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# Inhibition of Catalase by Aminotriazole *in vivo* Results in Reduction of Glucose-6-phosphate Dehydrogenase Activity in *Saccharomyces cerevisiae* Cells

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**Abstract**—The inhibitor of catalase 3-amino-1,2,4-triazole (AMT) was used to study the physiological role of catalase in the yeast *Saccharomyces cerevisiae* under starvation. It was shown that AMT at the concentration of 10 mM did not affect the growth of the yeast. *In vivo* and *in vitro* the degree of catalase inhibition by AMT was concentration- and time-dependent. Peroxisomal catalase in bakers' yeast was more sensitive to AMT than the cytosolic one. *In vivo* inhibition of catalase by AMT in *S. cerevisiae* caused a simultaneous decrease in glucose-6-phosphate dehydrogenase activity and an increase in glutathione reductase activity. At the same time, the level of protein carbonyls, a marker of oxidative modification, was not affected. Possible mechanisms compensating the negative effects caused by AMT inhibition of catalase are discussed.

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*Key words*: *Saccharomyces cerevisiae*, 3-amino-1,2,4-triazole, catalase, glucose-6-phosphate dehydrogenase

Reactive oxygen species (ROS) can be generated in cells either as by-products of aerobic metabolism and/or produced by specific enzymatic systems to destroy pathogenic organisms. There are other possible ways to form ROS, for instance, different external stresses, particularly xenobiotics, can be involved in the process. Superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($^{\cdot}OH$), which are the most studied ROS, can modify in fact any molecules, but oxidation of nucleic acids, proteins, and lipids is the most critical [1]. Cells possess special systems responsible for ROS detoxification. The first defense line includes antioxidant and associated enzymes. Antioxidant enzymes, namely superoxide dismutases, catalases, and peroxidases, directly deal with ROS. The second group consists of enzymes that assist the main enzymes detoxifying secondary products of free radical modification and maintaining necessary intracellular concentrations of reducing equivalents and low molecular mass antioxidants. For example, glutathione reductase and glucose-6-phosphate dehydrogenase, maintaining intracellular pool of reduced glutathione and NADPH, respectively.

The budding yeast *Saccharomyces cerevisiae* is probably the most studied eukaryotic organism employed as a model system to investigate physiological functions of antioxidant enzymes. This yeast possesses two catalase forms – A and T, encoded by different genes. Catalase A, the product of gene *CTA1*, is localized mainly in peroxisomes, and catalase T, the product of gene *CTT1*, is found mainly in cytosol [2]. Catalases disproportionate two molecules of hydrogen peroxide, resulting in molecular oxygen and water, and efficiently protect cellular constituents from oxidation. However, the physiological role of catalases in the cell is not associated with only this reaction. It depends highly on the expression of catalase isoenzymes. In addition, catalases in many eukaryotes possess peroxidase activity, using hydrogen peroxide as electron and proton acceptor. There are two different ways to investigate the role of catalases, as well as most other enzymes. The common feature of these methods is an investigation of

*Abbreviations*: AMT) 3-amino-1,2,4-triazole; CP) protein carbonyls; G6PDH) glucose-6-phosphate dehydrogenase; GR) glutathione reductase; ICDH) isocitrate dehydrogenase; LDH) lactate dehydrogenase; PMSF) phenylmethylsulfonyl fluoride; ROS) reactive oxygen species; SOD) superoxide dismutase; TEMED) N,N,N′,N′-tetramethylethylenediamine.

