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DEMONSTRATION OF ANAEROBIC CATALASE SYNTHESIS IN THE cz1 MUTANT OF SACCHAROMYCES CEREVISIAE [x]

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SUMMARY

Anaerobic synthesis of catalase T (a typical oxygen-inducible haem containing enzyme) in the cz1 mutant of yeast was demonstrated. The synthesis of catalase T in anaerobically grown mutant cells is stimulated by haemin under carbon--derepressed conditions of growth (galactose as carbon source) but not in glucose repressed cultures. Haem is practically undetectable in the anaerobically grown glucose repressed wild type strain and its level in derepressed cells amounts to 3% of the fully derepressed aerobically grown cells. In the cz1 mutant cultures grown in anoxia both on galactose and glucose the haem level usually exceeds 10% of that in the aerobic control.

INTRODUCTION

Molecular oxygen plays a dual role in haem protein formation. It serves as electron acceptor in the last two oxidative steps of haem synthesis (1-3). It is also known as a specific inducer of the respiratory adaptation process in which most of haem proteins are synthesized (4). Apoproteins of iso-2-cytochrome c and of cytochrome c peroxidase are present in anaerobically grown yeast (5,6) but only low peroxidase activity is detectable (7). The literature brings numerous controversies over the anaerobic synthesis of haem proteins. Besides the earlier reports on the presence of typical aerobic haem proteins in anoxia, some papers appeared describing poorly characterized haem proteins known only from anaerobic yeast (8). All these findings contradict the enzymological data demonstrating an obligatory requirement for molecular oxygen of the last oxidative reaction in the haem pathway (3). It is obvious that availability of haem is a prerequisite to haem protein formation, therefore the presence of any of the typical haem proteins

[x] This is the fourth paper in the series " Haemoprotein formation in yeast".

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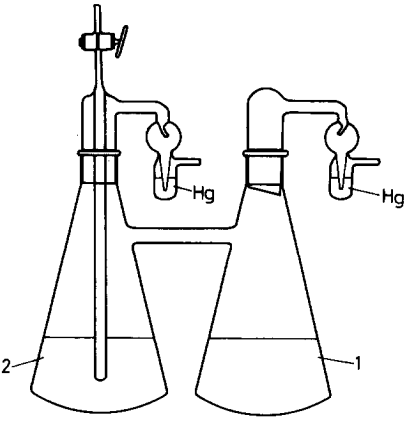


Figure 1.
Scheme of twin-bottles used for anaerobic yeast culture. 1,2 – cultures of mutant and wild type strains, Hg – mercury valve.

in anoxia is at the same time a direct proof of anaerobic haem synthesis.

This paper demonstrates that catalase T, a typical oxygen-inducible haem protein enzyme (9), is synthesized in strictly anaerobic conditions in the cz1 mutant of Saccharomyces cerevisiae.

MATERIALS AND METHODS

Organism. The parental SP4 rho+ (alpha leu1 arg) strain of S.cerevisiae was a haploid-heterothallic one. The mutant cz1 rho+ (alpha leu1 arg cgr4) strain was obtained after one-step EMS mutagenesis of the parental strain (10).
Media and culture conditions. Strains were grown on semisynthetic or YP medium. The semisynthetic (SS) medium was prepared as described by (11) and supplemented according to nutritional requirements. For experiments with haemin YP medium was used to avoid precipitation of haemin. To both kinds of medium 10% glucose or 2% galactose was added as carbon source. For anaerobic conditions 10 ml of a mixture of Tween 20, Tween 80 and ergosterol was added per 1 liter of medium. The mixture contained 1 part of Tween 20, 4 parts of Tween 80 and 20 mg of ergosterol per 1 ml of the mixture. For anaerobic cultures specially constructed twin-bottles were used (Fig.1). Two Erlenmayer flasks (á 700 ml) were connected above the liquid level by a 1 cm wide channel. Strictly anaerobic conditions were obtained after bubbling for at least 1 hour highly purified nitrogen through the medium. Commercial nitrogen was purified by passing through a system of four oxygen traps (two gas washing bottles filled with alkaline solution of pyrogallol and two columns containing copper chips heated to 220-250°C). The twin-bottles were closed with mercury valves. Cells were grown at 30°C in a rotatory shaker. For all experiments cultures were harvested in the late logarithmic phase of growth. Growth was stopped by addition of cycloheximide to a final

concentration of 50 ug/ml. Cells were harvested by centrifugation, then washed twice with 0.1 M phosphate buffer (pH=6.8) containing 50 ug of cycloheximide per 1 ml.

