JOURNAL OF BACTERIOLOGY, Nov. 1975, p. 693-703

Vol. 124, No. 2
Printed in U.S.A.

Enzymology and Genetic Regulation of a Cyclic Nucleotide-Binding Phosphodiesterase-Phosphomonoesterase from *Aspergillus nidulans*

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Received for publication 16 June 1975

A cyclic nucleotide-binding phosphohydrolase that possesses both a phosphomonoesterase and a phosphodiesterase catalytic function has been partially purified from *Aspergillus nidulans*. The enzyme hydrolyzes both *p*-nitrophenylphosphate and bis-(*p*-nitrophenyl)-phosphate. 3'-Nucleoside monophosphates are the best physiological phosphomonesterase substrates but 5'- and 2'-nucleoside monophosphates are also hydrolyzed. The enzyme catalyzes the hydrolysis of adenosine 5'-triphosphate, adenosine 5'-diphosphate, and 2',3'- and 3',5'-cyclic nucleotides, but not of ribonucleic acid, deoxyribonucleic acid, or nicotinamide adenine dinucleotide. The enzyme has acid pH optima and is not activated by divalent cations. Nucleosides and nucleotides inhibit the enzyme. Cyclic nucleotides are competitive inhibitors of the phosphodiesterase-phosphomonoesterase. The enzyme can occur extracellularly. The phosphodiesterase-phosphomonoesterase is present at high levels in nitrogen-starved mycelium, and it is strongly repressed during growth in media containing ammonium or glutamine and weakly repressed during growth in glutamate-containing medium. Experiments with various *areA* mutants show that this regulatory gene is involved in the control of the enzyme. No evidence for regulation of the enzyme by carbon or phosphorus starvation has been found.

Evidence is accumulating for the involvement of 3',5'-cyclic adenosine monophosphate (AMP) in regulation of metabolism, gene expression, and differentiation in fungal cells. Adenyl cyclase (13, 39, 40) and 3',5'-cyclic AMP phosphodiesterases (14, 27, 35, 37, 40) have been described in fungal cells, allowing the construction of regulatory models involving a transient increase in 3', 5'-cyclic AMP concentration as a biochemical signaling device. 3',5'-Cyclic AMP has been implicated in regulation of glycogenolysis in *Neurospora* (12), and the demonstration of a 3',5'-cyclic AMP-dependent protein kinase in *Saccharomyces* (38) suggests that phosphorylation of particular enzymes may be involved in metabolic regulation in fungal cells as in mammalian cells (24). In addition, fungal cells may possess a 3',5'-cyclic AMP-mediated process for transcription of catabolite-repressible genes of the kind found in procaryotic cells (29). A protein that cross-reacts with antibodies to the *Escherichia coli* 3',5'-cyclic AMP receptor protein has been detected in *Saccharomyces* (2) and physiological studies have indicated that 3',5'-cyclic AMP may be involved in the regulation of catabolite-sensitive enzyme synthesis (34). Extracellular 3',5'-cyclic AMP-dependent processes demonstrated in fungi include the promotion of conidial aggregation (42) and the promotion of citric acid accumulation (41).

Thus, it appears that several regulatory mechanisms involving 3',5'-cyclic AMP that have appeared to be peculiar to procaryotic or eucaryotic cells may be present together in fungal cells. This raises the problem of differential activation of these mechanisms at times of different 3',5'-cyclic AMP concentration within fungal cells. An additional complexity is introduced if one considers the genetic regulation of the synthesis of cyclic nucleotide-binding proteins, including those proteins that can modulate intraceilular (or extracellular) cyclic nucleotide concentration.

In the course of studying the universality of a cyclic nucleotide-binding phosphatase from higher plants that is very similar in many properties to cyclic AMP receptor protein (31, 32), a cyclic nucleotide-binding phosphomonoesterase was detected in *Aspergillus nidulans*. This enzyme also has a phosphodiesterase catalytic function. Many enzymes of nitrogen catabolism are repressed by ammonium and other nitrogen-containing metabolites (5, 8, 12, 16), and it has been shown that the *areA* gene is an impor-

tant regulatory gene involved in regulation of gene expression by such metabolites (5, 20). Since this phosphodiesterase-phosphomonoesterase can occur extracellularly and would be involved in extracellular processing of nucleotides, the regulation of this enzyme by nitrogen metabolites was studied. This paper describes the enzymology and genetic regulation of this *A. nidulans* cyclic nucleotide-binding phosphodiesterase-phosphomonoesterase.

MATERIALS AND METHODS

Strains. Details of the strains of *A. nidulans* used are described in Table 1.

Media and growth conditions. The standard minimal medium used was that of Cove (9), where 1% (wt/vol) glucose is the sole carbon source and no nitrogen source is present. Nitrogen sources and vitamin supplements were added to the medium as required. L-Glutamate was added as sodium L-glutamate, and ammonium was added as either ammonium chloride or ammonium tartrate. For carbon starvation treatments glucose was omitted from the medium. For the phosphate starvation treatments the phosphorus-free medium of Cohen (8) was used. Mycelium was grown at 30 C in a Gallenkamp orbital incubator as described previously (20). Harvested mycelium was stored frozen at −15 C. For extracellular enzyme determinations medium was collected and filtered once through Whatman no. 1 filter paper and stored at 4 C. A weighed sample of pressed dry mycelium was dried overnight at 45 C, the dry weight was determined, and the total dry weight of mycelium was calculated.

Enzyme assays. Enzymes were assayed at 30 C. Phosphatase was assayed in a standard medium (1 ml) containing 1 mM substrate in 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$. With *p*-nitrophenylphosphate as substrate, the reaction was terminated by addition of 2 ml of 0.1 M NaOH, and the *p*-nitrophenol was estimated from the absorbance at 400 nm. With other phosphate esters as substrates, reactions were terminated by addition of 12% perchloric acid and inorganic orthophosphate was measured by the method of Allen (1). Phosphodiesterase was assayed in the standard medium containing 1 mM bis-(*p*-nitrophenyl)phosphate. Reactions were terminated by the addition of alkali and the *p*-nitrophenol was determined as described above. Since *p*-nitrophenylphosphate is a product of this reaction and is a substrate for the monoesterase, phosphodiesterase could be overestimated except when initial rates were determined (see Table 3).