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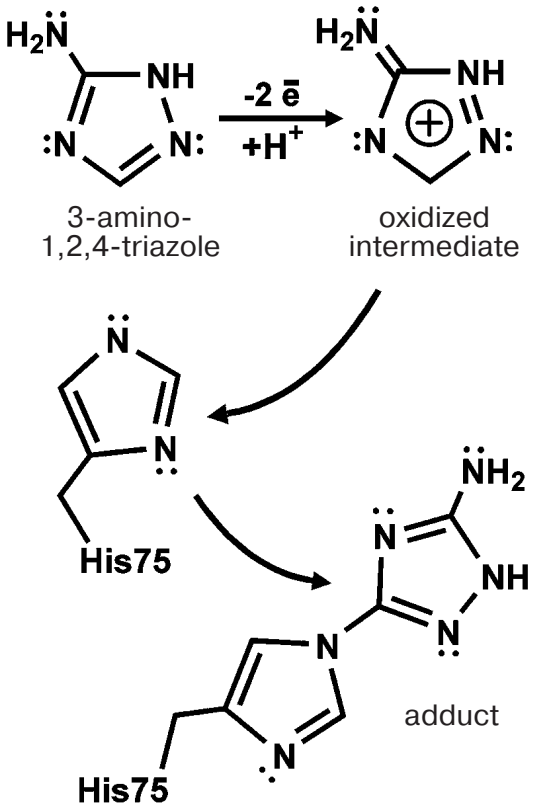


**Fig. 1.** Proposed scheme for reaction of AMT with His75 of human catalase [9].

physiology of cells lacking the activity of studied enzyme. The first way uses knockout isogenic strains and the second uses specific enzyme inhibitors. Both of these ways were applied earlier in our laboratory with different experimental models [3-7]. The use of *S. cerevisiae* catalase-deficient strains let us highlight the role of catalase isozymes in the protection of cellular proteins against oxidation [8]. A similar idea was exploited with superoxide dismutase (SOD)-deficient mutants [4]. Interesting results were obtained in the case with inhibition of Cu,Zn-SOD in *S. cerevisiae* by N,N′-diethyldithiocarbamate [9]. Both approaches have some positive sides and limitations, and therefore their combination is probably the most efficient way to elucidate the role of certain enzyme in cells.

In this work we used 3-amino-1,2,4-triazole (AMT), a known catalase inhibitor. It inactivates catalases by modifying the binding site for hydrogen peroxide between histidine and asparagine residues via covalent interaction with His75 in human catalase [10]. In according with the proposed scheme (Fig. 1), two-electron oxidation of AMT generates a common resonance-stabilized intermediate, followed by deprotonation. The nucleophile formed attacks His75 resulting in formation of non-coplanar adduct [10].

In active yeast cells, AMT affects many functions, but the most prominent one is the inhibition of amino acid biosynthesis, and therefore it is used also to induce histidine starvation [11]. In order to avoid interference with biosynthetic pathways, we used a model of starving yeast, where the mentioned processes are inhibited and cells are very tolerant to different stresses.

## MATERIALS AND METHODS

**Chemicals and yeast strains.** Glucose-6-phosphate, phenylmethylsulfonyl fluoride (PMSF), isocitrate, EDTA, and AMT were obtained from Sigma (USA); NADP(H), NAD(H), 2,4-dinitrophenylhydrazine, quercetin, oxidized glutathione, and N,N,N′,N′-tetramethylethylenediamine (TEMED) were from Reanal (Hungary), and guanidine-HCl was from Fluka (Germany). Inorganic chemicals were obtained from Reakhim (Russia). All the other chemicals were of analytical grade. Yeast extract was from BioGene (Great Britain).

The *S. cerevisiae* strains used in this study were as follows: YPH250 (*MATa trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*), YTT7 (YPH250 *Δctt1::URA3*), YIT2 (YPH250 *Δcta1::TRP1*), and YWT1 (YPH250 *Δcta1::TRP1 Δctt1::URA3*). The strains were kindly provided by Professor Yoshiharu Inoue (Kyoto University, Japan).

**Growth conditions and cell incubation.** Cells were grown in medium containing 1% yeast extract, 2% peptone, and 1% glucose (YPD) at 28°C with shaking at 180 rpm for 24 h ($A_{600}$ ~1.6-1.7). The yeast were harvested by centrifugation (5 min, 7000*g*) and resuspended in 0.9% NaCl. Then appropriate amounts of AMT were added and cells were incubated at 28°C with shaking at 180 rpm for additional 24 h. In *in vitro* inhibition experiments, the appropriate amount of AMT was added directly to the supernatants prepared from stationary growing cells.

