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Cyclic AMP Phosphodiesterase in *Thermomonospora curvata*

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Cyclic AMP phosphodiesterase (PDE; EC 3.1.4.17) in *Thermomonospora curvata* was purified and characterized. Fractionation of cell extracts by ion-exchange and size-exclusion chromatography revealed four PDE isozymes, which differed markedly in molecular weight, theophylline sensitivity, pH optima, and substrate affinity. Although the enzyme was labile after purification, total recovery of PDE activity was fivefold that of the crude extract. PDE biosynthesis appeared sensitive to the growth phase, growth rate, and carbon source. PDE levels in batch cultures peaked and declined rapidly during mid-exponential-phase growth. In continuous culture, maximal PDE and cellulase production occurred at dilution rates yielding mean cell generation times of about 5 and 17 h, respectively. The addition of glucose to cellulose-grown cells caused declines in both cyclic AMP and PDE levels, suggesting that the enzyme was subject to, rather than the agent of, catabolite repression.

Cyclic nucleotides, particularly cyclic AMP (cAMP), appear to control the rates of catabolic enzyme biosynthesis in both bacteria (6) and fungi (25). In bacteria, this control is exerted through a cAMP receptor protein (termed CRP or CAP) which is a dimer with two identical subunits, each capable of binding one molecule of cAMP; the N-terminal domain of the CRP binds the cAMP, while the C-terminal domain binds to DNA at the promoter region of a cAMP-dependent operon and stimulates transcription by promoting the formation of a preinitiation complex between RNA polymerase and DNA (10, 16).

Intracellular cAMP levels are determined by the relative rates of synthesis, degradation, and excretion. Synthesis of cAMP is catalyzed by adenylate cyclase (EC 4.6.1.1), an enzyme regulated at both the genetic and catalytic levels (26, 33) to provide sensitive mechanisms for controlling cAMP content. Excretion also appears to be an important factor in the control of intracellular cAMP levels, since most of the cAMP is found in the culture fluid (12, 18, 20, 28). However, the significance of the role played by cAMP phosphodiesterase (PDE; EC 3.1.4.17) in the degradation and control of intracellular cAMP has been difficult to evaluate. Evidence for a regulatory role in bacteria has been reported (5, 12, 21, 22), but other studies (7, 13) have raised important questions as to the relationship of PDE activity to cAMP levels. There is no evidence for compartmentalization of PDE or cAMP, yet the nucleotide persists at high concentrations within cells that have enzyme sufficient to degrade it completely. Decreased PDE activity (brought about by mutation or the presence of PDE inhibitors) results in elevation of cAMP levels and stimulation of cAMP-dependent functions in *Salmonella* spp. (2, 7). In *Thermomonospora curvata*, inhibition of PDE activity stimulates the cAMP-dependent synthesis of cellulase (34). The PDE of this actinomycete has not been described, so a study was done to characterize its activity. Here we present evidence for multiple forms of PDE in *T. curvata* and demonstrate that the growth phase, growth rate, and carbon source influenced PDE levels. These characteristics of PDE activity are in marked contrast to those of the frequently studied gram-negative bacteria (7, 23, 29) and constitute the first report on this enzyme from a thermophilic bacterium.

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MATERIALS AND METHODS

Organism and growth conditions. The strain of *T. curvata* and the conditions for its growth in minimal medium were as previously described (30). Glucose, cellobioase, and microcrystalline cellulose (Avicel; FMC Corp.) were chosen as the carbon and energy sources to provide conditions ranging from minimal to maximal levels of cAMP and cellulase biosynthesis. Growth on soluble sugars was measured by the increase in A_{610} and calculated from a standard curve in which an A_{610} reading of 1.0 corresponded to 0.59 mg of cells (dry weight) per ml. Growth in cellulose-containing medium was estimated by insoluble nitrogen by using the cell nitrogen value obtained earlier (30).

