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EXOGENOUS ADENOSINE 3':5'-MONOPHOSPHATE CAN RELEASE YEAST FROM CATABOLITE REPRESSION

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SUMMARY

Cyclic AMP added to yeast cells maintained in a glucose medium can bring about their release from catabolite repression to an extent and with kinetics that compare favorably with the same parameters during active derepression. This was first demonstrated for the synthesis of δ-aminolevulinate dehydratase, the derepression of which becomes detectable within 30 min, and was then extended to a number of mitochondrial enzymes, which require several hours for derepression. The initiation of derepression has been correlated with a significant increase in intracellular cyclic AMP.

INTRODUCTION

The information bearing on the involvement of adenosine 3':5'-monophosphate (cyclic AMP, cAMP) in intracellular regulation in *Saccharomyces cerevisiae* is ambiguous and its interpretation controversial, particularly with respect to its significance in, and relevance to catabolite repression (for recent reviews and citations see 1-4): An effect of the molecule on respiratory adaptation is shared by a number of other nucleotides (5). Although a recent report describes some stimulation by cAMP of amino acid incorporation into the mitochondrially synthesized polypeptides of cytochrome oxidase during derepression, it does not address the details of the time course and specificity of this effect (6). Stimulation by cAMP of the elaboration of active transport systems for several solutes has also been observed, but it is restricted to a hyperrepressible mutant of *S. pombe*, and even there requires prior

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starvation of glycerol-grown cells (7). The kinetics of the increase in cAMP subsequent to a shift from glucose to maltose are not consistent with its postulated function as a positive regulator - in a manner analoguous to that known to be operative in bacteria (8) - for the synthesis of α-glucosidase or succinate dehydrogenase (9). The content of cAMP of wild type cells of *S. fragilis* (10) is higher on lactate than 10% glucose, and in *S. carlsbergensis* grown on 2% galactose its level is increased by 70% over that found after growth on 2% glucose. But a mutant of the same strain, partially resistant to catabolite repression for several enzymes, still exhibits wild type levels of the nucleotide on *either* carbon source (11). Similarly, in *S. fragilis* (10), no simple correlation emerged between nucleotide levels, or adenylyl cyclase activity. Finally, although cAMP-binding protein(s) have been shown to be present in several yeast strains, and have been purified from *S. fragilis*, these studies failed to establish a potential functional role for them (12). A further complication is provided by the observation that the degradation of trehalose, a storage carbohydrate in yeast, is stimulated by cAMP (13).

We have previously described a simple experimental protocol for determining the kinetics of release of enzyme synthesis from catabolite repression in the absence of cellular proliferation and have employed it to study this regulation of a number of mitochondrial enzymes (14,15), as well as of δ-aminolevulinate dehydratase (EC 4.2.1.24) (16). The synthesis of this enzyme, which is probably responsible for the regulation of heme synthesis during derepression, is susceptible to very rapid release, with the transcriptive events for the first detectable enzyme molecules completed in <10 min at 30°. It thus seemed opportune to re-open the question of the implication of cAMP in derepression. By using this rapid, sensitive and reproducible system as an indicator of possible short-term effects of the molecule, one might hope to detect its action before it could be hydrolyzed or otherwise rendered inactive upon longer incubations. The results presented in this report suggest that, under appropriate conditions, addition of exogenous cAMP to cells of *S.*

TABLE 1

EFFECTS OF cAMP ON DEREPRESSION OF ALV DEHYDRATASE[a]