**Preparation of cell-free extracts.** Cells from the experimental cultures were harvested as mentioned above and washed twice with 50 mM potassium phosphate buffer (pH 7.0). The yeast pellets were resuspended in lysis buffer containing the same buffer, 1 mM PMSF, and 0.5 mM EDTA. The yeast cells were disrupted by vortexing with equal volume of glass beads (450-500 μm) (Sigma) followed by 1 min of cooling on ice. Cell debris was removed by centrifugation for 15 min at 4°C and 15,000*g*. The cell extracts were kept on ice and used during the next 6 h.

**Measurement of protein carbonyls.** The content of carbonyl groups in proteins was measured by determining the amounts of 2,4-dinitrophenylhydrazone formed under reaction with 2,4-dinitrophenylhydrazine. Carbonyl content was calculated from the absorbance maximum of 2,4-dinitrophenylhydrazone measured at 370 nm using an extinction coefficient of 22 $mM^{-1} \cdot cm^{-1}$ [8]. The results are expressed in nmoles per mg of protein.

**Enzyme activity assay.** The activity of SOD was assayed at 406 nm as the inhibition of quercetin oxidation by superoxide anion [9] in medium containing 30 mM Tris-HCl buffer (pH 9.0), 0.5 mM EDTA, 0.8 mM TEMED, 50 μM quercetin, and 1-50 μl of cell extract in a final volume of 2 ml. One unit of SOD activity was defined as the amount of soluble protein of supernatant that inhibited the maximal rate of quercetin oxidation by 50%. The activity of glucose-6-phosphate dehydrogenase (G6PDH) was measured by monitoring NADP reduction in medium containing 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 5.0 mM $MgSO_4$, 0.2 mM NADP, 2.0 mM glucose-6-phosphate, and 30 μl of supernatant in a final volume of 1 ml [8]. The activity of glutathione reductase (GR) was measured by following the consumption of NADPH in medium containing 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 1 mM oxidized glutathione, 0.25 mM NADPH, and 50 μl of supernatant in a final volume of 1 ml [8]. The activity of isocitrate dehydrogenase (ICDH) was assayed by monitoring NAD reduction in medium containing 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 0.25 mM NAD, 0.5 mM isocitric acid, and 100 μl of cell extract in final volume of 1 ml [5]. The activity of lactate dehydrogenase (LDH) was assayed by monitoring NADH oxidation in medium containing 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 0.16 mM NADH, 1 mM pyruvate, and 60 μl of cell extract in final volume of 1 ml [12]. NADPH or NADH oxidation and NADP or NAD reduction by respective enzymes were registered at 340 nm, and an extinction coefficient for these coenzymes of 6.22 $mM^{-1}\cdot cm^{-1}$ was used. The activity of catalase was assayed in 2 ml of medium containing 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 10 mM hydrogen peroxide, and 100 μl of cell extract in a final volume of 2 ml [8]. Hydrogen peroxide consumption was measured at 240 nm using an extinction coefficient for hydrogen peroxide of 39.4 $M^{-1}\cdot cm^{-1}$.

One unit of G6PDH, GR, ICDH, LDH, and catalase activity is defined as the amount of supernatant protein that utilizes or produces 1 μmol of substrate or product per minute. All activities were measured at 25°C and expressed per milligram of soluble protein in supernatant.

**Protein concentration and statistical analysis.** Protein concentration was determined by the Coomassie Brilliant Blue G-250 dye-binding method [13] with bovine serum albumin as a standard. Experimental data are expressed as the mean value of 4-6 independent experiments ± the standard error of the mean (SEM), and statistical testing used Student's *t*-test.

## RESULTS AND DISCUSSION

The involvement of catalase in protection of cellular components in *S. cerevisiae* depends very much on cultivation conditions, phase of culture growth, etc. [2, 6, 14, 15]. In order to reduce the interference of AMT with other metabolic processes we used a starvation model in which metabolism is rather retarded and mainly focused on surviving.

