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Possible Involvement of Cyclic Adenosine 3′,5′-Monophosphate in the Regulation of NADP-/NAD-Glutamate Dehydrogenase Ratio and in Yeast-Mycelium Transition of *Benjaminiella poitrasii*†

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The effect of different adenine-containing compounds on the NADP-/NAD-glutamate dehydrogenase (GDH) ratio was studied as a function of yeast-mycelium transition in *Benjaminiella poitrasii*. Under in vivo conditions, at a 5.0 mM concentration, cyclic AMP (cAMP) and dibutyryl cAMP maintained the cells in the yeast form for up to 7 and 5 h, respectively, and this was reflected in the patterns of GDH ratios observed. In vitro studies of phosphorylation and dephosphorylation have also been carried out, and the results suggest a possible correlation between cAMP, the GDH ratio, and cell form in *B. poitrasii*.

The possible role of cyclic adenosine 3′,5′-monophosphate (cAMP) in the differentiation of fungi has been studied by several researchers (11, 14). Exogenous addition of cAMP in the growth medium was found to trigger diverse morphological changes in fungi. In dimorphic fungi such as those of the genus *Mucor*, high intracellular levels of cAMP are associated with yeast (Y)-like cells, while low levels are characteristic of mycelial (M) cells (9). Exogenous addition of cAMP as well as of dibutyryl cAMP (dbcAMP, a lipophilic analog of cAMP) in the culture medium maintained *Mucor racemosus* and *Mucor rouxii* cells in the Y form (9, 12, 13). Furthermore, during Y→M transition, these compounds prevented the formation of M cells under selective conditions (2% glucose and an aerobic environment). In *M. racemosus*, the lowering of NAD-dependent glutamate dehydrogenase (NAD-GDH) to levels characteristic of the Y form is observed in the presence of cAMP and dbcAMP (13). It has been reported that monomorphic *Mucor bacilliformis* Y-form mutants had lower levels of NAD-GDH and higher levels of intracellular cAMP than M cells of the parent strain (15).

The earlier work on the dimorphic behavior of *Benjaminiella poitrasii* and its morphological mutants (Y-2 and Y-5) has shown that the NADP-/NAD-GDH ratio (GDH ratio) is correlated with the Y↔M transition (7). A low GDH ratio (<0.15) was associated with the M form while a higher GDH ratio (>0.15) was observed in the Y form, Y-2, and Y-5. A decrease in the GDH ratio (between 1 and 2 h) occurred prior to germ tube formation (>20% in 3 h). Parameters affecting the GDH ratio were found to affect the morphological state. For example, exogenous addition of compounds such as α-ketoglutarate (a substrate for NADP-GDH), cycloheximide (a protein synthesis inhibitor), and isophthalic acid (a competitive NAD-GDH inhibitor) yielded a high GDH ratio and delayed Y→M transition, resulting in the maintenance of the Y form. Under similar sets of conditions, the morphological mutants Y-2 and Y-5 displayed a high GDH ratio and maintained their Y morphology.

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The survey of data from other fungal species described above has already implicated the involvement of cAMP in Y→M transition as well as raised the possibility of a connection between cAMP and GDH activity levels (9, 11, 13). Study of the effects of various adenine-containing compounds and their possible involvement with GDH during transition in *B. poitrasii* was therefore relevant.

Y→M transition. Stock cultures of *B. poitrasii* parent and Y-form mutants (Y-2 and Y-5) were maintained on YPG agar slants (6). Transition studies (Y→M) and determination of morphology were carried out as described earlier (7). Preparation of cell extract and enzyme assays of NAD- and NADP-dependent GDH by reductive amination of α-ketoglutarate as well as protein determination were also carried out as described previously (7). The adenine-containing compounds were obtained from Sigma Chemical Co., St. Louis, Mo., while alkaline intestinal phosphatase of bovine origin was obtained from Armour Laboratories, Chicago, Ill. All of the other chemicals were of analytical grade.

