THE DEREPRESSION OF Δ-AMINOLEVULINATE SYNTHETASE IN YEAST

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SUMMARY

A simple and reproducible paradigm for the study of derepression of respiratory enzymes in yeast in the absence of growth is described. With it we have shown that δ-aminolevulinate synthetase undergoes an unusual cyclical pattern centering at t = 5 h after transfer to a derepression medium and extending for ~2.5 h in both directions. It can be explained by the induction, followed by the decay, of a novel activity, probably synthesized in the cytosol but also requiring the participation of (a) mitochondrially synthesized component(s).

INTRODUCTION

We are interested in the regulatory regime that must come into play when cells of a facultatively anaerobic yeast such as *Saccharomyces cerevisiae* are forced to substitute a non-fermentable carbon (and energy source) for a fermentable one. This transition from growth on glucose to e.g. ethanol is known as derepression, or release from (catabolite) repression, and results in a fundamental rearrangement of the cell and its mitochondria from a fermentative, glycolytic to an oxidative, respiratory pattern of metabolism. For this reason it has long been a favorite subject for studies on the biogenesis of respiratory enzymes and their regulation (for recent reviews see Refs. 1-4). Usually such investigations have used as the experimental triggering and timing device the exhaustion of glucose intrinsic to cells inoculated into and growing exponentially on this carbon source at a limiting concentration of say 10 g/l; growth leads to conversion of glucose carbon into cellular mass, ethanol plus CO_2, and derepression becomes manifest at a

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time when continued - much slower and almost stationary - growth demands the substitution of ethanol for the by now rapidly diminishing glucose. More recently we (4,5) have been able to dissociate derepression from the cellular growth cycle by the simple device of transferring repressed (i.e. grown on 5% glucose) exponential phase cells to a derepression medium containing 0.25% glucose - 3% ethanol as the carbon source. This paradigm has two obvious advantages: it permits precise timing control, and insures reproducibility of the events during derepression under conditions where there is virtually no increase in cellular mass (<30% in dry weight), protein (<10%) and bud emergence (constant at <5% budded cells).

We are using this system to explore various regulatory devices or requirements in derepression such as the synthesis of nuclear and mitochondrial DNA (5,6). In this note we wish to explore the possible implication of δ-aminolevulinate synthetase (*ALA Synth*) in such a scheme. *ALA Synth* is an interesting mitochondrial protein. As the enzyme catalyzing the first committed step in heme biosynthesis one would expect it to be subject to and participate in a variety of regulatory interactions (7-9), and this expectation appears to be borne out in a number of instances: in mammalian liver, activity of the enzyme controls the level of several cytochromes (10-12); it exhibits a very short half-life and its synthesis is subject to repression by glucose (13) and by heme (14,15), and to induction by a variety of agents (11,16,17). In yeast, at least three sets of interactions have been reported: i) several mutants appear to be pleiotropically deficient both in heme and lipid synthesis (18-20) with the former constituting the primary lesion (18); ii) although present in ρ^- mutants (which produce cytochrome c), the simultaneous presence of a recessive nuclear mutation - which by itself reduces but does not abolish heme synthesis in wild type - leads to a complete abolition of all heme and cytochrome synthesis (21); iii) in total cell homogenates there is an indication of an anomalous pattern of biosynthesis of the enzyme during derepression (22).

METHODS

Strains, Culture, Isolation: We used strain A364A (23), a *S. cerevisiae* haploid, genotype P ρ^+ (*a* ade_1 ade_2 ura_1 his_1 lys_2 gal_1) because of its potential utility as the parent of numerous *ts* mutants for macromolecular synthesis (23,24). Cells were grown at 30° in YEPD medium, containing (in g per 1) yeast extract (Difco) - 10; peptone (Difco) - 20; glucose - 20, to an A_{600} = 0.40, harvested by centrifugation, washed with water and resuspended at an A_{600} = 2.0 in a derepression medium consisting of YM-1 medium (23), supplemented with 2.5 g glucose plus 30 g ethanol per 1. Qualitatively similar results were obtained with 30 g glycerol plus 30 g ethanol per 1. Cells (usually 350 ml per sample) were harvested by centrifugation at 4000 rpm x 10 min at 4°, washed 2 x with sterile, distilled H_2O and once with buffer M (0.44 M sucrose containing 10 mM $MgCl_2$, 100 mM NH_4Cl, 10 mM Tris, all at pH 7.4) and were broken in this medium by homogenization with glass beads (4). The resulting homogenate was decanted, the beads washed 2 x with buffer M and the washings added to the homogenate, which was then centrifuged at 600 x *g* as many times as necessary to remove all material sedimentable under these conditions. The supernatant was centrifuged at 20,000 x *g* for 20 min with the pellet constituting the mitochondrial fraction which was suspended in 2 ml of buffer M.

