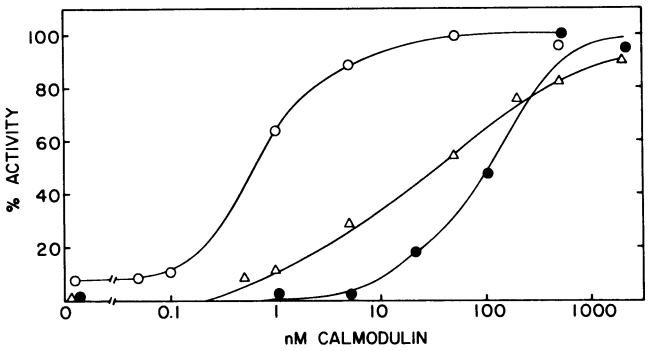

FIG. 6. Calmodulin dose-response curves. High- and low-molecular-weight enzyme were purified through the phenyl-Sepharose, DE52, and AcA34 steps described in the text. The separated fractions were assayed in the presence of increasing amounts of calmodulin. The calcium concentration was 10 μM. After a 10-min preincubation at 37°C, the assay was initiated by the addition of substrate. Whole cells were prepared by growing a culture to stationary phase, adding 15% glycerol, and freezing at −80°C. Before the assay, the cells were thawed and centrifuged at 7,000 rpm in an SS34 rotor. The pellet was suspended in 1 ml of 50 mM Tris (pH 8.0)–0.02% sodium azide. Samples (10 μl) were assayed with increasing calmodulin concentrations. All points represent the averages of triplicate assays. Symbols: ○, low-molecular-weight enzyme; ●, high-molecular-weight enzyme; △, enzyme on the surface of whole cells.

nomeric form with several high-salt treatments but did preferentially appear in lower-molecular-weight forms after treatment with Triton X-100 argues for the interaction being hydrophobic at least in part. The nature of the spontaneous breakdown from the high- to the low-molecular-weight form is unknown. Concentrated low-molecular-weight enzyme retained its low molecular weight when rechromatographed. We postulate that in the breakdown process, a hydrophobic domain responsible for aggregation may be lost. Such a situation is consistent with the results of SDS gel renaturation experiments, which showed two different molecular weights for enzyme derived from the high-molecular-weight enzyme but only one for enzyme that behaved as the low-molecular-weight form during gel filtration (Fig. 4). A test of the crude and purified preparations for protease activity proved negative.

The SDS gel protein pattern shown in Fig. 4A was relatively simple when stained with Coomassie brilliant blue: the high-molecular-weight entity contained one major protein. Two peaks of activity were found when the enzyme was eluted from the gel, one of which comigrated with the major stained band and one of which did not. Recovery was approximately 4%, and the renatured enzyme required calmodulin for activity. The same purified preparation of high-molecular-weight enzyme was subjected to gel electrophoresis in 8 M urea, and in this case there was no comigration of the Coomassie blue-stained bands and enzyme activity (Fig. 5A). Recovery was about 25%, and the enzyme again required calmodulin for activity. Silver staining the SDS gel showed minor bands in the region of the

adenylate cyclase activity. It is difficult to determine the specific activity, but we estimate at least 100,000 nmol/min per mg of protein. This is several orders of magnitude higher than the 142 nmol/min per mg of protein reported by Hewlett and Wolff (6) for the purified enzyme. Hewlett and Wolff (6) mentioned that SDS gels show a single band with an M_r of 69,000. This is not unlike the result shown in Fig. 4A. Since the result shown in Fig. 5A demonstrates that this protein is not adenylate cyclase, it is unlikely that the adenylate cyclase produced by *Bordetella pertussis* has been purified to homogeneity.

