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Regulation of intracellular cyclic AMP levels in the white-rot fungus *Phanerochaete chrysosporium* during the onset of idiophasic metabolism

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Abstract. Adenylate cyclase activity in *Phanerochaete chrysosporium* was present in cell fractions sedimenting at $1{,}000 \times g$, $15{,}000 \times g$ and $150{,}000 \times g$, and in the $150{,}000 \times g$ supernatant. A small amount of activity in the $1{,}000 \times g$ pellet could be solubilised by treatment with Triton X-100, and the enzyme in all fractions required an ATP-Mn^{2+} substrate. Adenylate cyclase activity in the $150{,}000 \times g$ pellet was low (0.003 nmol/mg protein · min) and may have resulted from contamination by other fractions. Highest adenylate cyclase specific activity (0.37 nmol/mg protein · min) was recorded in the $150{,}000 \times g$ supernatant at the onset of idiophasic metabolism. During this growth phase, adenylate cyclase activity also increased in the $1{,}000 \times g$ pellet and was maximally 4.5-fold greater than that in primary phase cultures. No significant cAMP-phosphodiesterase activity could be detected during growth in any of the cell fractions or in the growth medium with either Mn^{2+}, Mg^{2+}, or Ca^{2+} as added cations. The extracellular cAMP concentration increased logarithmically during primary growth; however, in cultures in idiophasic metabolism cAMP levels remained constant and relatively low. We suggest that excretion into the medium is the principal means by which intracellular cAMP levels are decreased in *P. chrysosporium*.

Key words: Adenylate cyclase – Cyclic AMP-phosphodiesterase – Idiophasic metabolism – *Phanerochaete chrysosporium* – White-rot fungus – Extracellular cAMP

The onset of lignin degradation and idiophasic metabolism in the white-rot fungus *Phanerochaete chrysosporium* are synchronous events (Keyser et al. 1978). However, the mechanisms which control the switch from pathways of primary to idiophasic metabolism have yet to be elucidated. We have observed that intracellular levels of cAMP increase dramatically prior to the onset of idiophasic activities, and that levels of this nucleotide decrease as the fungus re-enters a non-ligninolytic growth phase (MacDonald et al. 1984). Enzymes controlling intracellular cAMP levels may therefore have a central role in the regulation of metabolism in *P. chrysosporium*.

The synthesis of cAMP is catalysed by adenylate cyclase (EC 4.6.1.1) and the enzyme is generally regarded as the most important regulator of cAMP levels. Fungal adenylate cyclase activity has been found in association with cell membranes (Flawia and Torres 1972), although in some species only soluble activity has been detected (Uno and Ishikawa 1973; Wold and Suzuki 1974). Cyclic AMP can be enzymatically removed from the intracellular pool by the action of cAMP-phosphodiesterases. These enzymes are typically membrane-bound in fungi and their activity is dependent upon Mn^{2+} or Mg^{2+} (Pall 1981).

As part of the attempt to evaluate the role of cAMP and related enzymes in the growth and differentiation of *P. chrysosporium*, the activities of adenylate cyclase and cAMP-phosphodiesterase during growth were studied. In addition, extracellular levels of cAMP were examined since excretion of the nucleotide may also form part of its cellular regulation.

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Abbreviation: EB, extraction buffer

Materials and methods

Organism and culture conditions. Stocks of *Phanerochaete chrysosporium* Burdsall ME-446 (ATCC 34541) were maintained on 2% (w/v) malt extract slopes at 4 °C, following initial growth and sporulation at 37° C for 5 days. It had previously been determined that in a medium containing 2.4 mM $NH_4H_2PO_4$ the transition to idiophasic metabolism takes place after 4–5 days growth; with 24 mM $NH_4H_2PO_4$, idiophasic metabolism was not induced (MacDonald et al. 1984). For growth studies, each 250 ml culture flask contained 50 ml of medium consisting of 56 mM glucose, 1.47 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.09 mM $CaCl_2 \cdot 2H_2O$, 10 mM sodium 2,2-dimethylsuccinate, 25 µl of vitamin solution (Kirk et al. 1978), 25 µl of mineral salts solution (Jeffries et al. 1981), with either 2.4 or 24 mM $NH_4H_2PO_4$. After sterilization the media were inoculated with 1 ml of a spore suspension (approx. 3×10^5 spores/ml) in distilled water, and cultures were incubated standing at 37° C prior to cell fractionation.