To determine the sublethal doses of AMT, the yeast cells were cultivated in the presence of different concentrations of the inhibitor. It has been shown that 1-10 mM AMT in fact did not affect the growth of *S. cerevisiae* YPH250 wild strain (Fig. 2). However, in previous studies we have found that AMT-treatment at concentrations higher than 20 mM resulted in 20% decrease of survival of starved yeast cells. Therefore, in further experiments with starved yeast, we used the concentrations of AMT up to 10 mM, which demonstrated virtually no toxicity.

Figure 3 demonstrates the *in vivo* effect of various AMT concentrations on catalase activity in YPH250 wild-type cells. As expected, the activity of catalase decreased with an increase of the inhibitor concentration. The exposure of the yeast to 1 mM AMT resulted in about 1.5-fold reduction of catalase activity, and approximately 3-fold inhibition was found under the treatment with 10 mM AMT. Further we studied the influence of different concentrations of AMT as well as time of exposure on catalase activity in the parental and single-mutant strains *in vitro* (Fig. 4). As seen in Fig. 4a, cell treatment with 10 mM AMT for 10 h caused about 4-fold decrease in catalase activity of the wild strain. It should be noted that catalase activity in either YPH250 (wild type) or YTT7 (*ΔCTT1*) cells similarly depended on time of exposure to AMT (Fig. 4, a and c) and was more sensitive to inhibitor as compared to YIT2 (*ΔCTA1*) (Fig. 4b). At the same time, under 20-50 mM AMT treatment the degree of enzyme inhibition in parent cells (Fig. 4a) was similar to

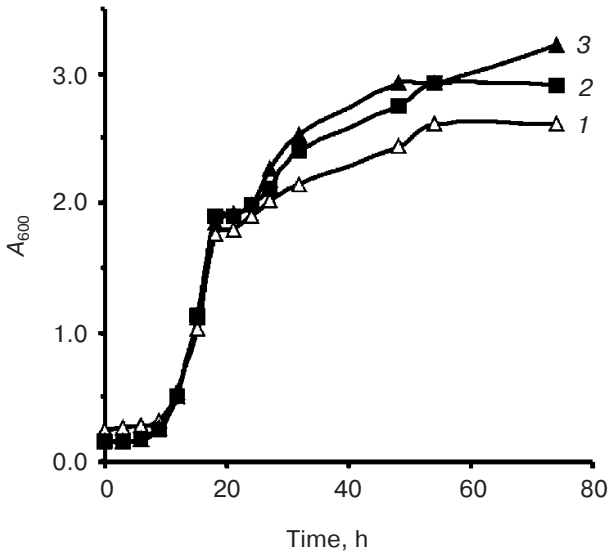


**Fig. 2.** Growth curves of *S. cerevisiae* wild type strain YPH250 growing at the presence of AMT during 24 h: *1*) control (without AMT); *2*) 1 mM AMT; *3*) 10 mM AMT.

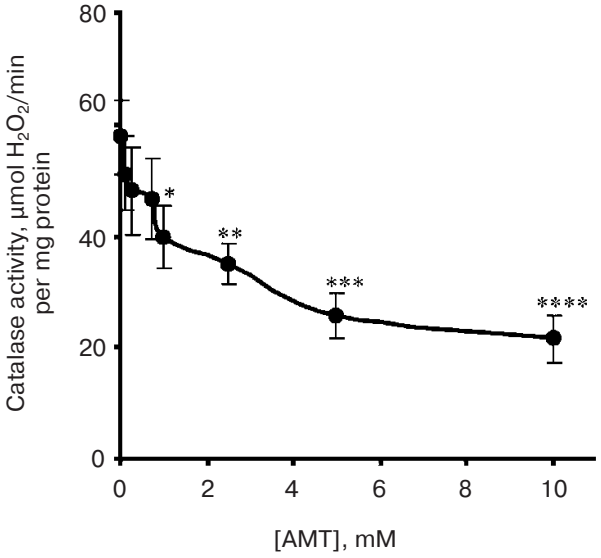


**Fig. 3.** Catalase activity in extracts from wild type strain cells treated with different AMT concentrations for 24 h at 28°C. Results are shown as means ± SEM ($n$ = 5-6). * Significantly different from respective control values with $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$, and **** $P < 0.001$.

that in YIT2 (*ΔCTA1*) strain (Fig. 4b). The highest degree of catalase inhibition was found in the extracts obtained from YTT7 cells (expressing only catalase A) treated by 50 mM AMT (Fig. 4c). In this case, the extracts completely lost catalase activity during 5 h of incubation with 50 mM AMT, whereas extracts from strain YIT2 (possessing only catalase T) lost approximately 80% of the activity within the same period of exposure.