B. poitrasii undergoes a shift from the Y to the M form by

TABLE 1. Effect of exogenous addition of adenine-containing compounds on Y→M transition in *B. poitrasii*[a]

Addition (5.0 mM)	% Germ tube formation at[b]:					
	2 h	3 h	4 h	5 h	6 h	7 h
None (control)	—[c]	22	43	78	82	87
Adenine	—	—	—	—	21	46
Adenosine	—	—	—	—	18	32
3′-AMP	—	14	40	67	82	84
5′-AMP	—	18	38	51	80	88
ADP	—	8	16	40	58	61
ATP	—	13	21	35	46	69
cAMP	—	—	—	—	—	10
dbcAMP	—	—	—	15	26	49

[a] The inoculum was grown for 48 h at 37°C in YPG medium, and 8 × 10^6 cells per 50 ml of medium were inoculated into the same medium containing the adenine compounds described above at a concentration of 5.0 mM and were incubated at 28°C.
[b] Cells were harvested at the times indicated, and the percentage of germ tube formation was calculated.
[c] —, no germ tube formation.

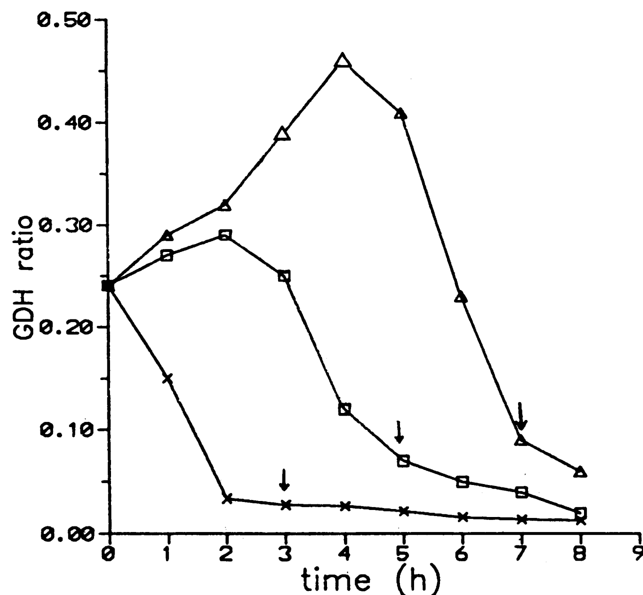

FIG. 1. Effect of 5.0 mM cAMP and 5.0 mM dbcAMP on the GDH ratio during the Y→M transition in *B. poitrasii*. Symbols: x, control; △, cAMP; □, dbcAMP; ↓, germ tube formation.

3 h when the incubation temperature is changed from 37°C to 28°C. The various adenine-containing compounds tested were added exogenously to YPG medium (containing 0.1% glucose) at the time of the shift (i.e., from 37 to 28°C). Concentrations of adenine-containing compounds below 5.0 mM had little effect on the Y→M transition. However, concentrations above 5.0 mM inhibited germ tube formation to various extents, resulting in the maintenance of the Y form. At 5.0 mM, the maximum suppression was obtained by cAMP (up to 7 h) while dbcAMP was found to be less effective (5 h) (Table 1). On increasing the cAMP concentration to 7.5 and 10.0 mM, the germ tube formation was delayed up to 9 and 11 h, respectively. The corresponding GDH ratios observed were 0.57 and 0.62. This observation is in contrast to that with *M. racemosus* (9, 13), in which 3.0 mM dbcAMP could effectively maintain the culture in the Y form. Studies of samples grown in the 5.0 mM concentration of cAMP and dbcAMP indicate an initial increase in the GDH ratio, which rapidly declines prior to germ tube formation (Fig. 1). This lowering of the GDH ratio (from 0.24 to 0.004 for control, 0.28 to 0.007 for dbcAMP, and 0.46 to 0.008 for cAMP), preceding germ tube formation, to a level characteristic of M-type growth in the presence of an external parameter is in agreement with our previous observation (7); i.e., the biochemical correlate (GDH ratio) should be sensitive to any signal(s) (cAMP in the present study) affecting the morphological outcome. Exogenous addition of adenine as well as adenosine also prevented germ tube formation up to 5 h. The addition of nucleotides AMP, ADP, and ATP at similar concentrations did not show a significant effect on germ tube formation per se (Table 1). In contrast, for *Candida albicans* it has been reported that exogenous addition of ATP delayed germ tube formation during Y→M transition (16).