TABLE 1. *Details of A. nidulans areA strains used*

Strain	Genotype[a]	Reference
Wild type	*biA1; niiA4*	30
areA102	*biA1; areA102; niiA4*	22
areA217	*yA1; areA217; riboB2*	(M. J. Hynes,
areA200	*biA1; areA200; niiA4*	Aust. J. Biol. Sci., in press)
areA19	*biA1; areA19; niiA4*	17

[a] *biA1*, Biotin auxotrophy; *niiA4*, lack of nitrite reductase; *yA1*, yellow conidia; *riboB2*, riboflavin auxotrophy.

3′,5′-Cyclic AMP phosphodiesterase was determined in a 100-μl medium containing 1 mM 3′,5′-cyclic AMP, 0.1 M acetate (Na^+, pH 5.0), 4 mM $MgCl_2$, and 5 μCi of 3′,5′-cyclic [8-^{3}H]AMP (specific activity 27.5 Ci/mmol) per ml. Samples (10 μl) were taken at various times after addition of enzyme and spotted onto polyethyleneimine thin-layer sheets. The polyethyleneimine thin-layer sheets were prepared as described by Randerath and Randerath (33). The thin-layer sheets were first developed by ascending chromatography with distilled water to separate adenosine from adenine nucleotides. The sheets were dried and then developed by ascending chromatography with a solvent composed of 5% boric acid in 0.5 M ammonium acetate, pH 7.5. The overall chromatographic procedure satisfactorily resolves 5′-AMP, 3′-AMP, 3′,5′-cyclic AMP, and adenosine from each other. Spots were detected under ultraviolet light, cut out, and counted at 6% efficiency using a scintillation fluid containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazolyl)benzene in toluene. 3′,5′-Cyclic guanosine 5′-monophosphate (GMP) phosphodiesterase was assayed in the same fashion.

Phosphodiesterase activity with nicotinamide adenine dinucleotide (NAD^+) as substrate was assayed in the standard medium containing 1 mM NAD^+ and 3.1 μCi of [U-^{14}C]NAD^+ per ml (specific activity 274 mCi/mmol). Samples (10 μl) were spotted onto polyethyleneimine thin-layer sheets which were then developed by ascending chromatography in 5% boric acid-0.5 M ammonium acetate, pH 7.5. The AMP and NAD^+ spots were detected, cut out, and counted as described above.

Phosphodiesterase activities with 2′,3′-cyclic AMP and 2′,3′-cyclic GMP as substrates were determined using assay media containing 1 mM substrate in the standard assay medium. Samples were spotted onto polyethyleneimine thin layers, and the products of the reaction were resolved by ascending chromatography with water and then with 0.5 M ammonium acetate, pH 7.5. The products were eluted from the thin layers with 1 ml of 0.7 M $MgCl_2$ in 2 mM tris(hydroxymethyl)aminomethane (Tris) (Cl^-, pH 7.5), and nucleotide and nucleoside concentrations were determined spectrophotometrically.

Ribonuclease was assayed by the method of Shortman (36), using yeast ribonucleic acid (0.1 mg/ml) as substrate in the standard assay medium. Deoxyribonuclease was assayed by the method of Kunitz (25), using calf thymus deoxyribonucleic acid (0.1 mg/ml) as substrate in the standard assay medium.

All enzyme assays were routinely conducted in duplicate and were corrected by the application of appropriate zero time controls and other controls as applicable. Protein was determined by the method of Lowry et al. (28), using bovine serum albumin as a standard. Rates of substrate hydrolysis were constant during the incubation periods employed in the enzyme assays.

Starch gel electrophoresis. Starch gel electro-

phoresis was carried out as described previously (32). The buffers used in the cathode and anode chambers were 0.2 M glycine-0.2 M Tris (pH 8.8) or 0.2 M borate-0.2 M ammediol (pH 8.8). Fivefold dilutions of these buffers were used to prepare the corresponding starch gels.

Extraction and analysis of enzymes in genetic experiments. Mycelia were initially thawed in 20 to 40 ml of a medium composed of 0.5 M ammonium sulfate, 1 mM sodium ethylenediaminetetraacetate (EDTA), 0.03% 2-mercaptoethanol, and 10 mM Tris (Cl^-, pH 7.5). The mycelia were dispersed by blending with an Ultra Turrax blender (Janke and Kunkel KG, Staufen, West Germany) for 15 s and were then disrupted at a pressure of 62×10^6 N/m^2 in a French press (Douglas Engineering, Melbourne, Australia). The resulting suspensions were centrifuged at 30,000 × *g* for 15 min at 0 to 4 C and the supernatants were recovered for enzymatic analysis.

Phosphodiesterase was routinely assayed with 1 mM bis-(*p*-nitrophenyl)phosphate as substrate in 0.1 M acetate (Na^+, pH 4.0) and 4 mM $MgCl_2$. Phosphomonoesterase was routinely assayed with 1 mM *p*-nitrophenylphosphate as substrate in 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$. Phosphodiesterase and phosphomonoesterase were assayed in growth experiments in the presence and absence of 0.5 mM 3',5'-cyclic AMP and 5 mM theophylline. Intracellular levels of phosphodiesterase and phosphomonoesterase were determined as specific activities on a protein basis, extracellular levels as specific activities on a dry weight basis. Absolute phosphodiesterase activities could be overestimated by this procedure as mentioned previously.

Materials. All nucleotides, nucleosides, phosphate esters, polynucleotides, and staining reagents were obtained from the Sigma Chemical Company. Sephadex G-150 was obtained from Pharmacia, and agarose A15 (50 to 100 mesh) and phosphocellulose (Cellex-P, 0.93 meq/g) from Bio Rad Laboratories. 3',5'-Cyclic [8-^{3}H]AMP (27.5 Ci/mmol), 3',5'-cyclic [8-^{3}H]GMP (13.0 Ci/mmol), and [U-^{14}C]NAD^+ (274 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. All other reagents were of analytical reagent grade. Miracloth was obtained from Calbiochem, polyethyleneimine (Polymin P) from B.A.S.F. (Australia), Avicel PH-101 cellulose from J. Beith and Co., Melbourne, and electrostarch from Electrostarch Co., Madison, Wisc.

