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Heme synthesis in yeast does not require oxygen as an obligatory electron acceptor✪

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In a previous paper (Krawiec, Z., Biliński, T., Schüller, C. & Ruis, H., 2000, *Acta Biochim. Polon.* 47, 201–207) we have shown that catalase T holoenzyme is synthesized in the absence of oxygen after treatment of anaerobic yeast cultures with 0.3 M. NaCl, or during heat shock. This finding suggests that heme moiety of the enzyme can either be formed *de novo* in the absence of oxygen, or derives from the preexisting heme pool present in cells used as inoculum. The strain bearing *hem1* mutation, resulting in inability to form δ-aminolevulinate (ALA), the first committed precursor of heme, was used in order to form heme-depleted cells used as inocula. The cultures were supplemented with ALA at the end of anaerobic growth prior the stress treatment. The appearance of active catalase T in the stressed cells strongly suggests that heme moiety of catalase T is formed in the absence of oxygen. This finding suggests the necessity to reconsider current opinions concerning mechanisms of heme synthesis and the role of heme as an oxygen sensor.

Anaerobically grown yeast cells do not possess typical heme proteins, including both yeast catalases A and T. The lack of catalase activity is one of the criteria of anoxia, because neither mRNAs nor apoenzymes of cytosolic catalase T and peroxisomal catalase A were reported to be produced in the absence of oxygen [1]. *In vitro* studies on the mecha-

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Abbreviation: ALA, δ-aminolevulinic acid.

nisms of heme synthesis have shown that two enzymes of heme pathway, coproporphyrinogen III oxidase and protoporphyrinogen IX oxidase, require molecular oxygen as an electron acceptor [2, 3]. As a consequence, it has been generally accepted that heme cannot be synthesized in anoxia. Heme is necessary as the prosthetic group of catalases, and as a regulatory molecule for the expression of catalases [4] and some other genes encoding various aerobic enzymes [5–7]. Heme could also be involved in posttranscriptional regulation of catalase T gene expression [8]. Thus, three independent factors should prevent the synthesis of active yeast catalases in the absence of oxygen. Earlier reports on anaerobic synthesis of small amounts of catalase T in yeast strains bearing the *cas1* and *cgr4* mutations suggested that some other electron acceptors could replace oxygen in heme synthesis *in vivo* [9, 10]. These findings, however, could result from the presence of residual amounts of heme in anaerobic cultures, adequate for the formation of small amounts of catalase T. In the previous paper [11] we showed that active catalase T is synthesized in the absence of oxygen, both in the *cgr4* mutant and in the wild type strain after treatment of anaerobic cultures with 0.3 M NaCl, or during heat shock. In those experiments the activity of the enzyme after stress treatment in anoxia were high and comparable to the levels observed in the presence of oxygen. These results did not explain definitively whether heme, which is necessary for catalase T apoenzyme synthesis, and ultimately for the formation of the active enzyme comes from the preexisting heme pool, or is synthesized *de novo* in anoxia. The aim of the present work was to approach this question.

MATERIALS AND METHODS

The following strains of *Saccharomyces cerevisiae* were used: CTT1hem- (*Mat***a** *trp1 ura3 arg4 hem1 CTT1 cta1-2* , DCAG-85A) diploid strain obtained from a cross between 85A (*Mat***a** *leu1 his4 cir1 cig1 ctta*) [12] and SP-4 (*Matα leu1 arg4*) [10], and DCA4-7ApCTA1-lacZ-*MAT***a** *arg4 his4 leu2 ura3 ctt1-1 cig1/pCTA1-lacZ/::URA3* [13] (kindly supplied by Dr. M. Skoneczny).

Experimental procedures assuring strict anaerobicity described in the previous paper were applied [11]. The double mutant strain CTT1hem-, bearing the *cta1* [14] mutation leading to the inability to synthesize catalase A, and the *hem1* [15] mutation precluding heme formation in the absence of its precursor δ-aminolevulinic acid (ALA) was constructed. In this strain the only catalase activity observed after ALA supplementation corresponds to catalase T. Cells of this mutant can grow in the absence of ALA in rich media, if supplemented with a source of unsaturated fatty acids and ergosterol. In order to remove detectable amounts of heme, this mutant was grown, prior to the experiment, for at least 20 generations in the absence of ALA under aerobic conditions. These heme- and catalase-depleted cells were used as inoculum for aerobic and anaerobic growth. At the end of anaerobic growth at 22°C, ALA solution was added from the side arm of the anaerobic vessel to reach its final concentration equal to 15 μg/ml. Control samples were withdrawn two hours after ALA administration and then the culture was warmed to 37°C or treated with concentrated NaCl solution to reach the final concentration of NaCl of 0.3 M. Cells were incubated anaerobically for one hour after stress treatment. In aerobic control experiments cells were grown in Erlenmeyer flasks closed with cotton stoppers to assure access of air. Cells were grown aerobically in complete medium (1% Yeast Extract, 1% Bacto-Peptone, 2% glucose) on a rotatory New Brunswick G-10 shaker and incubated at 22°C until they reached late exponential phase of growth. Anaerobic medium was supplemented with 0.5% Tween 80 and 0.0025% ergosterol. Before opening culture vessels were cooled by placing them on ice, not deeper than the level of growth medium.