The dynamics of catalase inhibition by 50 mM AMT in cell-free extracts of three strains studied is given in Table 1. The inhibition constants $I_{50}$ dropped with increase of time exposure. However, at the first period of incubation the decrease of $I_{50}$ values was more pronounced in the extracts from YTT7 (*ΔCTT1*) cells. The sharpest reduction of $I_{50}$ (2-3-fold) was found in the period between 2.5 and 5 h incubation for all strains used. In this case, the extracts from wild-type cells demonstrated the highest inhibition of catalase. For cytosolic catalase (YIT2 strain), we found about 2-fold reduction of $I_{50}$ value in every 2.5 h of exposure. Catalase of this strain was the most resistant to AMT. It is interesting to note that changes in the inhibition constant found for parental strain is not the simple sum of the constants of the respective single mutants.

Our data on inhibition of catalase in whole yeast cells and cell extracts are consistent with previous data on microorganisms [16, 17] and animals [3, 5, 18-20]. The inhibition of catalase resulted in generation of higher amounts of hydrogen peroxide in *S. cerevisiae* cells [16], which in turn increased the chance of free radical damage to different cell components.

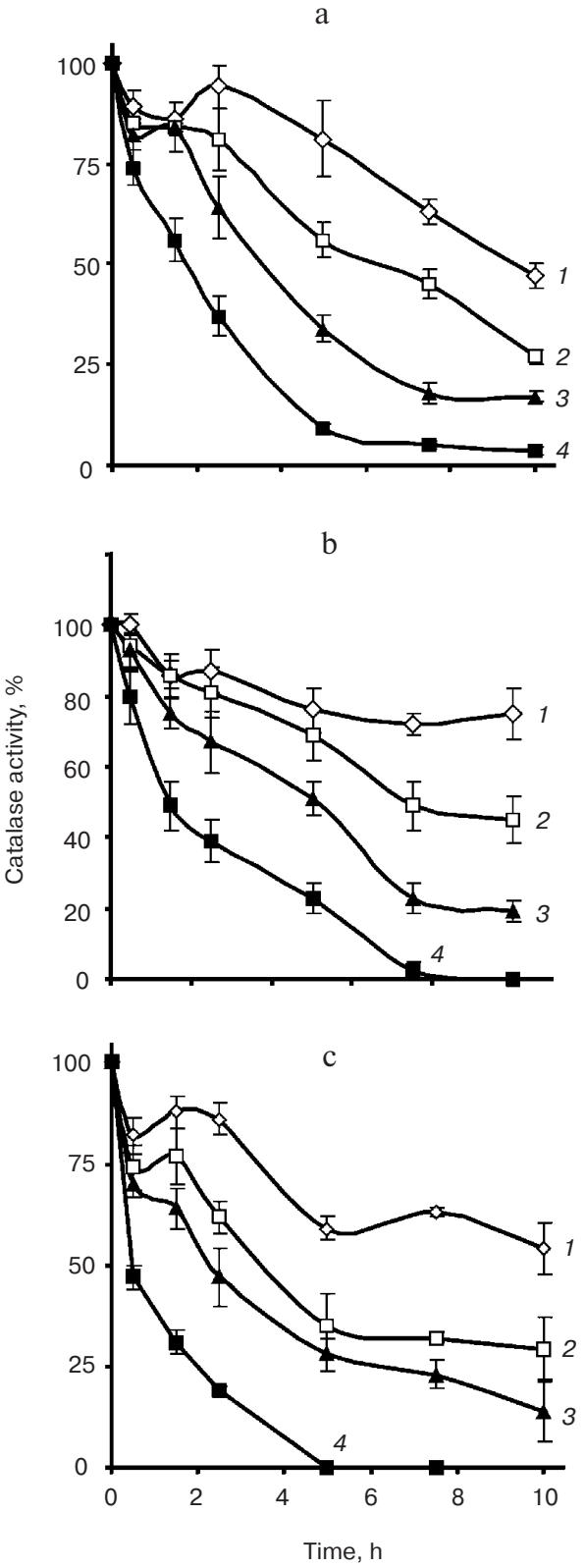


**Fig. 4.** Effect of AMT on the activity of catalase in extracts from different *S. cerevisiae* strains. Concentrations of AMT: 5 mM (*1*), 10 mM (*2*), 20 mM (*3*), and 50 mM (*4*). Strains: YPH250 (wild strain) (a), YIT2 (Δ*CTA1*) (b), YTT7 (Δ*CTT1*) (c). Results are shown as means ± SEM ($n$ = 4-5).

**Table 1.** Constants of inhibition ($I_{50}$) of catalases by 50 mM AMT (*in vitro*) in *S. cerevisiae* YPH250 (wild type), YIT2 (*ΔCTA1*), and YTT7 (*ΔCTT1*)

| Time, h | Strain | | |
|---|---|---|---|
| | YPH250 | YIT2 | YTT7 |
| 1.5 | 50.7 ± 5.6 | 56.5 ± 17.4 | 28.4 ± 4.0[1] |
| 2.5 | 26.3 ± 2.3 | 35.4 ± 8.9 | 11.5 ± 1.4[3] |
| 5.0 | 9.3 ± 0.7 | 16.4 ± 2.8[3] | 5.5 ± 0.9[2,b] |
| 7.5 | 8.5 ± 1.1 | 7.7 ± 0.8 | 5.5 ± 0.1[3,c] |
| 10.0 | 4.0 ± 0.4 | 6.6 ± 0.5[2] | 3.5 ± 0.6[a] |

[1] Significantly different from the respective values of YPH250 with $P < 0.05$, [2] $P < 0.01$, and [3] $P < 0.025$.
[a] Significantly different from the respective values of YIT2 with $P < 0.05$, [b] $P < 0.01$, and [c] $P < 0.025$ ($n$ = 4-5).