Extracts. For catalase activity and polyacrylamide gel electrophoresis cells were suspended in 0.05 M phosphate buffer (pH=6.8) and disrupted with glass beads in a Braun Homogenizer at 4000 rpm for 2 min. Unbroken cells and cells debris were removed by centrifugation at 1000xg for 10 min. The supernatant was collected for catalase assay.

Catalase (E.C.1.11.1.6) activity assay. The disappaerance of H_2O_2 was followed spectrophotometrically at 240 nm (12). The activity was expressed in units per 1 mg of protein. One unit (U) is equal to one nanomole of hydrogen peroxide decomposed per minute. Polyacrylamide slab gel electrophoresis was carried out as described by (13).

Protein determination. Protein concentration was determined by the method of Lowry et al. (14).

Haem determination. Haem content was determined according to (15).

RESULTS

The mutant designated as cz1 was isolated after one-step EMS mutagenesis by the tetrasolium salt staining method (10). The procedure of isolation and the nature of mutation(s) will be discussed in a subsequent paper (16). In the cz1 mutant grown under aerobic conditions synthesis of catalase T as well as of mitochondrial cytochromes is resistant to glucose repression (17). Preliminary experiments revealed a high catalase activity in the mutant cells grown anaerobically in 10% glucose medium. The activities of cytochrome c oxidase (E.C.1.9.3.2) and succinate-cytochrome c reductase (E.C.1.3.99.1) were undetectable in these conditions. Liquid nitrogen spectra of the mutant cells show no traces of typical oxygen-dependent haem proteins. Therefore, according to all known criteria of strict anaerobiosis (with the exception of catalase activity) complete anoxia was achieved in our experiments. Manyfold replications of the experiments confirmed our preliminary results. No catalase activity was found in the anaerobic wild type cultures, this confirming data available in the literature (18).

A new system of anaerobic culture of yeast was developped for studying the phenomenon of anaerobic synthesis of catalase. Two types of experiments were performed with the use of twin-bottles constructed as described in Materials and Methods. Mutant and wild type cells were grown in twin-bottles in anoxia. The 1 cm wide channel assured easy exchange of gases between both cultures, so that an eventual leak of oxygen into the system would induce

the synthesis of catalase in both mutant and wild type cells. In all experiments catalase activity was found only in the mutant cells. The results obtained in these experiments confirmed that mutant cz1 is capable of synthesizing catalase in anoxia, whereas the lack of catalase activity in wild type cells ensured internal control of the anaerobic system used. These results could not, however, exclude the possibility that catalase in the mutant is formed from the apoenzyme synthesized during anaerobic growth and from haem group accummulated during the aerobic phase of growth. This possibility was ruled out in the second type of experiments in which mutant cells were grown in strict anoxia during at least 10–15 generations, and therefore the initial amount of haem should have been diluted many times. In these experiments one bottle was inoculated with mutant or standard strain and after removal of oxygen, yeast was grown until the early stationary phase. During this period growing yeast was able to exhaust all traces of oxygen from both bottles. Then, the system was bent down and a few drops of this culture were transferred through the air channel to the second bottle, this protecting against introduction of oxygen during inoculation. The yeast culture from the second bottle was harvested in the late logarithmic phase of growth after cooling and treatment with cycloheximide. The level of catalase activity in the second bottle was identical to that found in the first type of experiments. The possibility of instantaneous synthesis of catalase during harvesting was excluded in the experiments in which cycloheximide was administered before the system from the built–in vessel. This procedure was discontinued as unnecessary and troublesome, as no differences could be found between cultures treated with cycloheximide before or just after the system was opened. The second type of experiments was applied in all crucial investigations, but for some experiments first type of culture was satisfactory.