Purification of the phosphodiesterase-phosphomonoesterase. All operations were carried out at 0 to 4 C. Mycelium (4 to 12 g) of wild-type *A. nidulans* (Table 1) was grown on standard minimal medium containing ammonium for 16 h and then grown for 4 h in the absence of ammonium as described above. The harvested mycelia were dispersed in 50 to 100 ml of a buffer containing 0.5 M ammonium sulfate, 50 mM Tris (Cl^-, pH 7.5), 1 mM sodium EDTA, and 0.03% 2-mercaptoethanol by blending for 15 s, by using an Ultra Turrax blender. This suspension of mycelia was subjected to disruption in a French pressure cell at a pressure of 62×10^6 N/m^2. The resulting homogenate was centrifuged at 30,000 × *g* for 15 min in the SS-34 rotor of a Sorvall centrifuge, and the supernatant from this centrifugation was taken as the starting point for the purification procedure.

The supernatant was fractionated by ammonium sulfate precipitation and the phosphodiesterase-phosphomonoesterase was recovered in the 60 to 100% ammonium sulfate saturation fractions. The precipitated enzyme was dissolved in about 5 ml of buffer A (0.5 M ammonium sulfate, 10 mM Tris [Cl^-, pH 7.5], 1 mM sodium EDTA, and 0.03% 2-mercaptoethanol) and applied to an agarose A15 (50 to 100 mesh) column (2 by 75 cm) equilibrated with buffer A. The phosphodiesterase-phosphomonoesterase elutes as a single broad peak between the two protein peaks of very high-molecular-weight material (eluting at the void volume, the exclusion limit for agarose A15 being 15×10^6) and low-molecular-weight ($<10^5$) material (Fig. 1). The agarose A15 column was calibrated using Blue Dextran 2000, thyroglobulin, gamma globulin, bovine serum albumin, cytochrome *c*, and myoglobin as standards. The active fractions were pooled, the enzyme was precipitated by addition of ammonium sulfate to 100% saturation, and the precipitated protein was collected by centrifugation and taken up in 3 ml of buffer A. The concentrated enzyme was applied to a Sephadex G-150 column (2 by 75 cm) equilibrated with buffer A. The column was eluted with buffer A at the rate of 0.2 ml per min and the phosphodiesterase-phosphomonoesterase was eluted near the void volume of the column. The most active fractions (specific activities were in the range 6 to 12 μmol/mg of protein per min) were pooled and stored at 4 C for kinetic analysis. The purification schedule is shown in Table 2.

RESULTS

Partial purification of the phosphodiesterase-phosphomonoesterase. Application of the procedure described above results in a 244-fold purification of the enzyme and a 62% recovery from the initial high-speed supernatant. The success of the purification scheme derives from the molecular weight of the enzyme (about

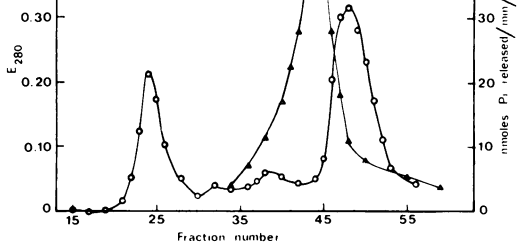

FIG. 1. *Elution of the phosphodiesterase-phosphomonoesterase from agarose A-15. Absorbance at 280 nm, ○; phosphatase, △.*

TABLE 2. *Purification of the A. nidulans phosphodiesterase-phosphomonoesterase*

Purification step	Total activity (μmol/min[a])	Total protein (mg)	Sp act (μmol/min per mg of protein)
Supernatant	10.0	194.9	0.05
60 to 100% $(NH_4)_2SO_4$ precipitate	6.9	24.9	0.28
Agarose (15 m)	7.6	10.6	0.72
Sephadex G-150	6.2	0.5	12.20

[a] The purification of the enzyme was followed by measuring the *p*-nitrophenylphosphatase inhibited by 1 mM 3',5'-cyclic AMP in the standard assay conditions described.

2×10^5) so that major purification from very high-molecular-weight proteins and nucleic acids and from low-molecular-weight ($<10^5$) proteins is achieved by gel filtration on agarose A15 (exclusion limit: 15×10^6) (Fig. 1). Attempts were made to purify the enzyme by ion-exchange chromatography. The enzyme is not retained by phosphocellulose at pH 8.0 but loses most of its catalytic activity after passage through a phosphocellulose column at low ionic strength. However this inactivation was reversible, and after concentrating by precipitation with ammonium sulfate, activity was restored. Another 3',5'-cyclic AMP-binding protein which undergoes reversible inactivation after phosphocellulose chromatography is the 3', 5'-cyclic AMP receptor protein of *E. coli* (3). The enzyme was not purified further because of the small amounts of protein remaining after the final step (Table 2) and because of this reversible inactivation after ion-exchange chromatography.

Only one phosphatase band was detected after electrophoresis of the final preparations of the enzyme in starch gels as described above. This zone of activity was inhibited by inclusion of theophylline or 3', 5'-cyclic AMP in the staining reagent. Only one peak of phosphatase was obtained after gel filtration of the final preparation of the enzyme through agarose A15; single and coincident peaks of 3',5'-cyclic AMP and theophylline-sensitive hydrolytic activity were obtained with *p*-nitrophenylphosphate and bis-(*p*-nitrophenyl)phosphate as substrates. These peaks also coincided exactly with peaks of hydrolytic activity with 3'-GMP, 5'-AMP, 2'-AMP, adenosine 5'-triphosphate, adenosine 5'-diphosphate, and 3',5'-cyclic AMP as substrates.