This precaution was taken to prevent the intake of air resulting from changes in gas pressure connected with lowering of its temperature. Cycloheximide solution was added to the cultures at the moment of opening of the vessels or when taking samples through the bottom stopcock, to prevent oxygen-induced protein synthesis. Catalase A and T activities were identified in extracts of the standard strains by specific staining of H_2O_2-treated polyacrylamide non-denaturing gel electrophoresis slabs with potassium ferrocyanide to detect unstained bands corresponding to catalase activity [16]. For catalase activity determinations and polyacrylamide gel electrophoresis yeast cells were harvested by centrifugation and suspended in 0.05 M buffer, pH 6.8, and disrupted with glass beads. All manipulations were performed at maximum 4°C in order to prevent possible formation of active enzyme from the preexisting apoenzyme. Unbroken cells and cell debris were removed by centrifugation at 1000 × ***g*** for 10 min. Catalase activity was determined according to a published method [17].

In situ catalase detection was performed by injecting 30% hydrogen peroxide solution trough the stopcock of anaerobic vessels, to reach its final concentration of 3%. The outlet of the hydraulic valve was connected with the opening of a measuring cylinder filled with water and placed vertically upside-down in a water bath in order to collect oxygen formed within the vessel. The time between the addition of hydrogen peroxide and the appearance of visible oxygen bubbles was noted. The treatment with hydrogen peroxide results in killing of all cells within 15 min, which was confirmed by plating on solid media.

Possible bacterial contamination of the cultures was prevented by addition of the antibacterial antibiotics penicillin (6 units/ml) and streptomycin (10 mg/ml) into the media. All cultures were inspected under the microscope in order to detect potential bacterial contamination.

Dithionite, resazurin, Tween 80, ergosterol, δ-aminolevulinic acid (ALA) and methylene blue were obtained from Sigma Co. Aldrich Oxiclear was used to purify nitrogen. Difco Yeast Extract, Bacto Peptone and Agar were used to prepare the media. The remaining chemicals were of laboratory grade.

RESULTS

The results of experiments presented in Table 1 show that catalase activity was not detected in anaerobic control samples, whereas in ALA-supplemented, shocked cells catalase T activity was always present. This suggests that heme necessary for catalase T formation in anoxia must have been synthesized *de novo*.

However, one could postulate that small amounts of heme necessary for catalase T for-

Table 1. Catalase T activity in the strain CTT1hem⁻ bearing the *hem1* and *cta1* mutations

Medium supplementation	Catalase activity (units/mg protein)	
Type of stress	Aerobic	Anoxia
None		
Control	0.0	0.0
Heat shock	0.0	0.0
Osmotic shock	0.0	0.0
+ δ-aminolevulinate		
Control	0.8	0.0
Heat shock	6.5	5.4
Osmotic shock	7.8	6.1

mation could be synthesized during the time of manipulations necessary for catalase assay. This explanation does not seem very probable, because the process of catalase formation requires time necessary for: i. transporting heme from mitochondria to cytosol, ii. its introduction into preexisting apoenzyme, and iii. formation of active tetramere by appropriate helper proteins. This complicated process could hardly proceed at 0°C during several minutes of manipulations.