Expt.	Medium	Specific Activity (nmol x mg^{-1} x h^{-1}) 0	60	120 min
1	Derepression (YM-1,0.25% Glc, 3% EtOH)	0.95 ± 0.06	2.3 ± 0.10	2.8 ± 0.13
	Repression (YM-1,2% Glc)	0.95 ± 0.06	0.97 ± 0.06	1.0 ± 0.08
	+ 20 mM NaF		1.4 ± 0.08	2.0 ± 0.06
	+ 5 mM cAMP		1.6 ± 0.08	2.2 ± 0.10
	+ 5 mM cAMP + 20 mM NaF		1.7 ± 0.06	2.6 ± 0.13
	+ 5 mM cGMP, or ADP, or ATP, or 5'AMP or 5'GMP		1.0 ± 0.08	1.0 ± 0.06
2	Derepression + 20 mM NaF	0.92 ± 0.06	2.0 ± 0.13	2.4 ± 0.13
	Repression + 20 mM NaF	0.92 ± 0.06	1.4 ± 0.08	2.1 ± 0.08
	+ 20 mM NaF + 5 mM cAMP		1.7 ± 0.06	2.6 ± 0.13
	+ NaF + cAMP, as above			
	+ 1 mM Theo[b]		1.8 ± 0.06	2.7 ± 0.16
	+ 1 mM Theo[b]		1.0 ± 0.10	1.1 ± 0.08
3	Repression	0.96 ± 0.01	0.98 ± 0.08	1.0 ± 0.06
	+ 5 mM cAMP (pH 4.5)		1.5 ± 0.06	2.1 ± 0.13
	+ 5 mM cAMP (pH 5.8)			1.5 ± 0.08

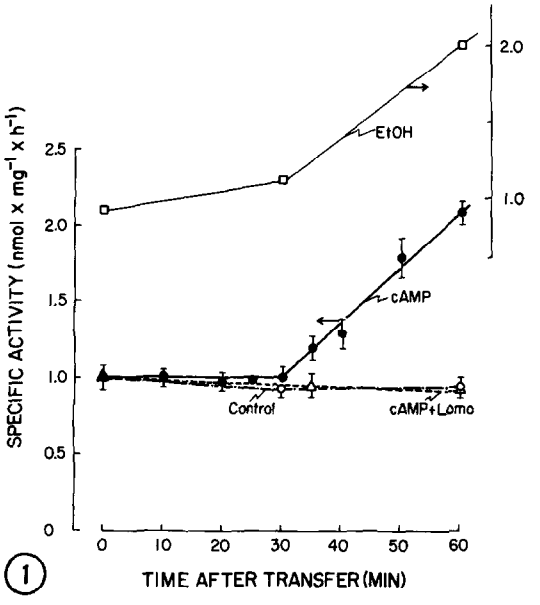
[a] all at pH 4.5, except for experiment 3 [b] Theophylline

cerevisiae maintained on 2% glucose produce effects similar to those observed upon transfer of such cells to 3% ethanol.

RESULTS AND DISCUSSION

Experimental Procedures: These have all been described previously (14-16).

Addition of cAMP Can Bring About Derepression: We reasoned that any potential effect of exogenous cAMP might be enhanced by facilitating its entry into the cells by lowering the pH to a region closer to its first pK. As shown by the results, summarized in Table 1, at pH 4.5 - but not nearly as effectively at the more usual pH of 5.8 (14-16) - addition of cAMP at a concentration of 5 mM is able to induce the formation of the enzyme in cells in 2% glucose to levels comparable to those characteristic of cells undergoing derepression. The effect appears to be specific for this particular nucleotide; the other nucleotides shown were found to be completely ineffective in two separate



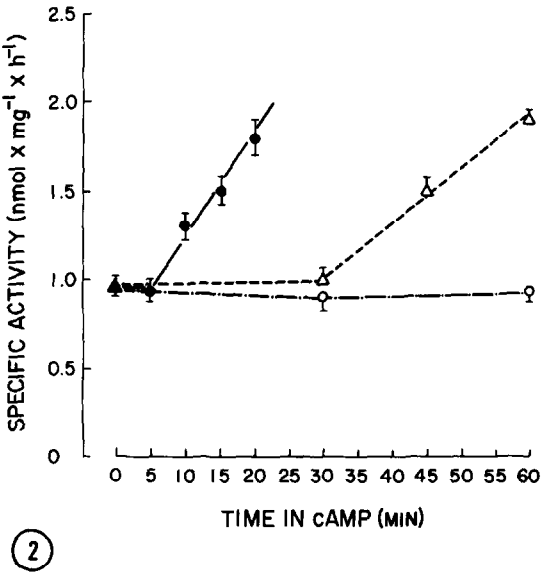


Fig. 1 (left) Kinetics of derepression of δ-aminolevulinate dehydratase (ALV-De) induced either by transfer from 2% glucose to 3% ethanol (□, right ordinate) or addition of 5 mM cAMP (●, left ordinate). The control (○) contained 2% glucose. Lomofungin (Lomo) was added to the cAMP-glucose medium at a concentration of 1 μg/ml. For other details see text. Error bars indicate standard deviations (n = 3).