The kinetic and physical data can be rationalized if one envisages a high-molecular-weight complex, held together by hydrophobic interactions, which is inactive without calmodulin and in which the sites which bind calmodulin are relatively inaccessible. Conversion to the low-molecular-weight form, by proteolysis or cleavage of a nonprotein hydrophobic element, would remove a hydrophobic domain, create a smaller molecule, and permit access so that activation could take place at lower calmodulin concentrations. In fact, we did detect a smaller molecule which required less calmodulin for activation and which did not tend to aggregate, even after concentration. This model predicts that the action of detergent and calmodulin should be synergistic. Wolff and Cook (17) have shown that crude extracellular adenylate cyclase is synergistically activated by nonionic detergents and calmodulin.

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ADDENDUM IN PROOF

After the submission of this paper, Shattuck et al. (R. L. Shattuck, D. J. Oldenburg, and D. R. Storm, Biochemistry **24**:6356–6362, 1985) presented data which complement our results. Using a purification protocol different from ours, these workers achieved a 250-fold purification from the culture medium, similar to results presented in this communication. The specific activities reported by Shattuck et al. are much higher than ours. We believe this is because an excess of calmodulin (2.4 μM) was present in their assay, in contrast to the 5 nM calmodulin present in our assay. Shattuck et al. did not observe a high-molecular-weight species.

LITERATURE CITED

1. **Confer, D. L., and J. W. Eaton.** 1982. Phagocyte impotence caused by an invasive bacterial adenylate cyclase. Science **217**:948–950.
2. **Franke, J., and R. H. Kessin.** 1981. The cyclic nucleotide phosphodiesterase inhibitory protein of *Dictyostelium discoideum*. Purification and characterization. J. Biol. Chem. **256**:7628–7637.
3. **Goldhammer, A., and J. Wolff.** 1982. Assay of calmodulin with *Bordetella pertussis* adenylate cyclase. Anal. Biochem. **124**: 45–52.
4. **Greenlee, D. V., T. J. Andreasen, and D. R. Storm.** 1982. Calcium-independent stimulation of *Bordetella pertussis* adenylate cyclase by calmodulin. Biochemistry **21**:2759–2764.
5. **Hanski, E., and Z. Farfel.** 1985. *Bordetella pertussis* invasive adenylate cyclase. Partial resolution and properties of its cellular penetration. J. Biol. Chem. **290**:5526–5532.
6. **Hewlett, E., and J. Wolff.** 1976. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*: purification and characterization. J. Bacteriol. **127**:890–898.
7. **Hewlett, E. L., M. A. Urban, C. R. Manclark, and J. Wolff.** 1976. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Natl. Acad. Sci. **73**:1926–1930.
8. **Kilhoffer, M. C., G. H. Cook, and J. Wolff.** 1983. Calcium-independent activation of adenylate cyclase by calmodulin. Eur. J. Biochem. **133**:11–15.
9. **Krishna, G., and L. Birnbaumer.** 1970. On the assay of adenyl cyclase. Anal. Biochem. **35**:393–397.
10. **Krishna, G., B. Weiss, and B. B. Brodie.** 1968. A simple, sensitive method for the assay of adenyl cyclase. J. Pharmacol. Exp. Therap. **163**:379–385.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**: 680–685.
12. **Leppla, S. H.** 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. Proc. Natl. Acad. Sci. USA **79**:3162–3166.
13. **Peterson, G. L.** 1977. A simplification of the protien assay method of Lowry *et al.* which is more generally applicable. Anal. Biochem. **83**:346–356.
14. **Stainer, D. W., and M. J. Scholte.** 1971. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63**:211–220.
15. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1984. Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis*. J. Infect. Dis. **150**:219–222.
16. **Wolff, J., and G. H. Cook.** 1973. Activation of thyroid membrane adenylate cyclase by purine nucleotides. J. Biol. Chem. **248**:350–355.
17. **Wolff, J., and G. H. Cook.** 1982. Amphiphile-mediated activation of soluble adenylate cyclase of *Bordetella pertussis*. Arch. Biochem. Biophys. **215**:524–531.
18. **Wolff, J., G. H. Cook, A. R. Goldhammer, and S. A. Berkowitz.** 1980. Calmodulin activates prokaryotic adenylate cyclase. Proc. Natl. Acad. Sci. USA **77**:3841–3844.