GUANOSINE 3', 5'-CYCLIC MONOPHOSPHATE PHOSPHODIESTERASE ACTIVITY OF Bacillus Licheniformis*

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SUMMARY

Guanosine 3', 5'-cyclic monophosphate phosphodiesterase activity was detected in extracts of Bacillus licheniformis strain A-5. The enzyme is associated with the 105,000 xg supernatant fraction of the extract, requires either Mn^{++} or Ca^{++} for full activity and has a pH optimum of 7.5-8.0. Two cyclic nucleotide phosphodiesterase fractions have been separated by diethylaminoethyl - cellulose chromatography, one of which exhibits a 14-fold increase in specificity toward guanosine 3', 5'-cyclic monophosphate as compared with adenosine 3', 5'-cyclic monophosphate.

Adenosine 3', 5'-cyclic monophosphate (cAMP) is now recognized as a key intracellular regulator of a number of physiological processes. In 1963, the occurence of a second cyclic nucleotide, guanosine 3', 5'-cyclic monophosphate (cGMP) was reported (1). In Escherichia coli, cAMP is apparently required for the transcription of the genome of inducible enzymes (2, 3, 4), but it has been demonstrated that cGMP can compete with cAMP for binding to a receptor protein and prevent cAMP dependent synthesis of β-galactosidase and galactokinase in vitro (5, 6, 7). In Bacillus licheniformis extracts, cAMP was not detected, while cGMP pools ranged from 0.5 nM to 8 nM at various growth stages of the cells (8). Thus cGMP may play some regulatory role in the physiology of these and other cells.

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Guanosine 3',5'-cyclic monophosphate is formed from guanosine 5'-triphosphate by guanylate cyclase (9) and hydrolyzed by cGMP phosphodiesterase. The latter enzyme has not been reported to occur in bacteria. We would like to report some preliminary observations on the characteristics of cGMP phosphodiesterase activity in B. licheniformis.

MATERIALS AND METHODS

Growth conditions: Bacillus licheniformis strain A-5 used throughout this investigation. The cells were grown in a minimal glucose-salts medium as previously reported (10), harvested at the end of exponential growth, and cells from two liters of culture were used for the preparation of extracts.

Preparation of extracts: Extraction of the enzyme from cells and all subsequent steps were performed at 0 to 5° C. Cells were harvested by centrifugation at 3000 xg for 10 minutes and were washed with a buffer containing 10 mM Tris-HCl pH 7.7, 2 mM $MgSO_4$, 0.25 mM ethlenediaminetetraacetic acid, 1.0 mM dithiothreitol and 10% glycerol (T buffer). Packed cells were suspended in 4.0 ml of T buffer and were broken by sonic treatment by three 30-second bursts in a 20.0 Kc MSE (Measuring and Scientific Equipment, London) sonic oscillator. The broken cell suspension was centrifuged at 105,000 xg for 60 minutes. The supernatant fraction was removed and applied to a 2.2 x 12 cm sephadex G-25 column equilibrated with 50 mM Tris-HCl buffer pH 7.5. The protein was eluted with Tris-HCl buffer and the first 14 ml after the void volume was collected. Extract prepared in this manner was used as crude extract. Protein concentration was determined by the method of Lowry, et al. (11) using bovine serum albumin as a standard.

Determination of cGMP phosphodiesterase and cAMP phosphodiesterase: The standard assay contained: 1-2000 μM cGMP, 1.0 μCi 8-(^{3}H)-cGMP

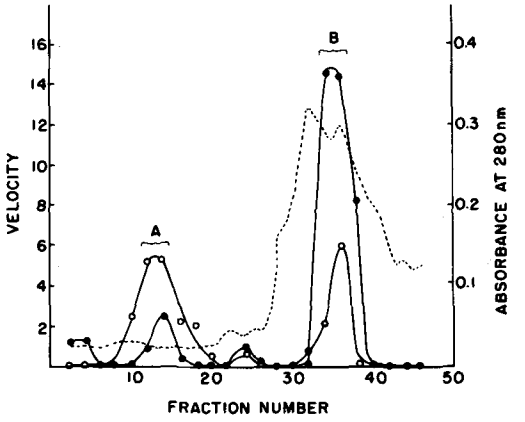

Figure 1. DEAE - cellulose chromatography of B. licheniformis cGMP phosphodiesterase and cAMP phosphodiesterase from 5 ml of crude fraction. Samples of 0.1 ml were assayed directly, each assay containing 0.1 mM cGMP or 0.1 mM cAMP. Reaction velocities are reported as pmoles cyclic nucleotide cleaved per minute: O——O, cyclic GMP phosphodiesterase; ●——● cyclic AMP phosphodiesterase; -----, absorbance at 280 nm.

(0.5 Ci/mM) purified by method of A.A. White and T.V. Zenser (12), 50 mM Tris-HCl buffer, pH 7.5, 1 mM $MnCl_2$, 2 mM $MgSO_4$, 1 unit/ml of 5'-nucleotidase and 300-900 µg of protein in a final volume of 100 to 150 µls. The assay was run for 60 min at 25° C and terminated by the addition of 2.0 ml of a 50% aqueous solution of AG 2-x8 resin. The resin was removed by centrifugation at 500 xg for 10 minutes, and radioactive guanosine in the supernatant fraction was determined using a Beckman LS-200 scintillation counter (9).