Table 1 presents the levels of catalase activity in wild type and mutant cells grown anaerobically in different media with or without haemin. As shown in Table 1 , the level of catalase activity in the mutant is much higher when galactose medium is supplemented with haemin. Addition of haemin to the glucose medium had no effect on catalase activity. The increase of catalase activity in haemin–supplemented media suggested that mutant cells accumulate the apoenzyme of catalase and addition of haemin enables formation of active enzyme.

TABLE 1.

Catalase activity in the cz1 mutant and SP4 wild type cells grown in different media in anoxia.

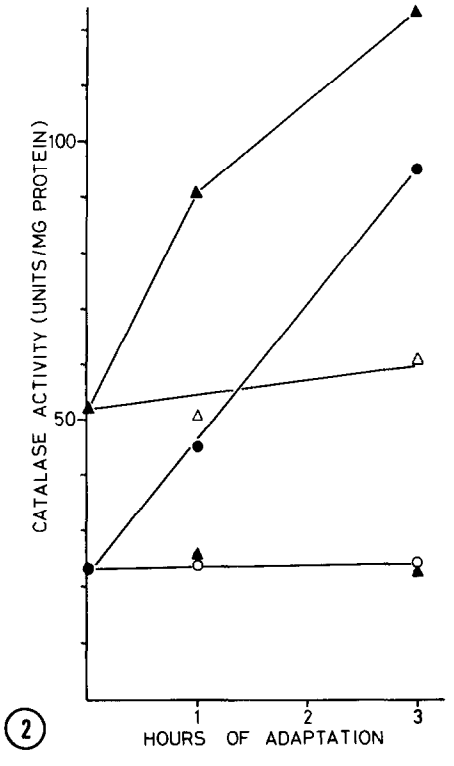
Strain	Carbon source	No addition	+ Haemin
SP4 WT	galactose 2%	0	0
	glucose 10%	0	0
cz1	galactose 2%	19.5	65.6
mutant	glucose 10%	60.1	60.7

The cells were grown anaerobically on SS or YP medium containing 2% galactose or 10% glucose as carbon source. Haemin (20 ug/ml) was added only to YP medium to avoid the precipitation of this compound. The levels of catalase activity in the cells grown on YP and SS medium did not show the differences. Data presented give values of at least 4 experiments. Catalase activity was expressed in units per 1 mg of protein.

If this asumption is true the formation of catalase should be independent of the de novo synthesis of protein after onset of aeration of anaerobically grown mutant cultures.

The results of oxygen-stimulated catalase formation in cz1 mutant pregrown on 2% galactose in anoxia are presented in Figure 2.The synthesis of catalase in samples without cycloheximide is almost linear during three hours of adaptation to oxygen. It is evident that catalase formation is almost completely inhibited in samples incubated with cycloheximide. The addition of haemin to the yeast pregrown without haemin and incubated without cycloheximide does not change the rate of catalase formation (data not shown). These results show that accumulation of catalase apoenzyme does not occur in mutant cells grown anaerobically in galactose medium.

The electrophoretic mobility of anaerobic catalase in polyacrylamide gels is identical to catalase T of aerobically grown both wild type and mutant strains as shown in Figure 3.