Preparation of cell fractionation for enzyme assays. The mycelia from each flask were gently filtered under suction and washed with 2×20 ml portions of prewarmed (37° C) extraction buffer (EB) containing 20 mM TES and 0.1% (v/v) 2-mercaptoethanol, pH 7.2. Portions of mycelium (approx. 1 g wet weight) were disrupted initially using a Teflon pestle and glass homogenizing vessel with 3 ml EB at 4° C, and then in a J4-3338 Aminco French pressure cell at 6894.76 kN/m^2. The lysate was centrifuged at $1{,}000 \times g$ for 5 min in an MSE Minor S centrifuge, and the pellet washed with 10 ml EB and centrifuged again at $1{,}000 \times g$ for 5 min.

The two supernatants were combined. The pellet was resuspended in 5 ml EB and termed the $1,000 \times g$ fraction; it was presumed to represent a fraction containing cell walls and associated plasma membranes. The $1,000 \times g$ supernatant was further centrifuged at $15,000 \times g$ for 20 min, and the pellet washed with 10 ml EB and centrifuged again for 20 min. The pellet was resuspended in 3 ml EB and termed the $15,000 \times g$ fraction; it was presumed to contain cell wall fragments and mitochondria. The $15,000 \times g$ supernatant was further centrifuged at $150,000 \times g$ for 2 h in a model L5-50B Beckman ultracentrifuge and the pellet, representing the $150,000 \times g$ fraction (unpurified membranes), was washed with 3×5 ml portions of EB. The pellet resulting from the final $150,000 \times g$ sedimentation was resuspended in 3 ml EB, and all particulate cellular fractions were stored at $-70°$ C in EB at protein concentrations of $5-20$ mg/ml prior to assay for adenylate cyclase and cAMP-phosphodiesterase activity.

Cyclic AMP assay. The concentration of cAMP in culture supernatants was determined using a cyclic AMP assay kit (Amersham). After removal of mycelium from the growth medium as described, 5 mM EDTA was immediately added to a 1 ml aliquot of the filtrates to prevent enzymatic degradation of cAMP, and the preparation was cooled to 4° C. The filtrates were then passed through Millipore filters (GSWP, 0.22 μm) to remove hyphal fragments, and stored at $-70°$ C prior to assay for cAMP. Concentrations of cAMP were assayed in duplicate.

Assay for adenylate cyclase. Adenylate cyclase was assayed as described previously (Houslay et al. 1976) in a mixture with final concentrations of 2.7 mM ATP, 9 mM $MnSO_4$, 18 mM theophylline, 1.8 mM EDTA, 52 mM of phosphocreatine, 1.8 mg creatine kinase/ml (EC 2.7.3.2; 72 units/mg), and 45 mM triethanolamine HCl, adjusted to a final pH of 7.4 with KOH. Samples for assay were prepared in triplicate.

Assay for cAMP-phosphodiesterase. This was carried out using the two-step radioassay method which has been described in detail by Marchmont and Houslay (1980). In the first stage, 100 μl of a reaction mixture containing (final concentrations), 5 mM $MgCl_2$, cyclic [^{3}H] AMP (approx 100,000 cpm), 40 mM Tris/HCl buffer, final pH 7.4, was used. The mixture was incubated at 30° C for 10 min and the reaction terminated by boiling for 3 min prior to the second stage conversion of 5′-AMP to adenosine by *Ophiophagus hannah* venom as described (Marchmont and Houslay 1980). All assays were carried out in duplicate.

Protein determination. Soluble and membrane-bound proteins were assayed with a Bio-Rad protein assay kit (Bradford 1976). Membrane proteins were solubilised by treatment with 0.045% (v/v) final concentration of sodium dodecyl sulphate (SDS). This concentration of SDS did not interfere with the protein assay. Serum albumin was used as the standard.