Assays. Cellulase production was measured as endo-1,4-beta-glucanase (EG; EC 3.2.1.4) activity in reaction mixtures containing 2.0% carboxymethyl cellulose (type 7L1; Hercules, Inc., Wilmington, Del.) buffered to pH 6.1 with 0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid] (U.S. Biochemical Corp.). Incubation time was 10 min at 65°C. An EG unit was defined as the liberation of 1 μmol of reducing sugar (as glucose equivalents) min^{-1}; the specific cellulase production rate (SCPR) was expressed as EG units milligram of cells (dry weight)$^{-1}$ (34). Reducing sugar was assayed by the Bernfeld method (4). The procedures for sampling of the cell mass for cAMP content, as well as for the verification of cAMP degradation to AMP in PDE reaction mixtures, were as previously described (34). PDE activity was measured by the method of Thompson et al. (32). Reaction mixtures were routinely incubated at pH 6.5 and 55°C to provide optimal conditions for PDE activity in *T. curvata* extracts. A PDE unit was defined as 1 pmol of cAMP hydrolyzed per min during 10 min of incubation. Crude cell extracts were obtained by disruption in a French press (American Instrument Co.) at 20,000 lb/in^2 in 0.1 M MES buffer (pH 6.5) containing 1 μg of DNase (Sigma Chemical Co.) per ml and clarified by centrifugation (17,000 × *g*, 10 min, 4°C). Protein was measured by the Bradford method (8). The glucose content of these extracts was determined by the coupled hexokinase method as described in Sigma Chemical Co. Diagnostics

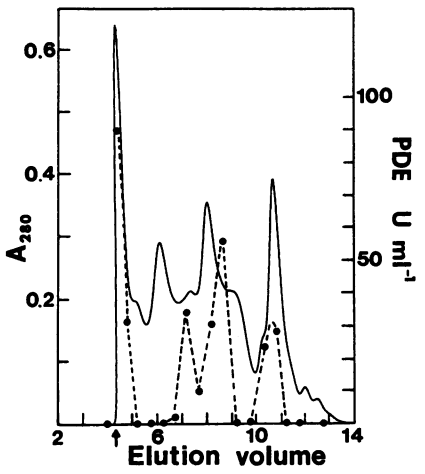

FIG. 1. Elution profile of PDE activity (●) and plotting integrator tracing (——) of A_{280} during fractionation of crude cell extracts by HPLC SEC. Column void volume was 4.4 ml (indicated by arrow). Based on results from six fractionation runs, protein recovery ranged from 92 to 98% of that applied. The PDE specific activity of purified fractions averaged 1,477 U/mg of protein (SD, ±14%) compared with 52 U/mg for the crude extract.

Research and Development Procedure no. 16-UV (Jan. 1986).

The standard deviations (SDs) for cAMP and PDE assays on a given sample were ±15.7 and ±9.0%, respectively. These values were obtained by analyzing samples three separate times, each time in duplicate. The SDs for cAMP content and PDE activity between replicate cultures were greater than those from a given sample, averaging ±20.6 and ±17.4%, respectively. Each sample was routinely assayed in triplicate. The SD was calculated, the value falling outside the SD range was discarded, and the other two values were averaged.

The presence of multiple PDE forms was determined by fractionation of clarified cell extracts by ion-exchange chromatography on a Bio-Rad DEAE-5PW column (7.5 by 75 mm) buffered to pH 7.0 with 0.1 M phosphate. Elution at 1 ml min^{-1} was by a linear 0 to 1.0 M NaCl gradient in the same buffer. Extracts were also fractionated by size-exclusion chromatography (SEC) on a Toyo Soda TSK G3000SW analytical column (7.5 by 300 mm) with 0.1 M NaCl in 0.1 M phosphate (pH 7.0) at 1 ml min^{-1}. The SEC column was calibrated for molecular weight determination by using protein standards in the Sigma MS-GF-200 marker kit. Both columns were run on a Perkin-Elmer series 4 high-performance liquid chromatography (HPLC) system with an LC-85 detector (Perkin-Elmer) set at 280 nm and an LCI-100 plotting integrator (Perkin-Elmer).

The depletion of specific sugars during growth was also measured by HPLC by using an ISCO high-pressure syringe pump set at a rate of 0.67 ml of distilled H_2O min^{-1}, a Bio-Rad HPX-87P column (300 by 7.9 mm) heated to 85°C, and an Erma ERC-7810 refractive-index detector. Peak height and area integration was done by using an LDC/Milton Roy CI-10 integrator. The limit of detection was about 150 μM in a 6-μl sample size. The average SD was ±7.6%.

Continuous culture. Operating culture volume was 330 ml stirred at 300 rpm in a New Brunswick Multigen unit kept at 52°C. Aeration rate was about 0.8 volume per volume per min. Modified minimal medium containing 1.5 mM cellobiose, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 8.2), and 0.01% gelatin (to retard wall growth) was delivered by a programmable peristaltic pump (ISCO WIZ series 1610-D04) at dilution rates (*D*) of 0.02 to 0.36 volume per volume per h. Samples (about 10 ml) were removed by suction through a tube leading to a sterile centrifuge tube. After sampling or after *D* change, at least five samples were taken to determine culture density constancy over a 24-h period before further sampling.