Effect in vitro of adenine-containing compounds. In vitro studies were carried out to ascertain the effect of adenine-containing compounds on the activity levels of NAD- and NADP-GDH (Table 2). In the presence of 10 μM cAMP and dbcAMP, the NAD-GDH activity of M cells increased 1.8- and 1.2-fold, respectively, compared with the activity of the control (contains no ATP). The increases in levels for NADP-GDH of the M form were 4.7- and 2.7-fold with cAMP and dbcAMP, respectively. Increasing the concentration of cAMP from 10 μM to 20 μM had no appreciable effect on M-form NAD-GDH (1,171.6 U/mg of protein), while NADP-GDH activity increased further (17.4 U/mg of protein). Similarly, addition of dbcAMP increased NAD-GDH to 837.6 U/mg of protein and NADP-GDH to 11.3 U/mg of protein. For the Y form, no significant effect on NAD-GDH or NADP-GDH was observed after the addition of either cAMP or dbcAMP. The NAD-GDH activity of M cells was inhibited (50%) by 5′-AMP, while NADP-GDH increased marginally (Table 2). No effect of 5′-AMP on either of the enzyme activities in the Y form was observed. Adenine, adenosine, and 3′-AMP were found to have no effect on either of the enzyme activity levels in the M or Y form, although under in vivo conditions, adenine and adenosine inhibited germ tube formation (Table 1). As reported for *Aureobasidium pullulans* (1), the possibility exists that both of these compounds are taken up by the cells and metabolized to cAMP under in vivo conditions, thereby affecting transition.

Phosphorylation-dephosphorylation and proteolysis. Protein phosphorylation and protein degradation are recognized as important mechanisms for controlling the activity of regulatory enzymes in eukaryotic cells (2, 5, 8, 10). It has been clearly demonstrated that NAD-GDH activity from *Candida utilis* (3) and *Saccharomyces cerevisiae* (4, 17) is regulated by phosphorylation and proteolysis.

TABLE 2. Effect of adenine-containing compounds on GDH activity in Y and M forms of *B. poitrasii*[a]

Addition (10 μM)[b]	GDH activity (U/mg of protein) in:			
	M phase		Y phase	
	NAD-GDH	NADP-GDH	NAD-GDH	NADP-GDH
None (control)	594.0 ± 82.1	2.8 ± 0.4	136.8 ± 21.1	22.9 ± 0.6
cAMP	1,153.3 ± 74.9	13.1 ± 1.2	154.1 ± 23.6	29.7 ± 2.6
dbcAMP	749.3 ± 78.3	9.5 ± 1.3	123.8 ± 14.5	29.7 ± 1.9
3′-AMP	611.4 ± 67.6	5.5 ± 0.8	142.5 ± 18.5	18.9 ± 2.0
5′-AMP	293.7 ± 58.5	3.9 ± 0.6	123.6 ± 16.4	18.0 ± 1.6
Adenine	587.8 ± 69.2	2.7 ± 0.2	148.2 ± 17.7	29.7 ± 3.3
Adenosine	565.5 ± 67.1	2.7 ± 0.2	153.9 ± 22.8	29.7 ± 2.4

[a] The average enzyme activities and standard deviations for triplicate experiments are shown.
[b] Except for the control, all of the additions contain ATP (1.0 mM) and the adenine nucleotide (10 μM) as indicated.