Results

Adenylate cyclase activity. Adenylate cyclase activity was present in those fractions which sedimented at $1,000 \times g$, $15,000 \times g$, and $150,000 \times g$, and in the $150,000 \times g$ supernatant. Table 1 shows the specific activity and subcellular distribution of the enzyme from 2 days old cultures growing in 24 mM $NH_4H_2PO_4$. More than 70% of the total activity was recovered in the $150,000 \times g$ supernatant, although some activity was also associated with cell wall material. The enzyme reaction in the pellets sedimenting at $1,000 \times g$ and $15,000 \times g$, and in the $150,000 \times g$ supernatant, required Mn^{2+} for maximal activity (Table 2) and 50 mM $MnCl_2$ was optimal; with 9 mM $MnCl_2$, activity was increased by between $20-30\%$ of that when 9 mM $MgCl_2$ was used. Treatment of the $1,000 \times g$ pellet with 0.5% Triton X-100 resulted in an 11% increase in adenylate cyclase specific activity. However, addition of the cardioactive diterpene forskolin, which has been shown to activate adenylate cyclase in some mammalian systems (Seamon and Daly 1983), did not affect activity significantly.

Highest adenylate cyclase activity ($0.28-0.37$ nmol/mg protein · min) was found associated with the $1,000 \times g$ pellet and $150,000 \times g$ supernatant during idiophasic growth (Fig. 1 A, B). In both fractions activity remained relatively constant during primary growth (those cultures which initially contained 24 mM $NH_4H_2PO_4$). However, at the onset of idiophasic metabolism (between days 4 and 5) there was a rise in adenylate cyclase activity; by day 7 activity was 4.5-fold greater than that of the primary phase cultures.

Adenylate cyclase activity in the $15,000 \times g$ and $150,000 \times g$ pellets ($0.004-0.01$ nmol/mg protein · min) was much less than that found in the other fractions, and levels were not affected significantly by the nitrogen regime (Fig. 1 C, D). In the $150,000 \times g$ pellet, highest activity was recorded during the early stages of growth (days 2 and 3), following which there was a rapid fall in levels; by day 7 activity had dropped to between 1.5 and 4.2% of that recorded initially. This low adenylate cyclase activity may have been due to contamination by the supernatant from the $150,000 \times g$ sedimentation despite washing the pellet in three changes of buffer to prevent this.

Cyclic AMP-phosphodiesterase activity. Cyclic AMP-phosphodiesterase activity could not be detected in any of the cell-associated fractions, or in the growth medium. Initially, a substrate concentration of 0.1 μM cAMP was used in the cAMP-phosphodiesterase assay. Subsequent attempts to detect enzyme activity by using cAMP concentrations of 1 μM, 50 μM and 250 μM proved unsuccessful. Neither could cAMP-phosphodiesterase activity be detected in any of the fractions when 5 mM $MgCl_2$ was replaced with 5 mM $MnCl_2$ or 5 mM $CaCl_2$.

To examine the possibility that inhibitors present in the cell fraction preparations were interfering with the assay, peripheral high affinity cAMP-phosphodiesterase [Km (cGMP)/Km (cAMP) = 170:1] from rat liver plasma membranes (Marchmont et al. 1981) was added to the sample preparations of each fraction, and the fractions were assayed for activity in the manner described. The cAMP-phosphodiesterase activity detected in the cell fractions was comparable to the controls (cAMP-phosphodiesterase with either EB or distilled water) confirming that no inhibitory substances, specific to this enzyme, were present in the sample preparation.

Extracellular cAMP levels. The extracellular concentration of cAMP in cultures with excess nitrogen increased with

Table 1. Specific activity and subcellular distribution of adenylate cyclase from *Phanerochaete chrysosporium*. Approximately 5 g wet wt of fungal mycelium from 2 days old cultures in growth medium initially containing 24 mM $NH_4H_2PO_4$ were homogenized and disrupted in a French pressure cell as described under Materials and methods. The crude extract thus obtained was submitted to differential centrifugation and each fraction was assayed for adenylate cyclase activity under standard conditions. The sum of the activities of fractions was taken as 100%

	Protein (mg)	Total enzyme (nmol/min)	Specific activity (nmol/mg protein · min)	Distribution (%)
1,000 × *g* pellet	2.43	0.19	0.08	24.9
15,000 × *g* pellet	1.92	0.01	0.005	1.54
150,000 × *g* pellet	1.26	0.005	0.004	0.57
150,000 × *g* supernatant	7.98	0.57	0.07	72.9

Table 2. Effect of assay conditions on adenylate cyclase activity in particulate cellular fractions sedimenting at 1,000 × *g*, 15,000 × *g*, and 150,000 × *g*, and the supernatant resulting from the 150,000 × *g* sedimentation. The fractions obtained after differential centrifugation of disrupted mycelia of 7 days old cultures in growth medium initially containing 2.4 mM $NH_4H_2PO_4$, were assayed for adenylate cyclase activity using standard incubation conditions, except for the indicated changes