RESULTS

Characterization of PDE enzymes. The PDE in crude extracts of exponential-phase *T. curvata* exhibited temperature dependence characteristic of thermophilic enzymes. The temperature optimum was 55°C under our assay conditions. No loss of activity occurred when crude extracts were held for 1 h at 55°C. However, purification rendered the enzyme unstable. Fractionation on ion-exchange HPLC yielded four PDE forms. The cumulative activity of the purified enzymes, when assayed within 1 h of elution, was about 10.2-fold that of the original crude extract applied to the column, but the purified enzymes lost about two-thirds of their activity overnight at either 4°C or −80°C (data not shown). Neither 10^{-5} M $FeCl_3$ nor 2 mM dithiothreitol (which stabilized the partially purified PDE from *Escherichia coli* [23]) was effective. Fractionation by SEC also yielded four forms (Fig. 1), which were designated PDE 1 to 4 in order of their elution. These partially purified isozymes were sufficiently stable to allow characterization (Table 1).

PDE 1 had an apparent molecular weight exceeding 300,000, suggesting possible association with membrane fragments or with other cellular debris. It had the highest substrate affinity and contributed the greatest share of activity in the purified fractions. PDE 2 was unusual in that theophylline stimulated rather than inhibited the enzyme; its K_m was severalfold higher than those of the other isozymes. PDE 3 and 4 were similar in their characteristics except for the exceedingly low apparent molecular weight of the latter. Binding of PDE 4 to the silica-based column packing may account for its retardation in elution and the subsequent appearance of low molecular weight.

Extracts were also prepared from stationary-phase cells for comparison of fractionation profiles with those described above. The profiles were quite similar, with two notable exceptions, i.e., PDE 1 (the enzyme contributing the greatest recovered activity from exponential-phase cells) was absent, and the PDE 4 peak was increased relative to those of PDE 2 and 3 (data not shown). These differences in fractionation profiles between exponential- and stationary-phase cells suggest changes in aggregation state or proteolytic inactivation (for a review, see reference 31). The average specific activity (units per milligram of protein) of fractions purified from stationary-phase cell extracts was

TABLE 1. Characteristics of *T. curvata* PDE enzymes after partial purification by SEC

PDE enzyme	Mol wt	Theophylline sensitivity[a]	pH optimum	K_m (μM cAMP)	% of crude-extract activity
1	300,000	0.73	5.5	14	214
2	90,000	2.22	6.5	227	88
3	32,000	0.34	7.3	30	89
4	11,000	0.42	7.5	59	135

[a] Calculated as the activity with 0.5 mM theophylline divided by the activity of the control. Values are averages of data obtained from six replicate fractionations.