Fig. 2 (right) Execution time for cAMP-induced release of ALV-De from repression. Cells were transferred from the cAMP (5 mM) glucose (2%, pH 4.5) medium (I) to a medium containing 10% glucose at pH 5.8 (II) and incubated for an additional 45 min at 30° in this medium. Two controls were used: cells maintained in I (Δ) and cells maintained in II throughout. Error bars indicate standard deviations (n = 3). For other details, see text.

experiments. The only agent exhibiting analoguous activity was fluoride ion; its addition had previously been shown by Wiseman and Lim (17) to be capable of bringing about a relatively slow derepression of α-glucosidase. The effects produced by cAMP and fluoride were neither additive nor synergistic, perhaps because they lead to the same outcome: fluoride has long been known for its ability to raise the levels of intracellular cAMP, believed to be due to its stimulatory effect on adenylyl cyclase (18-20). In contrast, addition of theophylline, an effective inhibitor of cyclic nucleotide phosphodiesterase,

was without effect.

Kinetics of the cAMP Effect: In our previous studies we demonstrated that the synthesis of new enzyme molecules during derepression first becomes manifest after 25 min at 30° and that this effect is completely abolished by low concentrations of lomofungin, a specific inhibitor of nuclear transcription in yeast (21). The results of similar experiments using cAMP added under repressing conditions are summarized in Fig. 1 and show a similar phenomenology.

If addition of cAMP is indeed able to overcome the effect of catabolite repression, one would expect a reciprocal relationship between the concentrations of effector and repressor. This was shown to be the case in experiments that compared the concentration of cAMP required to bring about derepression in the presence of varying concentrations of glucose in the medium: the concentrations of the nucleotide to produce derepression to half maximal levels in 1, 2; 5 and 10% glucose were approx. 1.5, 3, 7 and >>10 mM respectively, with maximal stimulations of 150, 115 and 100% at $\gtrsim$3, $\gtrsim$5 and $\gtrsim$9 mM, respectively.

The results described in the two previous paragraphs suggested an experimental protocol for the determination of the "execution time" (16,22) of the cAMP effect: we exposed cells to the nucleotide in a medium containing 2% glucose at pH 4.5 for varying lengths of time prior to their transfer to a medium containing 10% glucose at pH 5.8, conditions at which cAMP is totally inactive. We then continued the incubation for an additional 45 min and measured enzyme activity. The results of this study are presented in Fig. 2, and indicate that exposure to cAMP for $\gtrsim$5 min is both necessary and sufficient to overcome repression. However, they do not permit a distinction between two alternative interpretations: i) that this is the time required for the execution of (a series of) events induced by exposure to cAMP, or ii) that this time is necessary for the accumulation of cAMP in amounts sufficient to insure execution of these events.

A permissible inference based on the experiments described so far is that

exposure of repressed cells to *exogenous* cAMP promulgates an intracellular signal similar to the one released in such cells upon their transfer to a derepression medium. It says nothing about the identity of the signal(s), and, in particular, whether *intracellular* cAMP is an actual participant in the events leading to derepression. To do so it would be necessary, though not sufficient, to demonstrate the accumulation of increased amounts of the nucleotide inside cells challenged by *either* cAMP (or fluoride) *or* a derepression medium.

In experiments designed to test the second part of the hypothesis we found that the level of intracellular cAMP (measured as in 10,23) rose, within $\lesssim$5 min after transfer to a derepression medium, to a new plateau: In a representative experiment the initial and final values were 13 $\pm$ 0.5 and 32 $\pm$ 1.6 pmol x mg^{-1} cell protein, or as calculated in Ref. 9, 0.35 and 0.85 μM, respectively.