Substrate specificity of the phosphodiesterase-phosphomonesterase. The pH optima of the enzyme with bis-(*p*-nitrophenyl)phosphate, *p*-nitrophenylphosphate, and 3'-AMP as substrates are 4.0, 4.8, and 5.5, respectively. The pH activity profiles for all three substrates are very similar, though displaced along the pH axis: with all three substrates 50% of the maximum activity is observed at pH values 0.7 pH units above the pH optimum (Fig. 2). All substrates examined yielded acid pH optima. pH 5.0 was arbitrarily chosen as a compromise pH at which to examine the substrate specificity of the enzyme (Table 3).

3'-Ribonucleoside monophosphates are the best substrates at pH 5.0 and, with the exceptions of 5'-AMP, 5'-cytidine 5'-monophosphate, and 2'-AMP, 5'- and 2'-ribonucleoside monophosphates are hydrolyzed at very low rates compared to the rate of hydrolysis of *p*-nitrophenylphosphate in the conditions used. Although ribose 5'-phosphate is hydrolyzed by the enzyme, no phosphomonoesterase activity was observed with glucose 6'-phosphate as a substrate. The enzyme has a phosphodiesterase catalytic function, catalyzing the hydrolysis of bis-(*p*-nitrophenyl)phosphate, adenosine 5'-triphosphate, adenosine 5'-diphosphate, 2',3'-cyclic nucleotides, and 3',5'-cyclic nucleotides. However no activity was detected with NAD^+, ribonucleic acid, or deoxyribonucleic acid as substrates (Table 3).

3',5'-Cyclic AMP is hydrolyzed at a 10-fold greater rate than 3',5'-cyclic GMP (Table 3). With both of these substrates the immediate

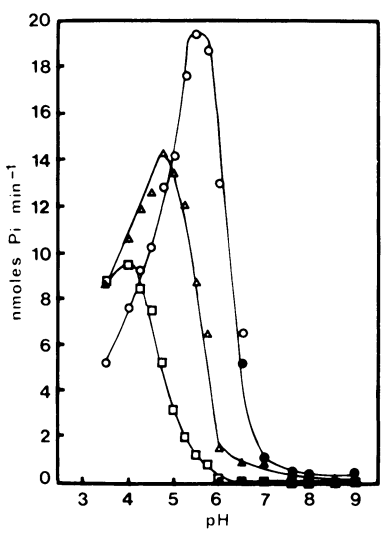

FIG. 2. *pH activity profiles for the phosphodiesterase-phosphomonoesterase. The pH of the assay medium was maintained using a 0.1 M acetate buffer (open symbols) or a 0.1 M Tris buffer (closed symbols). Substrates: bis-(p-nitrophenyl)phosphate* (□, ■); *p-nitrophenylphosphate* (△, ▲); *3'-AMP* (○, ●).

TABLE 3. *Substrate specificity of the A. nidulans phosphodiesterase-phosphomonoesterase*

Substrate[a]	Relative rate of hydrolysis[b]
p-Nitrophenylphosphate	100.0
3'-GMP	103.8
3'-CMP	93.4
3'-TMP	83.0
3'-AMP	82.0
3'-UMP	33.7
5'-AMP	37.9
5'-CMP	33.2
5'-UMP	3.5
5'-TMP	2.8
5'-IMP	2.1
5'-GMP	0.5
2'-AMP	15.6
2'-UMP	1.9
2'-CMP	1.8
2'-GMP	1.1
Ribose 5'-phosphate	2.7
Glucose 6'-phosphate	0.0
bis-(*p*-Nitrophenyl)phosphate	16.7
ATP	9.5
ADP	7.3
2',3'-cyclic AMP	11.4
2',3'-cyclic GMP	4.2
3',5'-cyclic AMP	2.3
3',5'-cyclic GMP	0.2
NAD^+	0.0
RNA	0.0
DNA	0.0

[a] All assays were conducted in a medium containing 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$ as described in Materials and Methods. The concentrations of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were both 0.1 mg/ml. All other substrates were present at 1 mM concentration. CMP, Cytidine monophosphate; TMP, thymidine monophosphate; UMP, uridine monophosphate; IMP, inosine monophosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

[b] Rates of hydrolysis are given relative to the rate of hydrolysis of *p*-nitrophenylphosphate in the same conditions. Initial rates of hydrolysis were determined from time courses of hydrolysis of the various substrates.

product of the reaction catalyzed by the phosphodiesterase-phosphomonoesterase is the corresponding 5'-ribonucleoside monophosphate.

Cation effects. The hydrolysis of *p*-nitrophenylphosphate by the phosphodiesterase-phosphomonoesterase at pH 5.0 was not stimulated by the addition of divalent cations (Table 4). Whereas inclusion of 1 mM Pb^{2+} in the assay had no effect on hydrolysis, inclusions of 1 mM concentrations of Cu^{2+}, Zn^{2+}, and Hg^{2+} were inhibitory. Inclusion of 1 mM sodium EDTA in the assay did not inhibit the enzyme.

Inhibitors of the phosphodiesterase-phosphomonesterase. Fluoride, arsenate, molybdate, diisopropylfluorophosphate, and phosphate inhibited both the phosphodiesterase and phosphomonoesterase functions of the enzyme, but pyrophosphate had a much smaller effect. The methyl xanthines theophylline, caffeine, and theobromine, which inhibit mammalian cyclic nucleotide phosphodiesterases (4), inhibited both catalytic functions of the enzyme (Table 5). Although nucleotidases from a variety of sources are inhibited by some amino acids or by tartrate (11), inhibition of the phosphodiesterase-phosphomonoesterase was not observed with inclusion of tartrate or any of the 20 L-amino acids in the assay. Neither 0.1 mM *N*-ethylmaleimide, iodoacetate, or 0.1 mM *p*-chloromercuribenzoate had any significant inhibitory effect, suggesting the absence of a functional thiol in the catalytic mechanism.