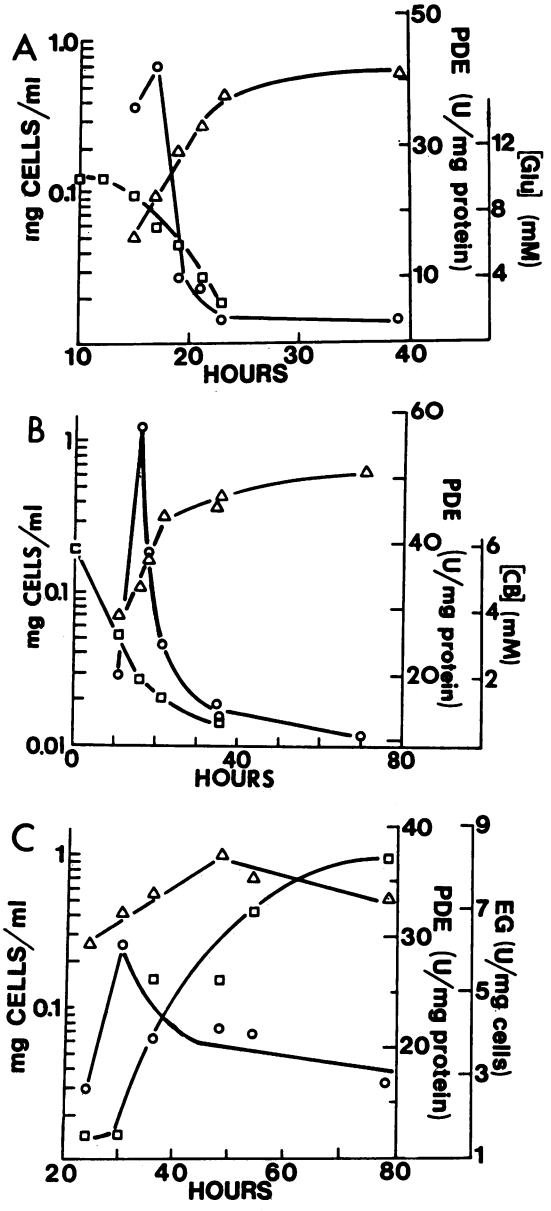

FIG. 2. Patterns of intracellular PDE levels (○) during growth (△) on glucose (A), cellobiose (B), or cellulose (C). Sugar depletion (□) in the medium is shown in panels A and B. During growth on cellulose (C), cellulase activity (□; measured as EG units per milligram of cells [dry weight]) reached high levels, but total detectable reducing sugar did not exceed 0.24 mM glucose equivalents. The extent of cellulose utilization by 78 h averaged 72%.

only 141 compared with 1,477 for those purified from exponential-phase cells. However, in both cases, total recovery of PDE activity was greater than that applied to the SEC column (5.1- and 2.7-fold for fractionations of exponential- and stationary-phase cells, respectively).

Influence of culture conditions on PDE levels. In our previous study (34), intracellular cAMP levels in *T. curvata* were highest (25 to 45 pmol/mg of cells [dry weight]) under conditions in which carbon and energy supply was growth limiting in batch cultures growing on cellulose or in continuous cultures fed a low cellobiose concentration. The cAMP levels were lowest (1 to 4 pmol/mg of cells [dry weight]) during rapid growth on soluble sugars. A comparison of PDE levels under these conditions is shown in Fig. 2.

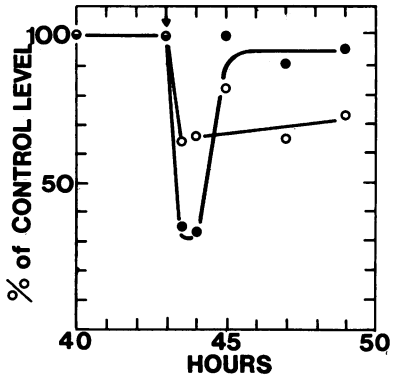

FIG. 3. Effect of glucose addition (final concentration, 10 mM [indicated by arrow]) on intracellular PDE specific activity (●) and cAMP levels (○) in *T. curvata* growing in minimal medium containing cellulose. A duplicate culture receiving no glucose was used as the control.

The growth phase had a marked influence on PDE content of *T. curvata* cells grown on soluble sugars but a lesser effect during growth on cellulose. The variations during growth on glucose and cellobiose, respectively, are shown in Fig. 2A and 2B. Peak PDE levels occurred during mid-exponential-phase growth (generation times, about 2.5 h), followed by a rapid decline. The drop in PDE content could not be attributed to carbon and energy source depletion since both glucose and cellobiose were still in ample supply (about 6 and 2 mM, respectively, as measured by HPLC) when the decrease began. SCPR values were less than 1 EG unit per mg of cells under these growth conditions (data not shown). During growth on cellulose (Fig. 2C), the SCPR reached a value of about 7 EG units per mg of cells by the end of exponential growth, but the recalcitrance of the cellulose to enzymatic attack severely limited growth (generation time, about 12 h). Under these conditions, the decrease in PDE levels was less pronounced.

During slow growth on cellulose, PDE specific activity appeared susceptible to a glucose effect (Fig. 3). After glucose addition, the following effects were apparent. (i) Intracellular PDE levels dropped rapidly. (ii) cAMP levels, although initially less affected, showed little recovery. (iii) Glucose uptake (measured by depletion in the medium as determined by HPLC analysis) persisted at a steady rate of 1.05 μmol ml^{-1} h^{-1} of culture (data not shown). The decrease in PDE specific activity in these cells after glucose addition could not be attributed to direct inhibition by the sugar since the addition of 2 mM glucose to PDE reaction mixtures containing extracts from cellulose-grown cells caused no detectable reduction in activity. Although the increase in the growth rate after glucose addition was not measured in these experiments, results from an earlier study (11) indicated that at least a doubling in growth rate would occur after glucose addition. To determine the influence of growth rate on intracellular PDE levels, we grew *T. curvata* in continuous culture with a severely limiting cellobiose concentration (approximating carbon source deprivation during growth on cellulose) and measured PDE specific activity over an 18-fold *D* range. As a comparison in this continuous-culture study, we also measured SCPR, which had earlier been shown to be *D* sensitive (34). The effect over a range of *D* from 0.02 to 0.36 is shown in Fig. 4. Over the