TABLE 3. Effect of phosphorylation and dephosphorylation on GDH activity in M and Y forms of the parent strain and mutants Y-2 and Y-5[a]

Additions	% of control activity in:							
	M form		Y form		Y-2		Y-5	
	NAD	NADP	NAD	NADP	NAD	NADP	NAD	NADP
None (control)	100	100	100	100	100	100	100	100
Phosphorylation								
ATP (1.0 mM)	120	210	106	103	110	104	114	102
ATP + 10 μM cAMP	180	450	118	130	131	108	124	103
Dephosphorylation								
$MgSO_4$ (10 mM)	19	103	45	ND[b]	81	ND	33	ND
$MgSO_4$ + 40 mM NaF	87	106	80	62	85	74	89	55
Alkaline phosphatase (1,000 U)	24	98	52	13	72	ND	61	ND

[a] For enzyme activities, cell extracts prepared in extraction buffer containing 1.0 mM phenylmethylsulfonyl fluoride were incubated for 10 min at 30°C. To all of the samples, NaF (40 mM) was added (except for $MgSO_4$, in which case NaF was added after the termination of reaction). Determination of the GDH enzyme activities was carried out as described by Khale et al. (7).

[b] ND, not detected.

In the case of *B. poitrasii*, incubation of crude cell extracts with 20 μg of trypsin type III (Sigma) per ml inactivated both NAD- and NADP-GDH, thereby showing no specific role for proteolysis in *B. poitrasii* dimorphism (data not shown).

For phosphorylation studies, cell extracts from Y and M cells grown for 48 h on YPG (containing 0.1% glucose) medium were incubated with the various components for 10 min prior to the GDH assay (Table 3). Addition of 1.0 mM ATP with 10 μM cAMP activated the NAD-GDH by 1.8-fold, while a 4.5-fold increase in the activity levels of NADP-GDH from M cells occurred (Table 3). No appreciable difference in the levels of either NAD- or NADP-enzyme after phosphorylation was observed for the Y form or the Y-2 or Y-5 mutants.

In contrast, for dephosphorylation, cell extracts when incubated with 10 mM $MgSO_4$ and alkaline phosphatase (1,000 U) exhibited loss of NAD-GDH activity to various degrees in M, Y, Y-2, and Y-5 cells, the maximum loss in enzyme activity occurring for the M form. Addition of 40 mM sodium fluoride (NaF) to incubation mixtures containing $MgSO_4$ largely prevented such an inactivation. The NADP enzyme from the Y form, Y-2, and Y-5 also showed loss of activity in the presence of $MgSO_4$ and alkaline phosphatase, which was also protected with the addition of NaF. However, no effect of the compounds described above was observed on the NADP-GDH enzyme from M cells.

Thus, from the results described above, it seems likely that the presence of ATP and cAMP is necessary for the activity of NADP-GDH enzyme from the M form to reach Y-form levels. In contrast, in the Y form of the parent strain as well as mutants Y-2 and Y-5, the in vitro inactivation of NADP enzyme seems to be mediated by an Mg^{2+}-requiring factor which in turn is inhibited by NaF. On the basis of the data described above, it is possible that, in the M form, NADP-GDH exists in a less active state (i.e., dephosphorylated), while in the Y form the enzyme occurs in an active state (phosphorylated). Because a loss in NAD-GDH enzyme activity occurs with dephosphorylation in all of the forms, the enzyme probably exists in an active phosphorylated state. Since the quantitative relationship between these two reaction rates is calculated as the GDH ratio (7), factors such as phosphorylation and dephosphorylation affecting the balance between these two enzymes would affect the GDH ratio. Furthermore, any change in the GDH ratio would in turn influence the morphological outcome, as is evident in Y-2 and Y-5 mutant cells.

In conclusion, the observed effect of exogenous addition of cAMP could possibly be correlated to the existence of NAD-GDH and/or NADP-GDH in an active phosphorylated state, in turn affecting the relative balance between NADP- and NAD-GDH (i.e., the GDH ratio) and thereby affecting the maintenance of a specific morphology in *B. poitrasii*. For instance, on the basis of our earlier observations (7), it can now be suggested that, in the M form, the NAD-GDH exists at relatively high levels in an active state (phosphorylated) while the NADP-GDH occurs in a less active state (dephosphorylated), resulting in a low GDH ratio (<0.15). Alternatively, in the Y form, Y-2, and Y-5, lower levels of an active NAD-GDH as well as an active (phosphorylated) NADP-GDH occur, which eventually gives rise to a characteristically high GDH ratio (>0.15) (7). These observations suggest that a possible correlation between cAMP, the GDH ratio, and cell morphology could exist in *B. poitrasii*.

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