Aminotriazole, which is used as a herbicide, is known to inhibit imidazole glycerol-phosphate dehydratase (IGPD) involved in the biosynthesis of histidine [21]. Therefore, the most pronounced effect of AMT is histidine starvation [10, 22-25]. For example, the use of AMT helped to demonstrate that starvation for histidine imitated calorie restriction in yeast that expended lifespan [26]. Although, it is believed that AMT affects histidine synthesis as a histidine analog [11], no clear evidences for this were found in the literature. Taking into account the abovementioned suggestion and data of others, at least two more possibilities can be proposed to explain the phenomenon we discussed above: (i) the inhibitor can interact with active site of IGPD; (ii) IGPD can be inactivated by highly reactive metabolites like $^{\bullet}OH$ due to increased $H_2O_2$ level resulting from catalase inhibition.

Both proposed hypotheses could be checked *in vitro* with purified enzyme. However, in the native cell confirmation of the first suggestion is impossible and examination of the second one seems to be complicated as well. For instance, not only IGPD inactivation but other effects associated with AMT can cause histidine starvation. In spite of abovementioned, we suppose that use of AMT as a catalase inhibitor to study physiological significance of the enzyme is suitable in comparison with other inhibitors.

Our work was designed to exclude maximally other processes connected with AMT presence and to clarify further the role of catalases in protection of cellular components *in vivo*. That is why the yeast cells were inoculated into 0.9% NaCl solution without any nutrients. It is known that similar conditions substantially increase replicative life cycle of the yeast [27, 28]. Most metabolic processes are substantially suppressed because resources are maximally directed to survive unfavorable conditions. Therefore, on one hand, the starvation state reduces interference of AMT treatment with other processes, and on the other hand, it makes cell highly resistant to different kinds of external stresses. That is due to an increase of the activity of antioxidant enzymes, particularly catalases, under starvation [2, 29].