Enzyme Assays: ALA Synth was assayed according to Marver *et al.* (25,26) on triplicate aliquots of the mitochondrial suspension: 0.2 ml of mitochondria was added to 0.8 ml of assay mixture (0.1 M glycine, 0.1 M Tris, 13 mM EDTA, 0.2 mM Pyridoxal phosphate, 1.16 mM α-oxoglutarate all at pH 7.4) and incubated for 60 min at 30° in air with shaking; 0.3 ml of 15% TCA was added and the protein removed by centrifugation and 0.9 ml of the supernatant transferred to a tube containing 0.6 ml of a mixture of 0.5 M Na acetate and 5% acetylacetone, heated in a water bath at 80° for 10 min and cooled. 0.3 ml of (0.5 M NaOH + 0.25 M Na_2HPO_4) was added and extracted with 3 ml of ethyl ether (9) and centrifuged. One ml of the aqueous layer was then vigorously mixed

with 1 ml of Ehrlich's mercury reagent, the A_{553} measured in a Zeiss spectrophotometer after 15 min, and the ALA concentration determined by comparison with authentic samples of the compound using zero time controls as blanks. One enzyme unit is defined as one nmole of product formed per hour.

Other Mitochondrial Enzymes: L-malate dehydrogenase was determined according to Ferguson *et al.* (27) while NADH:cytochrome c reductase and cytochrome c oxidase were assayed as before (4). Protein was measured by the method of Lowry *et al.* (28). Specific activity is defined as enzyme units per mg mitochondrial protein.

RESULTS AND DISCUSSION

Typical results on isolated mitochondria, obtained from cells derepressed by the paradigm described, are shown in Fig. 1; in the top panel (A) for three characteristic mitochondrial enzymes, and in the bottom, (B) for *ALA Synth.* Its cyclical pattern of biogenesis is quite unusual and is not shared by any of the other derepressible enzymes - whether located inside or outside the mitochondria - previously studied in our or other laboratories. The mitochondrial L-malate dehydrogenase (MDH), a matrix enzyme, and NADH:cytochrome c reductase (NADH:c), and cytochrome oxidase ($c:O_2$), characteristic activities of the inner membrane, are shown as examples. The pattern is, however, highly reproducible in both its qualitative and quantitative aspects (timing and extent). Similar patterns of rise and decay are also obtained when 3% glycerol is substituted for 0.25% glucose in the derepression medium, or when derepression is measured during the transition to stationary phase upon growth on 1% glucose (4). The effects of the two inhibitors added at the time of initiation of derepression can be summarized as follows: Addition of CAP prevents derepression while that of CHX not only blocks derepression but also leads to the destruction or inactivation of pre-existing enzyme with an apparent half life of 3 h. Furthermore (not shown), when CAP is present throughout the prior growth under repressing conditions, but removed at the time of transfer, there is *no* effect on the base line level, and the cyclic