However, such a possibility was tested by detecting catalase activity of the cells, directly within the culture vessels, at the moment of opening. This is possible, because both the substrate and the product of the reaction carried out by catalases easily penetrate cellular membranes, due to their small size and lack of electric charge. It was done by injecting a concentrated solution of hydrogen peroxide into the vessel and collecting the oxygen formed. Three samples were compared. The first one corresponded to control anaerobic culture, another anaerobic culture was subjected to heat shock. The third experiment was performed with the use of aerobic cultures of the wild type strain SP-4 showing high catalase activity. This culture, whose density was adjusted to values close to those of anaerobic ones, was transferred to anaerobic vessels and treated with hydrogen peroxide in a similar way. Figure 1 presents kinetics of gaseous oxygen formation by cultures. Evolution of oxygen takes place within two minutes after hydrogen peroxide administration both in anaerobic mutant culture subjected to the heat shock and in control aerobic cultures. Thus, the time of appearance of easily measurable amounts of oxygen is identical in the cells which evidently posses active catalase and in anaerobically grown, stress treated cells. During the experiment the formation of oxygen bubbles was seen within less than 20 s after H_2O_2 treatment, but in anaerobic control cells no formation of oxygen was observed during 30 min of incubation. The time close to 20 s after hydrogen peroxide administration is necessary for the substrate to enter the cell, the formation of the product, saturation of the intracellular solute with oxygen and finally forming oxygen bubbles.

Another type of experiment was performed with the *hem1* mutant grown without ALA and subjected to the same stress factors before cycloheximide treatment and subsequent supplementation with ALA. In no case was catalase activity detected. These results show that the absence of heme makes the formation of apoenzyme of catalase T, and/or the formation of auxiliary proteins impossible.

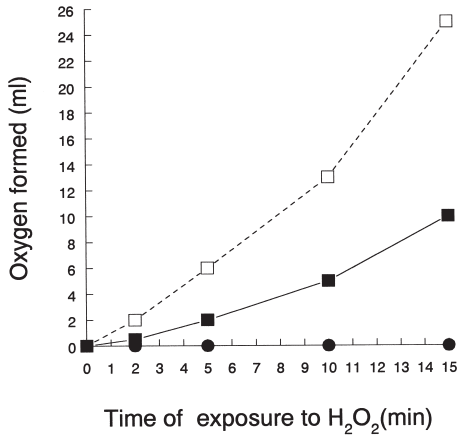

Figure 1. Kinetics of gaseous oxygen formation after treatment of aerobic and anaerobic cultures with 3% of hydrogen peroxide.

The amount of gaseous oxygen formed was measured by collecting oxygen bubbles in a measuring cylinder. ˜, Control anaerobic culture; £, control aerobic culture; ¢, anaerobic culture subjected to heat shock.

In the previous paper we showed that under the experimental conditions catalase T activity was always detected in the stressed cells, whereas catalase A activity was never detected in the tester strain DCA4-7ApCTA1-lacZ. In this strain the synthesis of four peroxisomal enzymes, including catalase A, is partly relieved from catabolic repression [13], due to the presence of the *cig1* mutation [12]. This strain expresses peroxisomal acetyl-CoA oxidase and a catalase A promoter-driven reporter gene, under anaerobic conditions, but is unable to form active catalase A. The results

suggested that the lack of heme in anoxia is the cause of the inability of cells to form active catalase A.

This discrepancy could result from differences between the strains in the regulation of heme synthesis, or its distribution, due to their different genetic backgrounds. It could make possible the formation of catalase T in one strain and prevent the synthesis of catalase A in the other.

In order to test this possibility a diploid strain bearing the *cig1* mutation and intact genes coding for both catalases was constructed. This was achieved by crossing the strain 85A with the wild type strain SP-4. The *cig1* mutation is semidominant [13], and catalase A synthesis in the diploid strain obtained from this cross is partly relieved from glucose repression. The diploid strain is therefore able to form aerobically both catalases in the presence of glucose. This diploid strain was used in anaerobic experiments and again only catalase T activity was detected (Fig. 2). It appears therefore, that after stress treatment in anoxia only catalase T can be formed. This result shows that the distribution of heme within the cell is very selective.

DISCUSSION

In our opinion control experiments excluded experimental error, i.e. bacterial contamination, oxygen leakage into the system, and the possibility that the heme necessary for catalase T formation came from a preexisting heme pool. The addition of cycloheximide to the cultures prevents *de novo* synthesis of catalase T apoenzyme and the synthesis of other proteins necessary for the formation of active catalase. Assuming that active catalase T is not synthesized in cells after heat shock, but only its apoenzyme is accumulated, the formation of holoenzyme could take place after opening of the anaerobic vessel. Immediate *in situ* assay for catalase activity makes this possibility rather improbable, because the kinetics of oxygen generation clearly suggest preexistence of active enzyme in anaerobic cultures. It is noteworthy that treatment of yeast cells with 3% hydrogen peroxide results in killing of all cells within 15 min after treatment. It seems therefore highly improbable that under such severe conditions heme synthesis, its transport to cytosol, and the introduction of heme into the protein moiety could take place within less than 20 s — the time when mass formation of oxygen bubbles is observed. Another argument supporting our postulate was obtained in aerobic experiments showing that the absence of heme prevents the synthesis or formation of catalase T apoenzyme after stress treatment. Thus, the results obtained strongly suggest that heme synthesis in yeast does not require oxygen as an obligatory electron acceptor, at least after heat shock or hyperosmotic stress.