Next, we studied the effect of AMT *in vivo* on the activity of different enzymes that are sensitive to oxidation and the level of protein carbonyl groups (CP)—a marker of oxidative protein modification in the cell. Table 2 demonstrates that AMT treatment did not significantly change the activity of SOD, ICDH, and LDH and the

**Table 2.** Activities of superoxide dismutase (units per mg of protein), glutathione reductase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, and isocitrate dehydrogenase (nmol NAD(P)H/min per mg of protein) and carbonyl protein levels (nmol per mg of protein) in *S. cerevisiae* YPH250 cells treated with different AMT concentrations *in vivo* for 24 h

| AMT, mM | Parameter | | | | | |
|---|---|---|---|---|---|---|
| | SOD | GR | G6PDH | LDH | ICDH | CP |
| 0 | 247 ± 35 | 33.4 ± 2.1 | 60.5 ± 3.1 | 11.9 ± 2.0 | 10.6 ± 2.0 | 2.63 ± 0.66 |
| 0.10 | 149 ± 34 | 28.9 ± 2.4 | 55.5 ± 3.0 | 7.8 ± 1.0 | 7.2 ± 1.0 | 2.10 ± 0.78 |
| 0.25 | 226 ± 50 | 30.0 ± 3.5 | 51.9 ± 1.2** | 8.9 ± 1.0 | 8.1 ± 1.0 | 2.17 ± 0.59 |
| 0.75 | 230 ± 54 | 33.5 ± 3.3 | 52.0 ± 4.6 | 9.6 ± 1.8 | 8.5 ± 1.8 | 2.55 ± 0.55 |
| 1.0 | 292 ± 18 | 31.8 ± 2.5 | 52.9 ± 3.6 | 10.7 ± 1.2 | 9.6 ± 1.4 | 2.73 ± 0.57 |
| 2.5 | 285 ± 61 | 28.2 ± 2.9 | 46.8 ± 3.8** | 7.4 ± 1.2 | 7.4 ± 1.2 | 2.18 ± 0.78 |
| 5.0 | 173 ± 47 | 33.1 ± 3.3 | 47.2 ± 3.2** | 9.7 ± 1.1 | 8.8 ± 1.3 | 2.95 ± 0.45 |
| 10.0 | 229 ± 70 | 45.5 ± 5.9* | 45.9 ± 1.8*** | 9.9 ± 1.1 | 9.7 ± 1.3 | 2.10 ± 0.35 |

* Significantly different from the respective control values with $P < 0.05$, ** $P < 0.001$, and *** $P < 0.025$ ($n$ = 4-5).

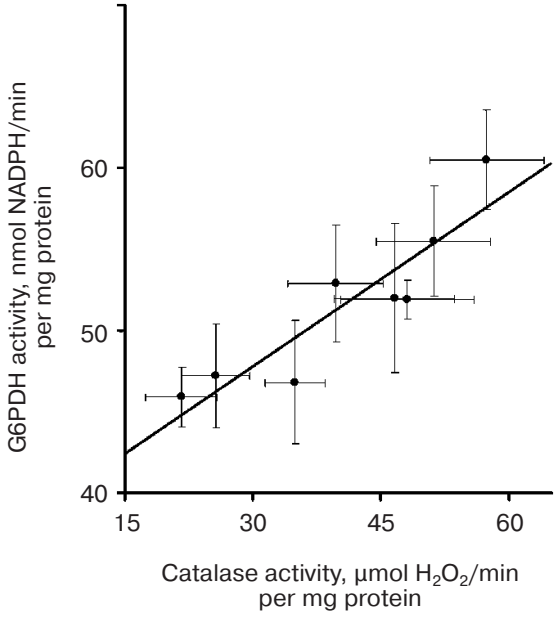


**Fig. 5.** Correlation between catalase and G6PDH activities in wild-type cells treated with different AMT concentrations during 24 h at 28°C. Results are shown as means ± SEM ($n$ = 5-6; $R^2$ = 0.83).

level of protein carbonyls. At the same time, GR activity was 1.4-fold increased by treatment with 10 mM AMT compared to the control. On the other hand, G6PDH activity gradually decreased in parallel with an increase of inhibitor concentration. When we plotted the activity of G6PDH against catalase activity at AMT exposure, rather strong positive correlation was found (Fig. 5).