There are two ways in which exogenous cAMP might produce an increase in intracellular nucleotide: either through uptake, or by stimulation of *de novo* synthesis, presumably with the intervention of a membrane-bound adenylyl cyclase (24). Cells (7.5 x 10^7 x ml^{-1}) suspended in YM-1 containing 2% glucose at pH 4.5 and exposed to labeled cAMP (total concentration = 1.0 x 10^{-4} M) are able to accumulate the nucleotide (25), at an initial ($\lesssim$9 min) linear rate of 15 pmol x min^{-1} x mg^{-1} cell protein. Provided that most of the molecules taken up remain in an undegraded form, this rate and the amount accumulated within the first 5 min are ample to produce levels of cAMP comparable to those generated in response to transfer to a derepression medium. This point was checked explicitly: in two experiments under the conditions outlined in Table 1, intracellular levels of the molecule rose from 0.27 $\pm$ 0.01 or 0.29 $\pm$ 0.006 μM (t=0, no cAMP) and 0.48 $\pm$ 0.01 μM (t=0, immediately after adding 5 mM cAMP), to 1.09 $\pm$ 0.10 or 1.25 $\pm$ 0.04 μM (t=6 min), respectively.

Effects on Derepression of Mitochondrial Enzymes: Release from catabolite repression is of particular importance and relevance to mitochondriogenesis

TABLE 2

EFFECTS OF EXOGENOUS CYCLIC AMP ON DEREPRESSION OF SOME MITOCHONDRIAL ENZYMES (pH 4.5)

Time (Hours)	Specific Activity (Mitochondrial Fraction) ALV Synthetase[a]	MDH[b]	Cyt Ox[b]	NADH:c[b]	Total Activity ALV-S	Mitochondrial Protein (mg/ml)
(A) 2% Glucose + 5 mM cAMP [Experimental]						
0	3.8 ± 0.08	11.6	1.15	0.78	32	4.17
3	5.7 ± 0.06	20.7	1.23	1.23	48	4.22
5	7.9 ± 0.10	45.8	2.06	2.63	65	4.08
(B) 0.25% Glucose + 3% Ethanol [Positive Control]						
0	3.8 ± 0.08	11.6	1.15	0.78	32	4.17
3	6.4 ± 0.10	28.4	1.38	2.09	54	4.25
5	10.2 ± 0.13	61.2	2.44	3.10	84	4.14
(C) 3% Glucose [Negative Control]						
0	3.8 ± 0.08	11.6	1.15	0.78	32	4.17
3	3.5 ± 0.06	12.3	1.09	0.85	29	4.10
5	4.1 ± 0.13	11.2	1.04	0.88	34	4.13

[a] nmol x mg^{-1} x h^{-1}

[b] nmol x mg^{-1} x min^{-1}

since the development of a fully functional, respiratorily competent organelle is both required for utilization and requires the presence of a weakly or non-repressing carbon source. The process usually extends over a period of several hours (1-5). Thus, it was not immediately obvious that exogenously added cAMP would be present in sufficiently high amounts over the critical time required to exert its effect, if any. We therefore modified the protocol slightly and added the nucleotide in two increments: the first on transfer to the derepression medium and the second 3 h later. Under these conditions (Table 2), addition of cAMP leads to an efficient lifting of the catabolite repression exerted by 2% glucose on the synthesis of four mitochondrial enzymes: L-Malate dehydrogenase (E.C. 1.1.1.34; MDH), alv synthetase (E.C. 2.3.1.37), cytochrome oxidase (E.C. 1.9.3.1; Cyt ox) and NADH:cytochrome *c* reductase (E.C. 1.6.2.1; NADH:*c*). These enzyme levels compare favorably with those produced under derepressing conditions. Of the four enzymes, the first two are localized in the mitochondrial matrix, while the remaining two form part of the inner membrane; all but the

first require not only a nucleo-cytoplasmic but also an intramitochondrial contribution to the biosynthesis of their constituent polypeptides (1-3).

Thus the signals transmitted or promulgated by cAMP during derepression affect the nucleo-cytoplasmic as well as the intramitochondrial system of gene expression. The two major unsolved questions, which we are currently addressing, are: i) the nature of the sensing device that transduces a change in the flux of catabolites into a change in intracellular cAMP, presumably with the intervention of adenylyl cyclase (26); and ii) the nature of the events through which cAMP controls transcription [or perhaps - in the mitochondrial case - translation (27)]: whether through direct, positive intervention (8), or indirectly by detachment of protein kinase from its regulatory subunit, permitting the phosphorylation of some target protein(s), that in turn govern the synthesis of new protein molecules (28).

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