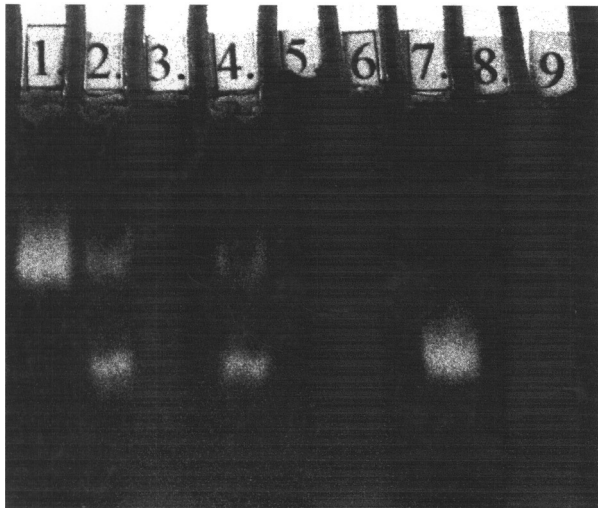

Figure 2. Identification of catalase activity in various strains of yeast under aerobic and anaerobic conditions.

Slot: 1. 85A, haploid (bearing *cig1* mutation), aerobic 22°C; 2. SP-4, standard haploid strain, aerobic 22°C; 3. SP-4, standard haploid strain, anoxia 22°C; 4. DCAG-85A, diploid strain, aerobic 22°C; 5. 85A, haploid (bearing *cig1* mutation), aerobic 22°C; 6. 85A, haploid (bearing *cig1* mutation), aerobic 22°C → 37°C; 7. DCAG-85A, diploid strain, anaerobic 22°C → 37°C; 8. DCAG-85A, diploid strain, anaerobic 22°C; 9. DCAG-85A, diploid strain, anaerobic 22°C.

Two independent mechanisms of the synthesis of δ-aminolevulinate, the first committed precursor of the tetrapyrrole ring, are known. One of them, the five-carbon or glutamate pathway, is found in the plant world [18] and dominates among prokaryotes [19, 20]. In non-plant eukaryotes including yeast, ALA is synthesized by ALA synthase from glycine and succinyl-CoA. The presence of ALA synthase in eukaryotes is a consequence of its distribution to mitochondria by the α-subclass of purple bacteria-like endosymbiont [21]. The coexistence of these two systems was found in *Euglena gracilis* [22]. In this organism the 5-carbon pathway provides ALA for chloroplast protoheme and chlorophyll. In contrast, ALA synthase provides ALA for non-plastid heme synthesis. Further steps of this ancient biosynthetic pathway seem to be very similar in all taxa with one notable exception. It is known that heme synthesis in bacteria may proceed in the absence of oxygen [23–25]. It is difficult to imagine, therefore, that any obstacles exist preventing the oxidation of porphyrinogens to porphyrins by electron acceptors other than molecular oxygen.

Opinions concerning oxygen dependence of heme formation in eukaryotes are generally accepted because they explain in a simple way the inability of yeast cells to form typical heme proteins in anoxia. The only straightforward experiments showing that oxidation of copro- and protoporphyrinogen to copro- and protoporphyrin requires the presence of oxygen, were performed only in *vitro* [2, 3]. Our results mean that either some cellular compounds could replace oxygen as an electron acceptor in the reaction carried out by appropriate oxidases, or more probably in yeast cells, similarly to *Salmonella typhimurium*, two independent mechanisms of porphyrinogen oxidation exist [24, 25]. One of them could be oxygen independent, and possibly stress inducible. In the database we have found the sequence YNL063w [26] with weak similarity to *Mycoplasma* protoporphyrinogen oxidase which could fulfill this role.

The postulated independence of heme synthesis of the presence of oxygen in yeast is supported by already published data. It is known that anaerobically grown yeast cells accumulate Zn-protoporphyrin in the stationary phase of growth [27]. This means that the last precursor of heme-protoporphyrin can be synthesized in anoxia, because the steps which are supposed to be oxygen dependent precede the formation of protoporphyrin. It is also postulated that the level of cytochrome P-450 is increased in anoxia when compared to aerobic conditions. The origin of heme necessary for its formation has never been explained. The ability of yeast cells to produce anaerobically Zn-protoporphyrin and cytochrome P-450 suggests that the lack of typical aerobic heme proteins results from the existence of specific regulatory mechanisms preventing their synthesis, rather than from oxygen dependence of protoporphyrin synthesis.