The phosphodiesterase is inhibited by a variety of nucleotides and their degradation products. A wide variety of pyrimidines and purines, including cytokinins, were tested as possible inhibitors of the enzyme in view of the inhibition of a cyclic nucleotide phosphodiesterase by cytokinins (15). None of these bases were inhibitory at 0.1 mM concentration. At 1 mM concentration, adenine and thymine inhibited the enzyme significantly but uracil, guanine, and cytosine had no effect (Table 6). In contrast, corresponding nucleosides could be much more effective inhibitors of the phosphodiesterase-phosphomonoesterase (Table 6). The order of effectiveness of the major ribonucleosides as inhibitors of the enzyme is G>A>C>I>U. Mod-

TABLE 4. *Effect of divalent cations on the A. nidulans phosphomonoesterase activity*

Cation added[a]	Relative activity[b]
None	100.0
Mn^{2+}	106.5
Mg^{2+}	87.5
Cu^{2+}	70.3
Ni^{2+}	104.2
Co^{2+}	101.0
Zn^{2+}	54.6
Fe^{2+}	91.5
Ba^{2+}	86.8
Ca^{2+}	89.3
Pb^{2+}	95.5
Hg^{2+}	37.0

[a] Cations were added to the assay medium as Cl^- salts to a final concentration of 1 mM.

[b] Rates of hydrolysis of *p*-nitrophenylphosphate were determined as described in Materials and Methods and are expressed relative to the rate with no added divalent cation.

TABLE 5. *Inhibition of the A. nidulans phosphodiesterase-phosphomonoesterase*

Addition	Relative phosphodiesterase activity[a]	Relative phosphomonoesterase activity[b]
None	100	100
Potassium pyrophosphate (1 mM)	82	95
Sodium phosphate (1 mM)	35	71
Sodium fluoride (1 mM)	8	11
Sodium arsenate (1 mM)	40	62
Ammonium molybdate (1 mM)	3	8
Theophylline (5 mM)	20	21
Caffeine (5 mM)	26	31
Theobromine (2.5 mM)	72	66
Diisopropylfluorophosphate (5 mM)	59	35

[a] Phosphodiesterase was assayed with 1 mM bis-(*p*-nitrophenyl)phosphate as substrate at pH 4.0 as described.

[b] Phosphomonoesterase was assayed with 1 mM *p*-nitrophenylphosphate as substrate at pH 5.0 as described.

ification of adenosine in the 6-position has a large effect on inhibitory effectiveness, thus adenosine > inosine > kinetin ribose > *N*-6-dimethylallyladenosine > *N*-6-dimethyladenosine > *N*-6-benzyladenosine and zeatin riboside in terms of inhibition of the enzyme by 1 mM concentrations of these nucleosides. Although the ribose moiety is crucial to the recognition of these compounds by the enzyme, modification of the ribose can have either little effect (cf. adenosine, 2'-deoxyadenosine, and 2',3'-isopropylideneadenosine) or a large effect (cf. guanosine and 2',3'-isopropylideneguanosine) depending upon the base moiety.

A wide range of nucleotides inhibit the phosphodiesterase-phosphomonoesterase (Table 7). With phosphorylation to various degrees in the 5'-position of nucleosides a common pattern of base-specificity is observed. Thus for nucleosides and for nucleoside 5'-monophosphates, diphosphates, and triphosphates, the order of effectiveness as inhibitors follows the same pattern of base specificity, namely G>A>C>I>U (Tables 6 and 7). A similar pattern (G>A>I >C>U) is obtained for the 3',5'-cyclic nucleotide series (Table 7). This pattern of base specificity is altered when the 2'- and 3'-hydroxyls are substituted or modified in nucleosides (Table 6) or in 2'-nucleoside monophosphates, 3'-nucleoside monophosphates, or 2'-deoxy 5'-nucleoside monophosphates (Table 7).

Kinetic parameters and competitive inhibition by cyclic nucleotides. The K_m values for *p*-nitrophenylphosphate and bis-(*p*-nitrophenyl)phosphate at pH 5.0 were 1.04 and 7.41 mM, respectively. 3',5'-Cyclic AMP and 3',5'-cyclic GMP act as competitive inhibitors. Plots of reciprocal velocity ($1/v$) against reciprocal of substrate concentration ($1/S$) in the presence or absence of these cyclic nucleotides have common intercepts on the $1/v$ axis. In addition, there is a linear relation between $1/v$ and cyclic nucleotide concentration at a given substrate concentration. The phosphodiesterase-phosphomonoesterase has K_i values of 18.6 and 12.70 μM for 3',5'-cyclic AMP and 3',5'-cyclic GMP, respectively.

Repression of the levels of the phosphodiesterase-phosphomonoesterase by nitrogen metabolites. Levels of the phosphodiesterase-phosphomonoesterase could be specifically quantitated in growth experiments by assaying for the two catalytic functions of the enzyme and the sensitivity of both activities to 3',5'-cyclic AMP and theophylline (Table 5). This

TABLE 6. *Inhibition of the phosphomonoesterase activity by purines, pyrimidines, and nucleosides*

Addition[a]	Relative activity[b]
None	100
Guanosine	14.7
Adenosine	22.1
2'-Deoxyadenosine	25.5
2'-Deoxycytidine	27.7
2',3'-Isopropylideneadenosine	30.1
Cytidine	31.3
Inosine	50.3
Kinetin riboside	65.6
N-6-dimethylallyladenosine	72.7
2',3'-Isopropylideneguanosine	76.4
N-6-dimethyladenosine	82.0
N-6-benzyladenosine	83.6
Zeatin riboside	84.0
Uridine	85.6
Thymidine	94.0
2'-Deoxyuridine	98.5
None	100
Adenine	52.3
Hypoxanthine	72.7
Thymine	75.4
Uracil	95.0
Guanine	99.3
Cytosine	103.8

[a] Purines, pyrimidines, and nucleosides were added to give a final concentration of 1 mM in the assay.

[b] Relative phosphomonoesterase activities with 1 mM *p*-nitrophenylphosphate as substrate were determined as described.