Assay supplements	Adenylate cyclase activity (nmol/mg protein · min)			
	1,000 × *g* pellet	15,000 × *g* pellet	150,000 × *g* pellet	150,000 × *g* supernatant
9 mM $MgCl_2$	0.67	0.003	0.001	0.89
9 mM $MnCl_2$	0.97	0.005	0.002	1.12
20 mM $MnCl_2$	2.32	0.005	0.002	2.82
50 mM $MnCl_2$	2.71	0.008	0.003	3.74
50 mM $MnCl_2$ + forskolin	2.70	0.008	0.003	3.60
50 mM $MnCl_2$ + 0.5% Triton X-100	3.01	0.009	0.003	–

growth and reached a maximum concentration of 0.27 µM after 5 days. No such increase was observed under conditions of nitrogen limitation where levels of cAMP, after a slight initial rise, remained constant (Fig. 2).

Discussion

We have studied the activities of adenylate cyclase and cAMP-phosphodiesterase, and changes in the extracellular cAMP concentration, during primary and idiophasic growth phases of *Phanerochaete chrysosporium*. Adenylate cyclase activity was located in all particulate cellular fractions and in the supernatant which resulted from the 150,000 × *g* sedimentation. Highest adenylate cyclase specific activity was present as a soluble form in the 150,000 × *g* supernatant. This activity is comparable to that found in other fungi (Flawia and Torres 1972; Gomes et al. 1978) and is of the same order of magnitude as the maximally stimulated mammalian adenylate cyclase (Steer and Wood 1979; Webster and Olsson 1981). In all mammalian tissues adenylate cyclase is considered to be membrane-bound and Mg^{2+}-dependent. However, its activity in *P. chrysosporium*, as in some other fungi (Uno and Ishikawa 1973; Wold and Suzuki 1974), requires an ATP-Mn^{2+} substrate and appears to be predominantly non-membrane associated.

The adenylate cyclase specific activity present in the particulate cellular fractions sedimenting at 1,000 × *g* and 15,000 × *g* was lower than that in the 150,000 × *g* supernatant. The 1,000 × *g* pellet consists mainly of cell wall fragments, a proportion of which may be associated with plasma membranes. The 15,000 × *g* pellet is presumed to contain some cell wall material as well as mitochondria. The adenylate cyclase activity present in these fractions was therefore most probably associated with a plasma membrane protein; a slight increase in enzyme activity resulting from Triton X-100 treatment of the 1,000 × *g* and 15,000 × *g* pellets suggests that some adenylate cyclase activity was truly membrane-associated. In *Mucor rouxii*, a similar treatment of the particulate cellular fraction sedimenting at low seed resulted in a 70% solubilisation of adenylate cyclase activity (Cantore et al. 1980).

Of particular significance were the observations that adenylate cyclase activity in the cell extract and cell wall fractions increased markedly in nitrogen-exhausted cultures. This rise in adenylate cyclase activity correlated closely with the increase in intracellular cAMP levels at the onset of idiophasic metabolism reported previously; total levels of the nucleotide in idiophasic cultures were 3–4 times higher than in cultures in primary metabolism (MacDonald et al. 1984). Therefore, it seems likely that cultures of *P. chrysosporium* entering idiophasic metabolism increase cAMP by increasing adenylate cyclase specific activity. In a study of cyto-differentiation in *Blastocladiella emersonii* (Gomes et al. 1978), adenylate cyclase activity was also found to fluctuate according to the phases of growth. Activity was barely detectable during vegetative growth and increased 70-fold at the onset of sporulation.

Cyclic AMP-phosphodiesterase activity was not detected in any of the cellular fractions nor in the growth medium of *P. chrysosporium*. The absence of cAMP-phosphodiesterase activity has been noted in other investigations of fractionated fungal cells, and may be common in some prokaryotes.

