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Mutants in orthophosphate-regulated cyclic phosphodiesterase showing rhythmic conidiation in *Neurospora*.

1. Orthophosphate regulated cyclic phosphodiesterase (cPDase)

In wild-type hyphae grown in low and high phosphate media, cPDase is detected in hyphal extracts and in culture filtrates (1, 2). The enzyme hydrolyzes bis-p-nitrophenyl phosphate, and cyclic 2',3'- and cyclic 3',5'-AMP, GMP, GMP and UMP, cPDase activity with cyclic 3',5'-AMP is most efficient at around pH 4 and is stimulated by Mn^{++} (1). With cyclic 3',5'-AMP as substrate, intracellular cPDase produced in wild-type hyphae grown in high phosphate medium shows a K_m of 1×10^{-5} M, whereas cPDases produced in hyphae grown in low phosphate medium have K_m 's of 1×10^{-5} , 2×10^{-3} and 1×10^{-2} M (2). These three enzymes are designated cPDase I, II and III, respectively. A regulatory mutant lacking the ability to assimilate phosphate from many sources, *nuc-1*, produces wild-type levels of specific activity of cPDase I in high phosphate medium, whereas in low phosphate medium the mutant does not produce all the molecular species of cPDases (2). In the mutant *pho-3* (3), the levels of specific activity of all species of cPDase are reduced. *pho-3* is a regulatory mutant of cPDases (2).

With cyclic 3',5'-AMP as substrate, extracellular cPDases produced under low phosphate conditions showed K_m 's of 10×10^{-5} M and 2×10^{-3} M, which corresponded to cPDase I and II. These enzyme activities were inhibited non-competitively by an intracellular regulatory protein designated neucraasin, whereas with cyclic 2',3'-AMP as substrate enzyme activities were stimulated by neucraasin in the presence of Mn^{++} (Shinohara, Furukawa and Hasunuma, in preparation).

2. Rhythmic conidiation in cPDase mutants.

Two groups of mutants, *cpd-1* and *cpd-2*, which show reduced growth in orthophosphate-free Fries medium supplemented with cyclic 3',5'-AMP, exhibit rhythmic growth in Fries minimal medium (4) and rhythmic conidiation on solid glycerol complete medium containing Fries salts (5). *cpd-1* was mapped on LG IVR, 19.4% distal to *pyr-2* and *cpd-2* was located on LG IL, 5.6% proximal to *arg-1* (6). The growth of both mutants on solid glycerol complete medium is sensitive to white light ($3.3 \text{ J/m}^2/\text{sec}$). A light pulse of only 10 min is sufficient to reduce linear extension of hyphae in race tubes and to cause phase shifting of conidiation. The period lengths of rhythmic conidiation of *cpd-1*, *cpd-2* and *bd* mutants are 22.7, 22.1 and 22.8 h respectively in continuous darkness (free-running), while in a 9:15 (light:dark) regime they are entrained to 24 h. In continuous darkness the period length of *cpd-2* varies from 23.0 to 24.2 h between 17 and 33° C, and that *cpd-2* varies from 21.4 to 23.6 h between 21 and 33° C. Conidiation rhythms in *cpd-1* and *cpd-2* were identified as being circadian, as in the *bd* strain (5).

Submerged mycelia of *cpd-1* and *cpd-2*, grown in liquid medium in test tubes were examined after drawing out onto a slide glass. Serial observation of dense and sparse regions was performed by light microscopy. Dense regions contain predominately submerged conidia, while sparse regions contain submerged mycelia (7). These strains, *cpd-1* and *cpd-2*, have reduced levels of cyclic 3',5'-AMP (6). The oscillation of levels of cyclic 3',5'-AMP under the control of the clock system may correlate with the rhythmic formation of aerial hyphae and conidia from hyphae on solid medium and the rhythmic formation of submerged conidia in liquid medium. This assumption was supported by direct measurement of the levels of cyclic 3',5'-AMP in the mycelia of wild-type, *cpd-1*, *cpd-2* and *bd* grown in liquid media. Cultures were sampled every three hours after white light irradiation. The four strains showed a small peak of cyclic 3',5'-AMP in continuous darkness after 15 h from the time of light off and a large peak after 24 h. A second small peak appeared 39 to 42 h after the start of continuous darkness and a second large peak appeared after 48 to 50 h. The levels of cyclic 3',5'-AMP in these four strains showed oscillations with a period length of about 24 h. The levels of cyclic 3',5'-AMP in *cpd-1* and *cpd-2* were 5 to 15% and in *bd* 50 to 70% of the wild-type level (7). Conidiation on solid media in a parallel experiment took place 2-3 h after the appearance of a large peak of cyclic 3',5'-AMP in *bd* but in *cpd-1* and *cpd-2* conidiation took place during the period of the large peak of cyclic 3',5'-AMP. Small peaks of cyclic 3',5'-AMP may correspond to the shoulders of conidiation bands. Reduction in the level of cyclic 3',5'-AMP may be a trigger for the change in morphogenesis from vegetative to aerial hyphae (conidiophore) and a subsequent increase in the level of cyclic 3',5'-AMP may be a trigger for the formation of conidia

(2, 5). A mutant of adenylate cyclase, cr-1, forms abundant conidia on solid media, but the levels of cyclic 3',5'-AMP in various ~~allelic~~ mutants of cr-1 were the same or rather higher than those of cpd-1 and cpd-2 (Hasunuma, unpublished). Conidia of cr-1 contain normal level of cyclic 3',5'-AMP (8). In cpd-1, cpd-2 and cr-1, localized accumulation of cyclic 3',5'-AMP at the tip of aerial hyphae may lead to the formation of conidia.