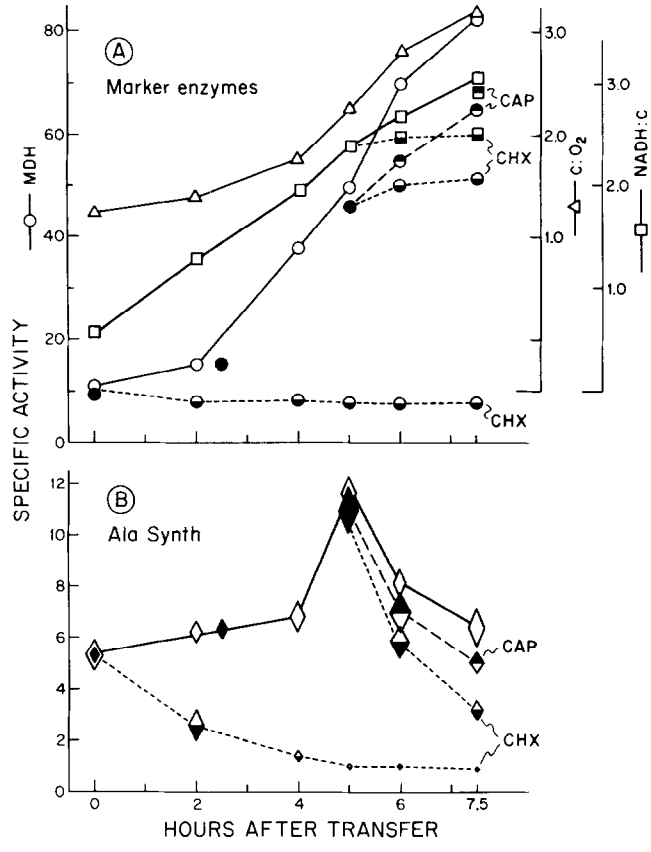

FIGURE 1

Derepression of mitochondrial enzymes. Marker enzymes on top (A); *ALA Synth* below (B). The procedure used is described under METHODS. Zero time is taken as the time of transfer into the derepression medium. Derepression was for a total of 7.5 h at 30° with constant aeration. In one experiment (open symbols) no inhibitors were added. In a second experiment the inhibitors CHX (10 µg/ml) or CAP (4 mg/ml) were added at t = 5 h; its results are shown as filled symbols for the control, as half-filled symbols for the experimental. In a third experiment the inhibitors were added at zero time; for the sake of clarity only the results with CHX are shown. With CAP the value for *ALA Synth* were equal to 5.0 ± 0.9 (standard deviation) at all times; for MDH and NADH:c they were within 20% of the controls; cytochrome oxidase was not tested. The size of the symbols in the bottom panel represents the standard deviation in the various determinations. For the reliability of the other assays see Ref. 4.

In the absence of inhibitors total protein in the mitochondrial fraction increases by 100 ± 20% during the first four hours and remains constant thereafter.

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pattern is conserved but with a delay of one hour (i.e. maximum derepression is at t = 6 h). When the inhibitor is added at the time of maximal derepression (t = 5 h) there is no significant alteration in the kinetics of the

decay of the enzyme; it drops to the base line level within 2.5 h. However, addition of CHX produces an enhancement in both the rate and extent of this decay. Finally (in other experiments), when either of the inhibitors is added during the initial 2.5 h after transfer and then removed, there is no derepression of *ALA Synth*, at least not during the subsequent 5 h, while the other enzymes *are* synthesized.

Our interpretations may be summarized as follows: the enzyme exists in two forms, a base-line and a derepressible variety. The latter is synthesized only during the initial (fermentative) phase of derepression, prior to the period when respiration by newly elaborated mitochondria comes to be of importance (4). Its synthesis probably continues for a period longer than 5 h into derepression but becomes masked at that point by a rapid and specific process of inactivation or degradation. Both forms also appear to be subject to rapid degradation (turnover), but at different rates, whenever their synthesis in the cytoplasm is interrupted by CHX. The derepressible - but not the base line enzyme - requires for its activity or integration the simultaneous presence of an entity synthesized by - or dependent on a product of - the mitochondrial translational system. This collaborative effect of both systems of cellular protein synthesis and specification for the elaboration of the enzyme under certain conditions confirms the genetic results of Sanders *et al.* (21), and is reminiscent of that obtained with the three other enzymes for which a mitochondrial contribution has previously been unambiguously demonstrated (reviewed in Refs. 29,30).

Cyclical responses in the biosynthesis of *ALA Synth* have previously been reported by Simon and Boell (31), Chan *et al.* (32) and Osanai and Rembold (32a) in developing chick liver, insect flight muscle and honey bee respectively. Furthermore, in mammalian liver the induced synthesis of the enzyme is subject to glucose repression (13). Our results on yeast mitochondria confirm and extend those of Jayaraman *et al.* (22), but both of these studies conflict with a claim by Labbe (33) concerning an absence of correlation between the

level of *ALA Synth* activity and degree of repression. However the rapidity of the rise and fall in the activity observed here might easily obscure or prevent its substantiation under certain circumstances. Our findings on the derepression of the enzyme are supported by the reports of Ycas and Drabkin (34) and of Barrett (35)on its *de novo* biosynthesis during respiratory adaptation.

The pattern of *Ala Synth* is strongly reminiscent of that of another enzyme also presumed to fulfill a key function during derepression, namely the mitochondrial DNA-dependent RNA polymerase (36). The uniquely cyclical nature in the levels of these two enzymes may go far in providing a regulatory device for the onset and particularly the *shut-down* (4) of derepression: what will need to be done next, is to inquire into possible mechanisms responsible for turning the synthesis of the derepressible version of these two enzymes on and off, as well as the one responsible for their specific degradation or inactivation.

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