In our previous work with *S. cerevisiae* grown on ethanol, we found that lower catalase activity was associated with higher level of CP [8]. A negative correlation found between catalase activities and CP levels was interpreted as possible protection of cellular proteins against oxidation by catalase in the yeast grown on ethanol. A similar pattern was found on inhibition of catalase by AMT in goldfish brain and kidney—the negative correlations between catalase activities and CP levels were very high [5, 20]. In contrast, we did not find changes in CP level under catalase inhibition by AMT in this work. This may reflect a negligible role of catalase in protection of proteins under conditions used in this study.

Usually low catalase activity is accompanied by decrease in activities of enzymes sensitive to oxidation. Many different enzymes, including antioxidant and associated with them, can be oxidatively inactivated in various organisms under different conditions [30]. In this study, we have checked SOD, G6PDH, GR, LDH, and ICDH susceptibility to oxidation. At the same time, it was interesting to examine the possible change in the activities of abovementioned enzyme, because the last four of them are responsible for maintaining the level of reducing coenzymes, which allow cells to survive oxidative stress. A reduction in the activity of these enzymes in parallel with catalase inactivation could be expected, but it is not necessary. For instance, LDH from animal tissues is an enzyme highly resistant to oxidation (Lushchak, unpublished data). Although in the yeast cells LDH activity is provided by the enzyme, which is not a homolog of LDH from animals, in our experiments we did not observe any decrease in LDH activity.

Earlier we found that the absence of catalase activity was associated with lower GR activity [8]. In contrast, GR activity increased on 10 mM AMT treatment in this work. This activation could serve as compensatory response in order to improve cell protection under catalase inhibition. Similar increase in GR activity after AMT injection into frogs was earlier described by a Spanish team [19]. The injection of AMT enhanced the activities of glutathione-S-transferase and glutathione peroxidase in brain [20] and kidney [5] of goldfish.

Finally, Table 2 shows that catalase inhibition paralleled the reduction of G6PDH activity and very high positive correlation was found between activities of these two enzymes (Fig. 5). In order to check if AMT can directly affect G6PDH activity, we examined the influence of the inhibitor on the enzyme activity in cell-free extracts *in vitro*. In contrast to catalase (Fig. 4), none of the AMT concentrations changed the activity of G6PDH in supernatants of disrupted cells (not shown). It can be concluded that AMT does not inhibit G6PDH directly. In addition, we have investigated the influence of AMT on G6PDH activity in the strain with both genes coding catalases inactivated. In contrast to wild-type cells, in this case we found no decrease in the enzyme activity in the presence of AMT (not shown). Hence, the reduction of G6PDH activity observed in yeast cells treated with AMT is associated with catalase inhibition. So, what are the possible mechanisms responsible for the decrease of G6PDH activity after yeast incubation with AMT? It is known that inhibition of catalase by AMT increases steady-state levels of reactive oxygen species in *S. cerevisiae* [16], which in turn can elevate the intensity of oxidative modification of proteins, for example G6PDH. Our previous results on free-radical inactivation of G6PDH in $Fe^{2+}/H_2O_2$ *in vitro* [31] confirm this suggestion. In addition, we have found a similar effect *in vivo* using single mutants deficient in catalases and wild-type cells grown on ethanol [8]. It was concluded that catalases are important for G6PDH protection against inactivation under mild oxidative stress, which takes place in cells cultivated on ethanol.

It can be concluded that AMT can be a useful "tool" to study the physiological role of catalases in *S. cerevisiae* cells. It is likely that treatment of yeast with AMT induces mild oxidative stress, but it does not affect the level of oxidized proteins and even does not change the activity of the enzymes known to be sensitive to ROS. At the same

time, under conditions used in this study G6PDH may serve as an indicator for evaluation of free radical oxidation with low intensity in live cells.

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