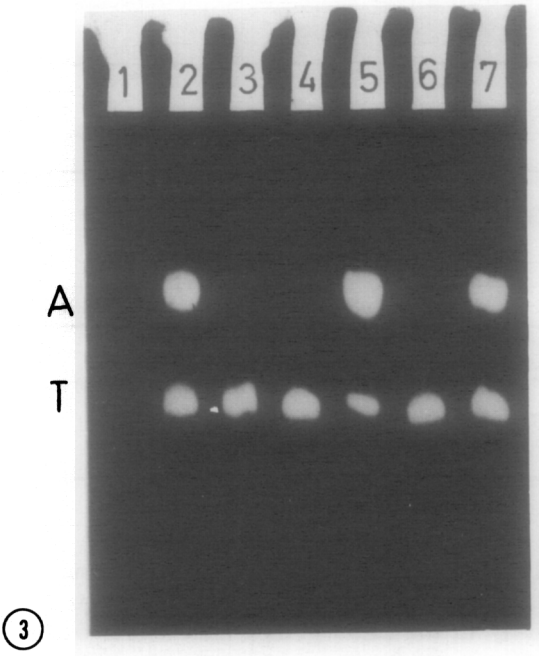


Figure 2.
Induction of catalase activity by oxygen in the cz1 mutant strain. The cells were grown anaerobically on YPGalactose medium with or without haemin (20 ug/ml). Anaerobic cultures containing haemin were diluted two times with the same medium and aerated during 3 hours with cycloheximide (CHX) △—△ or without CHX ▲—▲ . Anaerobic cultures not supplemented with haemin were divided into three parts and diluted with YPGal medium ●—● YPGal medium containing CHX O—O and YPGal containing CHX + haemin ▲—▲ ,respectively. The samples were taken every hour and catalase was assayed.

Figure 3.
Polyacrylamide slab gel electrophoresis of catalase A and T from extracts of wild type and mutant cells. The cells were grown anaerobically (N_2) and aerobically (O_2) on SS medium with 10% glucose or 2% galactose as carbon source. The extracts were prepared as described in Materials and Methods. The sample applied had a total catalase activity of 1.0 – 2.0 U in a maximal volume of 50 ul. Gel was specifically stained for catalase activity as described by (13). The following samples were applied on the gel : 1 – WT 2% galactose N_2, 2 – WT 2% galactose O_2, 3 – WT 10% glucose O_2, 4 – cz1 10% glucose N_2, 5 – cz1 2% galactose O_2, 6 – cz1 2% galactose N_2, 7 – WT 2% galactose O_2. A – catalase A, T – catalase T.

Haem content of yeast grown in anoxia.

The results presented in Table 1 show that the highest level of catalase

TABLE 2.

Haem content in mutant and parental strains in various growth conditions.

strain	growth conditions			
	aerobic		anaerobic	
	2% galactose	10% glucose	2% galactose	10% glucose
SP4 WT	287.7	83.7	8.6	traces
cz1	298.8	156.3	26.2	24.1

Haem content is expressed as nmole of haem/g of dry weight. Strains were cultured as described in Materials and Methods. The cells were harvested in late log phase of growth and haem content was determined as described in Materials and Methods.

activity in anoxia was observed in the mutant grown in 10% glucose medium. Addition of haemin does not augment catalase activity, as observed for cells grown on galactose. These results suggested differences in haem content in repressed and derepressed mutant cells. Thus haem content in wild type and mutant cells grown in different media was tested. These results presented in Table 2 demonstrate that wild type derepressed cells grown anaerobically contain no more than 3% of the haem present in fully derepressed aerobic cultures. Haem content is hardly detectable in wild type cells grown on 10% glucose in anoxia. In the cz1 mutant strain grown in anaerobic conditions the total amount of haem is much higher than in the parental strain. The differences in haem level between repressed and derepressed anaerobically grown mutant cells are very low, some factors other than total haem content influence the catalase level. In the derepressed cells availability of haem for catalase synthesis may be lower owing to the increased demand for haem evoked by the presence of other haem protein apoenzymes which are glucose repression sensitive and/or changes in compartmentation and transport of both compounds.

DISCUSSION

Formation of haem protein requires the simultaneous availability of the haem group and of apoproteins. As shown (19) , the haem availability is a prerequisite to the synthesis of one of the mitochondrially synthesized subunits of cytochrome c oxidase. The stimulation of anaerobic synthesis of catalase T in the cz1 mutant by haemin addition suggests a similar regulatory mechanism of apoprotein synthesis. In contrast Barlas et al. (20) have shown in another mutant low catalase T activity and accumulation of catalase apoprotein in anaerobic conditions. After starting aeration the formation of active catalase T in this mutant does not require de novo protein synthesis. All these facts suggest that the regulatory mechanism of catalase synthesis in anoxia consists of at least two different steps or levels.

The cz1 mutant described in this paper offers also the possibility of studying the alternate electron acceptors of the last oxidation steps of the haem pathway. The presence of electron acceptors others than oxygen has been shown for bacterial cells (21).

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