3. Enzymatic characterization of cpdase mutants.

The levels of extracellular cpdase in hyphal culture of cpd-1 and cpd-2 grown in low phosphate media were 19.2 and 9.8% that of wild-type, whereas in bd the levels were similar to wild-type. In ammonium sulfate precipitates of mycelial culture filtrates of wild-type, two unidentified proteins were detected by SDS-PAGE electrophoresis. One of these is missing in cpd-1, cpd-2 and bd and the other is missing in cpd-1 and bd. In crude extracts of hyphae grown in high phosphate media, the levels of cpdase I in cpd-1 and cpd-2 were about 20% that of wild-type. In bd, the level of cpdase I was only about 10% that of wild type. In crude extracts of hyphae grown in low phosphate medium, the levels of cpdase III in cpd-1 and the levels of cpdase II and III in cpd-2 are reduced. In bd, excess amounts of cpdase I, II and III are produced (6).

Since cpd-1, cpd-2 and bd showed reduced levels of cyclic 3',5'-AMP and reduced levels of cpdase I, these strains were suggested to be mutants showing either reduced activity of adenylate cyclase or over production of Mg^{++} -stimulated-cyclic-phosphodiesterases (9). In the presence of 10 μM GTP, adenylate cyclase activity of cpd-1, cpd-2 and bd were 69.3, 34.0 and 63.2% that of wild-type. Mg^{++} -stimulated-phosphodiesterase activity at intracellular concentrations (0.2 μM of cyclic 3',5'-AMP in cpd-1, cpd-2 and bd were 199, 137 and 329% that of wild type. Thus in cpd-1 and bd, a slight reduction in the activity of adenylate cyclase and higher levels of Mg^{++} -stimulated-cyclic-phosphodiesterase were observed. A mutant of adenylate cyclase, cr-1 (10), showed 20.3% of the activity of wild-type, while the activity of Mg^{++} -stimulated-cyclic-phosphodiesterase was 293% that of wild-type (6). Although the levels of adenylate cyclase in cr-1 mutants were reported to be 1-4% that of wild-type (11), it seems likely that the results were compounded by the enhanced activity of Mg^{++} -stimulated-cyclic-phosphodiesterase. The levels of cyclic 3',5'-AMP in cpd-1, cpd-2, cpd-3 and cr-1 were 9.2, 9.8, 16.3, 13.4% that of wild-type (FGSC #988). cpd-3 isolated with cpd-1 and cpd-2 (4) is very similar to cr-1 in morphology and is mapped on LG IC. The levels of adenylate cyclase and Mg^{++} -stimulated-cyclic-phosphodiesterase in cpd-3 were 31.1 and 253% those of wild type (Hasunuma, in preparation).

4. Conclusion.

Although cpd-1 and bd may be very leaky mutants of adenylate cyclase, cpd-2 may be a mutant of adenylate cyclase, comparable to cr-1. The reduction of the level of cyclic 3',5'-AMP in cpd-1 and bd may be the result of slightly reduced activities of adenylate cyclase and apparently enhanced activities of Mg^{++} -stimulated-cyclic-phosphodiesterase. The level of cyclic 3',5'-GMP in these strains is unaffected (6), whereas in cr-1 the level is reduced to 11-15% that of wild-type (10). Two types of adenylate cyclase may exist in *Neurospora*; the mutation cr-1 may affect the enzyme that shows activity with both adenylate cyclase and guanylate cyclase, while the other enzyme regulated by cpd-2 may only show activity with adenylate cyclase.

The reduction in the level of cyclic 3',5'-AMP, brought about by the reduction in the level of activity of adenylate cyclase in cpd-2 and by the increase in the level of activity of Mg^{++} -stimulated-cyclic-phosphodiesterase in cpd-1 and bd, did not affect the period length of conidiation. Using bd cr-1, Feldman et al. (12) ruled out the interaction of adenylate cyclase and a major pool of cyclic 3',5'-AMP in the mechanism of the clock system. From the analysis of cpd-1, cpd-2 and bd, however, mutants exhibiting rhythmic conidiation showed rhythmic oscillation of cyclic 3',5'-AMP, the levels of which were lower than in wild-type, and the peak of the level of cyclic 3',5'-AMP roughly corresponded to the conidiation band. The results strongly suggest a direct action of cyclic 3',5'-AMP in the clock mechanism. Rhythmic oscillation of energy charge (13) and of cyclic 3',5'-AMP (7), short period length of conidiation rhythm exhibited by oligomycin resistant (bd ol1) strain (14) and long period length caused by the inhibitor of Mg^{++} -stimulated-cyclic-phosphodiesterase (15) suggests a rhythmic flow of ATP from mitochondria to cyclic 3',5'-AMP.

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