TABLE 7. *Inhibition of the phosphomonoesterase activity by nucleotides*

Addition[a]	Relative activity[b]	Addition[a]	Relative activity[b]
None	100.0	None	100.0
2'-CMP	16.6	2'-d-5'-GMP	5.6
2'-UMP	22.0	2'-d-5'-CMP	19.4
2'-AMP	23.3	2'-d-5'-TMP	76.7
2'-GMP	44.6	2'-d-5'-UMP	81.9
		2'-d-5'-AMP	97.0
3'-AMP	14.4	2',3'-Cyclic CMP	46.8
3'-CMP	15.6	2',3'-Cyclic AMP	49.1
3'-GMP	18.8	2',3'-Cyclic GMP	49.6
3'-TMP	69.3	2',3'-Cyclic UMP	82.5
3'-UMP	78.2		
5'-GMP	12.0	GTP	14.1
5'-AMP	17.5	ATP	20.7
5'-CMP	33.5	CTP	36.5
5'-IMP	49.6	ITP	53.3
5'-TMP	76.7	XTP	93.7
5'-UMP	92.5	UTP	80.2
GDP	11.1	3',5'-Cyclic GMP	10.9
ADP	19.6	3',5'-Cyclic AMP	12.8
CDP	22.9	2'-Deoxy 3',5'-cyclic AMP	15.1
IDP	54.3	3',5'-Cyclic IMP	26.3
XDP	92.0	3',5'-Cyclic CMP	33.9
UDP	94.5	N^6,$O^{2'}$-dibutyryl cyclic AMP	76.0
		3',5'-Cyclic UMP	94.9
		3',5'-Cyclic TMP	99.3

[a] Nucleotides were added to give a final concentration of 1 mM in the assay. CMP, Cytidine monophosphate; TMP, thymidine monophosphate; UMP, uridine monophosphate; IMP, inosine monophosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GDP, guanosine diphosphate; CDP, cytidine diphosphate; IDP, inosine diphosphate; XDP, xanthine diphosphate; UDP, uridine diphosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; ITP, inosine triphosphate; XTP, xanthine triphosphate; UTP, uridine triphosphate.

[b] Relative phosphomonoesterase activities with 1 mM *p*-nitrophenylphosphate as substrate were determined as described.

assay procedure thus provided multiple checks in the derepression experiments to be described below.

When wild-type *A. nidulans* was transferred to a medium containing no nitrogen source, the level of extracellular phosphodiesterase-phosphomonoesterase rose to a maximum level over 4 to 6 h (Fig. 3a). This rise in extracellular levels was paralleled by a rise in intracellular levels of the enzyme over the same period (Fig. 3b). Both intracellularly and extracellularly, the changes in total phosphodiesterase-phosphomonoesterase are paralleled by the 3',5'-cyclic AMP- or theophylline-sensitive activities. When 10 mM NH_4^+ was included in the medium, the extracellular level of the enzyme rose over 16 h to approximately 25% of the level obtained when the mycelia grew for only 4 h in the absence of NH_4^+.

The effect of addition of NH_4^+ or cycloheximide to the growth medium in this type of experiment is shown in Fig. 4. Inclusion of NH_4^+ or cycloheximide from the beginning of the growth period blocked the increase in extracellular levels of the phosphodiesterase-phosphomonoesterase observed in the absence of NH_4^+. Addition of NH_4^+ or cycloheximide in the middle of the growth period largely prevents further increase in the extracellular level of the enzyme. The changes in the level of the enzyme as measured by 3',5'-cyclic AMP-sensitive phosphodiesterase (Fig. 4a) are paralleled by the changes in the levels of 3',5'-cyclic AMP-sensitive phosphatase (Fig. 4b). Neither NH_4^+ nor cycloheximide affects the catalytic activity of the enzyme in purified or crude preparations.

Growth of wild-type mycelium in medium in which glutamate was the sole source of nitro-

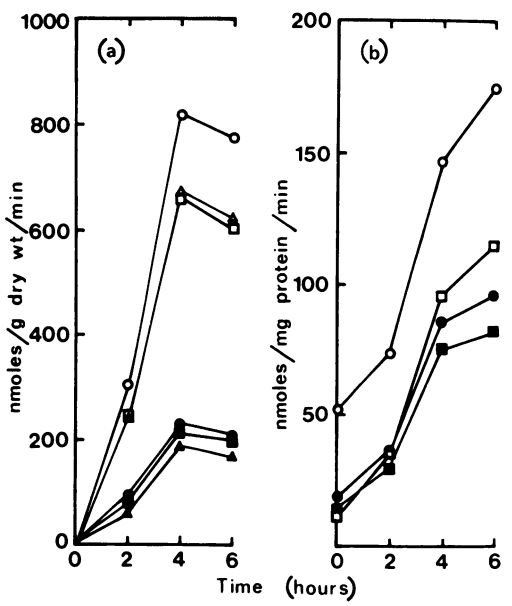

FIG. 3. *Induction of the phosphodiesterase-phosphomonoesterase in wild-type A. nidulans. The extracellular levels of the enzyme are shown in (a), the intracellular levels in (b). Open symbols, phosphomonoesterase; closed symbols, phosphodiesterase. Total activity, ○, ●; theophylline-inhibited activity, △, ▲; 3',5'-cyclic AMP-inhibited activity, □, ■. All assays were conducted in duplicate with appropriate duplicated controls. Mycelium was transferred to ammonium-free medium at zero hour.*