The activity of cAMP phosphodiesterase was assayed by replacing the cyclic guanylate nucleotide with the corresponding cyclic adenylate nucleotide in the method stated above.

Identification of enzyme reaction products: A two-dimensional thin layer chromatography (TLC) system was used for identification of the dephosphorylated tritiated product - guanosine. After spotting 10 µl of the assay mixture on a corner of a square of silica gel TLC (3 x 3 cm), the sheet was

developed for 1 hr using a solvent consisting of n-propanol: NH_4OH: H_2O (6:3:1). After drying for 1 hr the sheet was turned 90 degrees and developed for 1 hr using a solvent containing n-butanol: H_2O (86:14). This 2-dimensional system completely separated cGMP from guanosine and other gyanylate nucleotides. After the chromatograms were thoroughly dried, the compounds were detected with UV light and cut from the TLC sheet. Each piece was placed in a scintillation vial, 10 ml of scintillation fluid was added and the radioactivity of the sample was determined as above.

DEAE- cellulose column chromatography: The crude extract fraction (5 ml, 120 mg protein) was applied to a 1.5 x 20 cm DEAE-cellulose (DE-52) column equilibrated with T buffer. Using a flow rate of 8.0 ml/hr, the column was washed with 300 ml of T buffer, followed by a gradient ranging from 0-0.8 M of Na-acetate in T buffer. Protein was monitored by UV absorbance and fractions (2.5 ml) were assayed for cGMP phosphodiesterase and cAMP phosphodiesterase activity.

Materials: The tritiated cGMP and cAMP was purchased from Schwartz Bioresearch. Non-radioactive cGMP, cAMP and 5'-nucleotidase (Crotalus adamanteus venom) were purchased from Sigma Chemical Company. Sephadex G-25 resin was obtained from Pharmacia while AG 2-x8 resin was purchased from Bio-Rad Laboratories. DEAE-cellulose (DE-52) was obtained from Whatman. Silica gel TLC plates were obtained from Eastman Kodak Company.

RESULTS AND DISCUSSION

Guanosine 3', 5'-monophosphate phosphodiesterase activity in B. licheniformis cells resides primarily (>90%) in the 105,000 xg supernatant fraction. The enzyme activity was linear with respect to protein concentration and time of assay. Maximum activity of the enzyme is observed at a pH of 7.5 and the activity is highest when a Tris-HCl buffer is used. Significant

activity is observed when imidazole or glycylglycine buffer is used, but phosphate buffer inhibits the enzyme reaction.

Of the divalent cations tested, either Mn^{++} or Ca^{++} produces optimum activity of cGMP phosphodiesterase (12 pmoles x min^{-1} x mg^{-1} protein) when [cGMP] is 0.4 mM, while Mg^{++} supports only 25% of this optimum activity. Mn^{++} but not Ca^{++} supports optimum activity of cAMP phosphodiesterase. This is different from the mammalian system, where the cGMP phosphodiesterase activity is best satisfied by Mg^{++}, although Mn^{++} can substitute (13).

Column fractions from DEAE-cellulose chromatography were assayed for cGMP and cAMP phosphodiesterase activity and two major fractions were found having both enzyme activities (Figure 1). Fraction A eluted at 0.24 M and fraction B eluted at 0.61 M Na-acetate. The ratio of cGMP phosphodiesterase activity to cAMP phosphodiesterase activity in crude extract is 0.14. The ratio of the phosphodiesterases of fraction A is 2.06 whereas fraction B is 0.42. Thus fraction A appears to be purified toward cGMP phosphodiesterase activity.

Rat liver (14) appears to contain an activity capable of hydrolyzing cGMP but not the more prevalent nucleotide cAMP. It has been reported that partially purified cAMP phosphodiesterase preparations also hydrolyze cGMP (15, 16, 17) and that cGMP can affect the rate of hydrolysis of cAMP (18, 19). In certain cases, cAMP inhibited the hydrolysis of cGMP and it was suggested that the two cyclic nucleotides were hydrolyzed by one enzyme that existed in several forms with different affinities for cGMP and cAMP (17, 20). From our results of metal ion requirement and DEAE-cellulose column fraction enzyme activities, it is possible that B. licheniformis contains 2 forms of

phosphodiesterases, one of which can be purified toward an increased specificity for cGMP.

In crude extracts, cGMP phosphodiesterase activity was found to have apparent K_m values ranging from 92-973 μM. A variable K_m value of cGMP phosphodiesterase was found in bovine heart (18, 21), bovine liver (22) and rat liver (14).

Using fraction A (Figure 1), cAMP at 2 mM inhibited cGMP phosphodiesterase activity at various cGMP concentrations (0.02 to 2 mM), exhibiting a 50% inhibition at 20 μM cGMP. The enzyme activity was also inhibited (at 0.5 mM cGMP) by 0.1 mM AMP, fructose-6-phosphate, or fructose-1, 6-bisphosphate but not inhibited by glutamine, L-glutamate, α-keto-glutarate, citrate, succinate at 1 mM and phosphenolpyruvate at 0.1 mM.

The levels of both guanylate cyclase and cGMP phosphodiesterase activity in B. licheniformis remain constant throughout the growth cycle (23). Since the intracellular pool concentration of cGMP in B. licheniformis ranges from 0.5-8.0 nM (8), regulation of cGMP pools by regulation of cGMP phosphodiesterase (K_m >90 μM) is unlikely. Thus, regulation of the activity of the guanylate cyclase of this microorganism (9) is presently under further investigation.

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