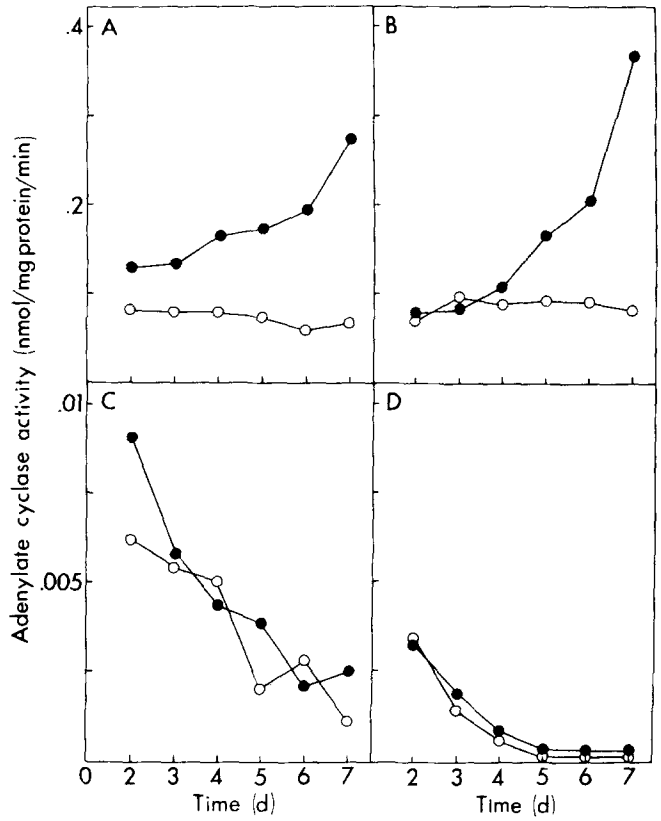

Fig. 1A–D. Adenylate cyclase activity in subcellular fractions during growth of *Phanerochaete chrysosporium* in 2.4 mM $NH_4H_2PO_4$ medium (●) and 24 mM $NH_4H_2PO_4$ medium (○): **A** 1,000 × *g* pellet; **B** 150,000 × *g* supernatant; **C** 15,000 × *g* pellet; **D** 150,000 × *g* pellet

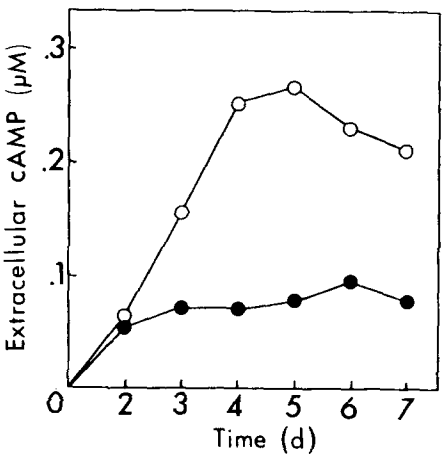

Fig. 2. Extracellular cAMP production by *P. chrysosporium* during growth in 2.4 mM $NH_4H_2PO_4$ medium (●) and 24 mM $NH_4H_2PO_4$ medium (○)

Those organisms which lack a cAMP-phosphodiesterase may regulate internal cAMP levels by extracellular secretion of cAMP. Thus far, there is no evidence for the existence of phosphodiesterase in Streptomycetes and it is noteworthy that in *Streptomyces granaticolor* 90% of the total cAMP was found in the growth medium (Dobrová et al. 1984).

In *P. chrysosporium*, we found that the extracellular concentration of cAMP increased logarithmically during primary growth. The increase in extracellular cAMP levels followed the increase in mycelial dry weight that we observed previously (MacDonald et al. 1984) so that cAMP was excreted at a constant rate per unit cell mass. No such increase was evident under conditions of nitrogen exhaustion when extracellular cAMP remained at relatively low constant levels during idiophasic growth. The levels of cAMP recorded in the growth medium probably represent the total cAMP lost from the cells since no cAMP-phosphodiesterase activity was detected in the medium during the growth period. In addition, there was no significant loss of cAMP when the filtered growth medium was incubated at 37° C for 48 h (data not shown). We can assume, therefore, that the low levels of extracellular cAMP observed in nitrogen-exhausted cultures are the result of a decrease in the rate of cAMP efflux from cells. How the rate of cAMP efflux is regulated in response to depletion of exogenous nitrogen, and the possible extracellular function of the nucleotide, are points for further study.

Our observations suggest that both adenylate cyclase and the release of cAMP into the medium participate in the regulation of cAMP levels during the onset of idiophasic metabolism. In the absence of evidence for the existence of cAMP-phosphodiesterase activity in *P. chrysosporium*, we propose that excretion into the medium is the principal means by which intracellular cAMP levels are decreased.

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