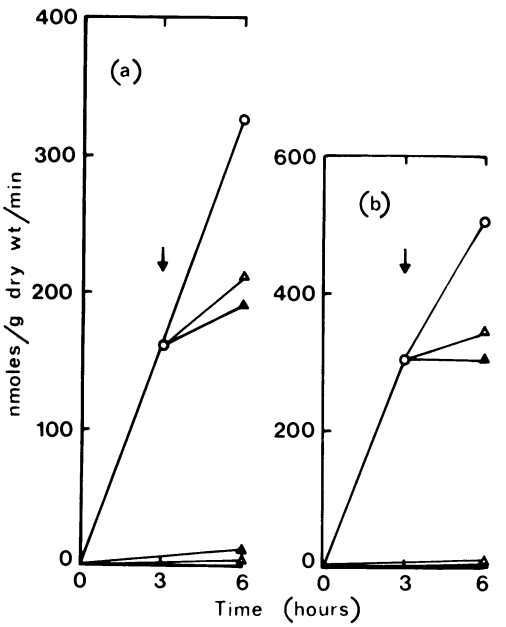

FIG. 4. *Effect of added ammonium ions and cycloheximide on the extracellular levels of the phosphodiesterase-phosphomonoesterase of wild-type A. nidulans grown in conditions of ammonium deprivation. Extracellular 3',5'-cyclic AMP-inhibited phosphodiesterase (a); extracellular 3',5'-cyclic AMP-inhibited phosphomonoesterase (b). Treatments: ○, no ammonium; △, 10 mM ammonium added at zero time or at 3 h (arrowed); ▲, cycloheximide (15 μg/ml) added at zero time or at 3 h (arrowed). All assays were conducted in duplicate.*

gen led to approximately 55 to 70% lower enzyme levels than present in nitrogen-starved mycelium. When glutamine was the sole nitrogen source extremely strong repression was observed (Table 8).

Effects of areA lesions on enzyme levels. Since it has been shown that the *areA* gene is an important regulatory gene involved in regulation of gene expression in *Aspergillus* by nitrogen-containing metabolites (5, 20), the level of the phosphodiesterase-phosphomonoesterase in a variety of *areA* mutant strains was examined. When strain *areA217* was grown in the same ammonium-deprivation conditions as the wild-type control in the experiment shown in Fig. 3, no significant increases in the extracellular or intracellular levels of 3',5'-cyclic AMP- or theophylline-sensitive phosphodiesterase-phosphomonoesterase were detected over the same 6-h period. As shown in Table 8, nitrogen starvation failed to increase the intracellular or extracellular levels of the phosphodiesterase-phosphomonoesterase in the mutants *areA102*, *areA217*, and *areA19* compared with the wild-type control. However, with mutant *areA200* nitrogen starvation caused a major increase in the levels of the enzyme, although to a lower level than obtained with the wild-type control (Table 8).

Effect of carbon starvation on the phosphodiesterase-phosphomonoesterase. The levels of some enzymes of *A. nidulans* have been observed to increase when mycelium is starved for carbon (8, 16–18). Therefore the effect of carbon starvation on the enzyme was investigated and it was found that ammonium repression still occurred in the absence of a carbon source (Table 8). Under conditions of starvation for both carbon and nitrogen, the enzyme level was increased but did not reach the levels present in mycelium starved for nitrogen only (Table 8). A mutant *cre*-4, apparently altered in some aspects of catabolite repression (Hynes, unpublished data), produced similar levels of the enzyme to the wild-type strain (data not shown). Therefore, there is no evidence for regulation of the enzyme by catabolite repression.

Effect of phosphate starvation on the phosphodiesterase-phosphomonoesterase. *A. nidulans* has been observed to synthesize a number of acid and alkaline phosphohydrolases under conditions of phosphate starvation (10). However, phosphate starvation in the presence of ammonium did not cause any increase in the intracellular or extracellular levels of the phosphodiesterase-phosphomonoesterase above those observed with 10 mM NH_4^+ or cycloheximide added to the medium (Table 8). This failure of phosphate deprivation to derepress the enzyme in the presence of ammonium was observed with the wild-type and with the *areA102* and *areA217* strains.

A component of the large phosphatase activity induced in these experiments by phosphate starvation was inhibited by 0.5 mM 3',5'-cyclic AMP (Table 8) but this represents only ~5% of the total phosphatase. It remains to be determined whether this component represents a phosphatase that is very sensitive to 3',5'-cyclic AMP or merely a small inhibition of the major phosphatases that are induced by phosphate deprivation.

Further experiments involving phosphate starvation of wild-type, *areA102*, and *areA217* strains failed to demonstrate increases in the levels of the phosphodiesterase-phosphomonoesterase either in the medium or intracellularly. Starch gel electrophoresis of growth media or mycelial extracts from such experiments failed to reveal any of the enzyme. Therefore, it appears that the phosphodiesterase-phosphomonoesterase, unlike other *Aspergillus* phospha-

TABLE 8. *Effect of growth conditions on the levels of intracellular and extracellular phosphodiesterase-phosphomonoesterase in various areA mutant strains*

Final growth conditions[a]	Cyclic AMP-inhibited phosphodiesterase[b]		Cyclic AMP-inhibited phosphomonoesterase[c]		Final growth conditions[a]	Cyclic AMP-inhibited phosphodiesterase[b]		Cyclic AMP-inhibited phosphomonoesterase[c]	
	Extracellular[d]	Intracellular[e]	Extracellular[d]	Intracellular[e]		Extracellular[d]	Intracellular[e]	Extracellular[d]	Intracellular[e]
Wild type					No carbon source + 10 mM NH_4^+	5	1	0	1
10 mM NH_4^+	19	2	4	6	Phosphate starvation + 10 mM NH_4^+	5	—	2,082	—
No nitrogen source	625	155	990	208	*areA217*				
No carbon source + 10 mM NH_4^+	23	6	25	8	No nitrogen source	7	3	0	4
Phosphate starvation + 10 mM NH_4^+	14	—	1,255	—	No carbon source + 10 mM NH_4^+	17	3	15	1
No carbon or nitrogen source	138	19	231	40	Phosphate starvation + 10 mM NH_4^+	19	—	1,541	—
10 mM L-glutamate	265	51	478	72	*areA19*				
10 mM L-glutamine	6	3	8	4	No nitrogen source	3	1	1	3
No nitrogen source + cycloheximide	5	7	7	9	*areA200*				
areA102					No nitrogen source	168	58	347	84
No nitrogen source	4	1	2	2					

[a] Mycelium was grown for 16 h in 10 mM NH_4^+ medium and then transferred to this final medium for 4 h before harvesting mycelium and collecting medium. In the cases of phosphate starvation treatments mycelium was grown for 16 h in 10 mM NH_4^+ medium with low phosphate (8) and then transferred to medium lacking phosphate for 4 h before collecting medium. NH_4^+ was added as ammonium chloride. In media with no carbon source, glucose was not present in the medium. Cycloheximide was present at a concentration of 15 μg per ml.