Our conclusions are also strongly supported by recent studies on catalase synthesis in *Schizosaccharomyces pombe* which showed that the levels of catalase in anoxia are higher than in aerobic cells. Strikingly, this was observed in a mutant resistant to oxidative stress [28]. This finding creates another link between the stress response and catalase (and heme) synthesis.

The results presented suggest that all enzymes necessary for heme synthesis are not only present, but also able to function in anaerobically grown cells, at least when cells are subjected to thermal or osmotic stress. The formation and distribution of the final product – heme is therefore strictly regulated in standard yeast strains, because the activity of catalase T is undetectable in anoxia under "physiological conditions". However, in strains bearing the *cas1* and *cgr4* mutations catalase T synthesis takes place also under "physiological conditions" [9, 10].

One of the explanations of our results is that the formation of measurable amounts of heme from protoporphyrin by ferrochelatase is possible only when heme is removed from the

place of its formation by appropriate heme transporters, because large scale accumulation of heme was never observed. Accumulation of heme in any cellular compartment could be very dangerous, because this compound is a potent source of oxygen free radicals. The postulated regulatory mechanisms preventing the accumulation of easily measurable "free heme pool" could act through the regulation of the synthesis, or degree of saturation of appropriate heme transporters. A lack of demand should prevent heme formation by means of full saturation of these transporters by heme.

It has been proposed [29] that oxygen-dependence of heme synthesis makes this molecule an ideal sensor of oxygen, which plays a crucial role in the expression of numerous genes coding for various proteins, including non-heme proteins [7]. Some authors [30–32] however, are aware of the fact that the precise role of heme in oxygen sensing is still unclear. The ability to form heme in anoxia shown by our studies suggests that the appearance of the heme molecule *per se* cannot be considered as a signal of the presence of oxygen. A signaling role could be rather played by redox or spin state changes of heme iron in a hemoprotein oxygen sensor [33]. It is also not obvious that a common oxygen sensor exists, or whether heme is its active group. The studies on the regulation of yeast peroxisomal enzymes suggest that heme is not involved in the induction of their expression by oxygen [34]. Such a role could be played by iron sulfur clusters, as it was found in bacteria [35].

Our results suggest that oxygen could merely increase the ability of cells to form heme as a preferred electron acceptor. Its presence augments demand for this molecule, by inducing expression of genes encoding typical aerobic heme proteins. The presence of oxygen could also raise the "regulatory pool" of heme, which in turn influences the expression of various sets of genes.

Heme is necessary for activating the heme-responsive transcriptional activator HAP1 involved in the expression of numerous genes including the catalase T gene [4, 5]. The question arises whether the heme control element [4] of the CTT1 gene promoter could be compromised during anaerobic induction of its expression by stress factors. As shown, cells deficient in heme are not able to form catalase T apoenzyme and/or other proteins necessary for its formation. Our results suggest that the amount of heme produced during stress in anoxia is high enough both for the activation of HAP1 and the formation of active catalase T. HAP1 forms an inactive complex with four other proteins including Hsp90 [36] and preexists in a dimer form [37] which is a prerequisite for transcriptional activation of genes. If heme, mutation or maybe stress, disrupt the complex, HAP1 becomes activated. HAP1 activation under experimental conditions could have important consequences for the synthesis of other heme-dependent proteins, because HAP1 binds to at least two independent classes of DNA elements and makes possible differential transcription at these sites [38].

Our results do not explain whether heme synthesis in anoxia is possible under "physiological conditions", although the presence of catalase T in *cgr4* and *cas1* mutants suggests a positive answer to this question. Its formation could depend on demand resulting from the synthesis of heme protein apoenzymes and the presence of other auxiliary proteins. These results do not explain the reason why catalase A activity is absent in anaerobically grown diploid strain bearing the *cig1* mutation. In these cells heme is available only for the formation of the cytosolic enzyme, but not for the peroxisomal one. Different localization of these enzymes could mean that their formation requires different sets of auxiliary proteins. Recent studies showing a lack of detectable "free heme pool" in yeast peroxisomes [39] also suggest that such a "free heme pool" does not exist and heme is supplied to this organellum on demand only in amounts necessary for "immediate use".

The controlling mechanisms are still not known in yeast.

Our knowledge concerning heme transport and the mechanisms controlling the availability of heme for the synthesis of various heme proteins in yeast cells is still incomplete [40], in contrast to the knowledge of bacterial systems [41, 42].

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