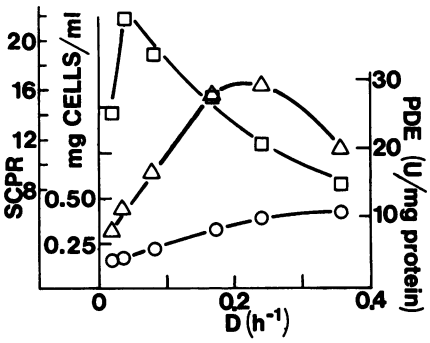

FIG. 4. Comparison of cell mass (○), SCPR (□), and intracellular PDE specific activity (△) as influenced by *D* during growth of *T. curvata* in continuous culture receiving a limiting cellobiose concentration (1.5 mM).

full range, the reducing sugar concentration of the effluent fluid remained below the level of detection (20 μg/ml). Beyond 0.36, culture washout became apparent. Theoretically, steady-state cell mass levels should have remained constant over the *D* range until washout was approached (15). However, we observed an increasing cell mass with increasing *D*. We believe this departure from the theoretical is due to the heavy energy drain imposed on the cells by biosynthesis of extracellular protein during cellulase induction. In the *D* range (0.04 to 0.08) supporting maximal cellulase induction, the concentration of soluble protein in the culture fluid was in the range of 0.36 to 0.47 mg of protein per mg of cells (dry weight) (data for extracellular protein not shown in Fig. 4). Total culture product (if taken as cell mass plus extracellular protein) was constant throughout the *D* range. The maximal SCPR and PDE specific activities occurred at mean cell generation times of about 17 and 5 h, respectively. Over the *D* range tested, the differences between the minimal and maximal SCPR and PDE specific activities were about 2.5- and 4-fold, respectively.

DISCUSSION

The existence of structurally unrelated multiple PDE forms in eucaryotes is well known (for a review, see reference 3). However, previous studies on a variety of bacteria (7, 17, 23, 24, 29) have revealed only one PDE form in each species. Therefore, the demonstration of four forms of the enzyme in *T. curvata* is unusual. These forms differ from each other in several characteristics. One of the most notable differences is that of substrate affinity; PDE 1, which contributed the greatest proportion of activity in the purified fractions, had a K_m about 1/16 that of PDE 2 and about 1/50 of the average K_m reported for the gram-negative bacteria (7, 23, 24). Since PDE 1 was not detectable in stationary-phase *T. curvata* cells, its elimination could account for a large part of the marked decrease in total PDE activity compared with that observed in mid-exponential phase. This pattern of diminished PDE specific activity in *T. curvata* is the converse of that observed in *Klebsiella* species, in which PDE specific activity increases severalfold from mid-exponential to stationary phases (9, 29). The converse nature of these patterns cannot be explained by differences in sensitivity to glucose inhibition. In *T. curvata*, as in *E. coli* (27), the catalytic activity of PDE is insensitive to glucose inhibition. PDE activity in *Klebsiella* spp. is stimulated by growth in glucose-containing medium, yet is lowest during early exponential growth, when glucose availability is maximal (29). The drop in PDE specific activity caused by glucose addition to cellulose-grown *T. curvata* cells could be explained if adenylate cyclase in this actinomycete were inhibited by glucose (as it is in *E. coli* [27]) and if PDE were inducible by cAMP (as suggested by Aboud and Burger [1]). Glucose inhibition of adenylate cyclase would lower intracellular cAMP levels and subsequently decrease the rate of PDE biosynthesis.

In carbon-limited continuous culture, the enzymatic composition of bacterial cells can be markedly altered by changes in *D*, and therefore, the technique is useful in determining the influence of growth rate on enzyme biosynthesis (19). However, to the best of our knowledge, the present report is the first to show the influence of growth rate on PDE levels in continuous culture. The establishment of an optimal growth rate for maximal PDE activity in *T. curvata* is in contrast to the currently held concept, recently expressed by Gottschalk (14), that PDE levels in bacteria remain constant under a variety of culture conditions.

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