[b] Cyclic AMP-inhibited phosphodiesterase was measured at 30 C in an assay medium containing 1 mM bis-(*p*-nitrophenyl)phosphate, 0.1 M acetate (Na^+, pH 4.0), and 4 mM $MgCl_2$ as described. Assays were conducted in the presence and absence of 0.5 mM 3',5'-cyclic AMP.

[c] Cyclic AMP-inhibited phosphomonoesterase was measured at 30 C in an assay medium containing 1 mM *p*-nitrophenylphosphate, 0.1 M acetate (Na^+, pH 5.0), and 4 mM $MgCl_2$, as described. Assays were conducted in the presence and absence of 0.5 mM 3',5'-cyclic AMP.

[d] The extracellular specific activity is expressed as nanomoles per gram (dry weight) per minute.

[e] The intracellular specific activity is expressed as nanomoles per milligram of protein per minute.

tases, is not subject to phosphate repression. In some phosphate-deprivation experiments a phosphodiesterase activity was induced that was inhibited by 3',5'-cyclic AMP but not by theophylline, and that was not associated with 3',5'-cyclic AMP- or theophylline-sensitive phosphomonoesterase. The nature of this particular enzyme remains to be resolved.

DISCUSSION

The *A. nidulans* phosphodiesterase-phosphomonoesterase is very similar in its properties to acid phosphatase II from the phytopathogenic fungus *Fusarium moniliforme* (14). Both enzymes have phosphodiesterase and phosphomonoesterase catalytic functions which are not activated by divalent cations, have acid pH optima, and are inhibited by fluoride, arsenate, molybdate, phosphate, and diisopropylfluorophosphate but not by reagents that react with sulfhydryl groups. The two enzymes have very similar substrate specificities and are inhibited by a wide range of polynucleotide degradation products. Both enzymes are secreted into the culture medium.

The *A. nidulans* and *F. moniliforme* enzymes are inhibited by methyl xanthines as are cyclic nucleotide phosphodiesterases from *Saccharomyces carlsbergensis* (27, 37) and *Neurospora crassa* (35). However the latter enzymes have pH optima in the range 7.4 to 8.5 and have not been reported to catalyze a phosphomonoesterase reaction. Extracellular phosphodiesterases that also have phosphomonoesterase activity have been isolated from bacterial growth media (7); however, these enzymes do not appear to be homologous to the phosphodiesterase-phosphomonoesterase from *A. nidulans*.

3',5'-Cyclic AMP-binding phosphomonoesterases are present in higher plants (31, 32) and in *E. coli* (Polya, unpublished data), but these enzymes have no phosphodiesterase catalytic function.

Results described in this paper demonstrate that synthesis of the *A. nidulans* phosphodiesterase-phosphomonoesterase is subject to ammonium repression and is regulated by the product of the *areA* regulatory gene. Arst and Cove (5) and Hynes (20) have proposed that the *areA* gene product is required for the synthesis of nitrogen-catabolic enzymes and that ammonium repression involves a blocking of the positive regulatory function of the *areA* gene product. Three *areA* mutants do not produce significant amounts of enzyme whereas a fourth mutant, *areA200*, has significant levels, although these are not as high as those of the wild type. Similar differential effects on the levels of ammonium-repressible enzymes have been observed with other *areA* mutants (5; M. J. Hynes, Aust. J. Biol. Sci., in press). The enzyme is regulated by the amino acids glutamate and glutamine in a similar way to a number of enzymes of nitrogen catabolism (20).

No evidence was found for catabolite repression of the enzyme. However, the carbon starvation experiments yielded a particularly interesting result in that ammonium repression still occurred in the absence of a carbon source. This contrasts with the situation for some other catabolic enzymes. Two amidase enzymes and a glutamate uptake system are not subject to ammonium repression in the absence of a carbon source (16–18), whereas a third amidase enzyme (Hynes, J. Gen. Microbiol., in press), histidase (Polkinghorne and Hynes, Genet. Res., in press) and nitrate reductase (19), are apparently not synthesized in the absence of a source of carbon. The fact that some enzymes are synthesized in the absence of a carbon source makes it unlikely that this is a general physiological effect. It has been observed that $NADP^+$-specific L-glutamate dehydrogenase activities of *A. nidulans* are rapidly lost with carbon starvation (21). This enzyme has been implicated in ammonium repression (6, 20, 23). Therefore, the possibility that carbon starvation, in itself, abolishes ammonium repression has been raised (21). The results presented here concerning ammonium repression of the phosphodiesterase-phosphomonoesterase now make this rather unlikely. No regulation of the enzyme by phosphorus starvation could be demonstrated. Thus, this particular phosphatase is under distinct and separate regulation from other fungal phosphatases that are subject to phosphorus regulation (10, 26). The possibility that some of these described phosphatases are subject to multiple controls by carbon and/or nitrogen as well as phosphorus should perhaps be considered.

The physiological rationale for nitrogen metabolite control of this enzyme may be that the enzyme functions extracellularly to provide nucleosides for translocation into the fungal cell in conditions of low nitrogen status. Like the phosphatase II of *F. moniliforme* (14), the *A. nidulans* phosphodiesterase-phosphomonoesterase is secreted into the medium. Under conditions of nitrogen starvation approximately 40% of this enzyme produced by wild-type *A. nidulans* is secreted into the culture medium. Extracellular 3',5'-cyclic AMP promotes aggregation of conidia (42) and citric acid accumulation by *A. niger* (41). However it is not yet known to what extent the type of fungal phosphodiesterase described in this paper is significant in modulating extracellular or intracellular 3',5'-cyclic AMP concentrations. The physiological role of this enzyme and the enzymology and genetic regulation of other cyclic nucleotide phosphodiesterases in *A. nidulans* are being further investigated.

ACKNOWLEDGMENTS

This work was supported by grants to G.M.P. and M.J.H. from the Australian Research Grants Committee.

Acknowledgment is also due to Lefi Petrou, Jan Dickinson, and Christine Mitchem for technical assistance.

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