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Regulatory Protein Yap1 Is Involved in Response of Yeast *Saccharomyces cerevisiae* to Nitrosative Stress

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Abstract—The goal of this work was to investigate the possible involvement of protein transcription factor Yap1 in regulation of activity of antioxidant enzymes superoxide dismutase and catalase during yeast response to nitrosative stress. It was found that the inactivation of the *YAP1* gene, encoding Yap1p, cancelled the activation of superoxide dismutase and catalase by NO-donors. Then, using chimeric protein Yap1–GFP, we found the accumulation of Yap1p in the nucleus in response to nitrosative stress. Therefore, we conclude that these results in combination with previous data clearly demonstrate the involvement of Yap1p in upregulation of superoxide dismutase and catalase in yeast cells in response to nitrosative stress.

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Key words: yeast, *Saccharomyces cerevisiae*, nitrosative stress, Yap1p, regulatory protein, fluorescence

The role of nitric oxide (˙NO) in biological systems has been studied since the 1970s, when its effects as vasodilator and later as neurotransmitter were described [1]. To investigate the role of ˙NO, several donors, particularly sodium nitroprusside (SNP), are used in research and have even been introduced into medical practice [2]. Being of low toxicity, SNP is used in clinics as a vasodilator [3]. Nitric oxide regulates many processes in mammalian cells. For example, it activates soluble guanylate cyclase, and in this way it regulates different pathways, particularly apoptosis [3-6]. In animals and plants ˙NO is produced by NO-synthase (NOS), the operation of which is delicately controlled by the cell [7, 8]. The immune system uses ˙NO and superoxide anion ($O_2^{\cdot-}$) that can be spontaneously or enzymatically transformed into secondary products. For example, interaction of ˙NO with $O_2^{\cdot-}$ yields peroxynitrite, and $O_2^{\cdot-}$ catabolism gives hydrogen peroxide (H_2O_2) and hydroxyl radical (˙OH). Nitric oxide and its derivatives are collectively called reactive nitrogen species (RNS), whereas $O_2^{\cdot-}$, H_2O_2, ˙OH, and some other oxygen-containing compounds are called reactive oxygen species (ROS). In the cells of the immune and other systems, ˙NO is produced by inducible NO-synthase (iNOS), and $O_2^{\cdot-}$ by the membrane system of NADH-oxidase [1, 9]. As mentioned above, interaction of ˙NO with $O_2^{\cdot-}$ results in the formation of the very powerful oxidant peroxynitrite, a very powerful antibacterial compound. Peroxynitrite is also a very efficient nitrifying agent [10].

Although microorganisms do not possess NOS, they have several mechanisms of protection against ROS and NOS. For example, the enterobacterium *Escherichia coli* enhances antioxidant potential in response to exposure to reactive species. This response of *E. coli* to the stress induced by ROS and NOS is coordinated by two well studied pathways—*OxyR* and *SoxRS* [11, 12]. In this organism, ˙NO activates the *SoxRS* regulon [12]. In addition, bacteria possess systems converting ˙NO into less toxic compounds. One of the systems relies on flavohemoglobin, which under aerobic conditions operates as NO-dioxygenase [13-15]. Flavohemoglobin has been found in other bacteria and yeasts. Information on the effects of RNS on yeasts is scarce ([16-18] and references cited therein). Especially little is known about their effects on the antioxidant system in *Saccharomyces cerevisiae*. This system is especially sensitive to ˙NO and per-

Abbreviations: GFP, green fluorescent protein; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GSH/GSSG, reduced/oxidized glutathione; GSNO, S-nitrosoglutathione; IDH, isocitrate dehydrogenase; (i)NOS, (inducible) NO-synthase; MDH, malate dehydrogenase; ROS (RNS), reactive oxygen (nitrogen) species; SNP, sodium nitroprusside; SOD, superoxide dismutase.

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oxynitrite, and the use of mutants defective in antioxidant enzymes and glutathione biosynthesis raised the idea of the involvement of this system in yeast defense against RNS [16]. Recently we have carried out works aimed to study the role of antioxidant enzymes in defense against RNS and found that these enzymes modified yeast response to this stress [19-21]. It is known that increase in activity of catalase under oxidative and nitrosative stresses involves the synthesis of new molecules of the enzyme, and transcription factor protein Yap1 coordinates bakers' yeast response to oxidative stress [19, 21]. Because of the analogy with ROS-induced stress, we suggested that Yap1p might be involved in regulation of the yeast antioxidant system in response to nitrosative stress induced by two nitric oxide donors—SNP and S-nitrosoglutathione (GSNO). To answer this question we used a yeast strain defective in the *YAP1* gene, and a transducent possessing a plasmid encoding the chimeric fluorescent protein Yap1–GFP (green fluorescent protein). These two approaches confirmed the involvement of Yap1 protein in increase in superoxide dismutase (SOD) and catalase activities in response to nitrosative stress.

MATERIALS AND METHODS

Materials. Sodium nitroprusside (SNP), phenylmethylsulfonyl fluoride (PMSF), 5,5′-dithio-*bis*-(2-nitrobenzioc acid) (DTNB), sulfosalicylic acid, 2-vinylpiridin, reduced and oxidized glutathione (GSH/GSSG), N,N,N′,N′-tetramethylenediamine (TEMED), and quercetin were obtained from Sigma-Aldrich GmbH (Germany), NADPH from Reanal (Hungary), and peptone and yeast extract from Fluka (Germany). All other chemicals were from local suppliers. GSNO was synthesized by the method described in [22].

Strains and yeast culture. In this work yeast strains *S. cerevisiae YPH250* and its isogenic derivative defective in the gene encoding Yap1p were used. To investigate localization of Yap1p in yeast cells, a strain transformed by a plasmid encoding Yap1p with green fluorescent protein GFP was used.

Yeast culture was grown to stationary phase (72 h) at 28°C on an orbital shaker (175 rpm) in liquid media YPD containing 1% (w/v) yeast extract, 2% (w/v) bactopeptone, and 2% (w/v) glucose. However, to prevent elimination of the plasmid encoding the chimeric protein, the cell culture was grown in synthetic media (SD) containing 0.69% (w/v) yeast nitrogen bases, 0.192% (w/v) of mixture of amino acids excluding tryptophan, and 2% (w/v) glucose. The cell cultures grown for 12-16 h in YPD or SD media with initial cell concentration $0.3 \cdot 10^6$ cells/ml were used to inoculate the main cultures.

Yeast treatment. Yeast cells of the strains used were treated in cultivation media for 1 h with 0.5, 1, and 2.5 mM SNP and GSNO.

Preparation of cell-free extracts. Yeast cells were disintegrated by shaking with glass beads (0.4-0.5 mm) followed by centrifugation as described earlier [20, 21]. The resulting supernatants were kept on ice before used.

Measurement of activities of antioxidant enzymes. The activity of SOD was measured at 406 nm as the inhibition of quercetin oxidation by superoxide anion as described earlier [20, 21]. One unit of SOD activity was defined as the amount of supernatant protein that decreased the maximal speed of quercetin oxidation by 50%. The activities of glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) were measured as described earlier [20, 21]. The consumption of NADPH was registered at 340 nm using molar extinction coefficient 6220 $M^{-1} \cdot cm^{-1}$. The dismutation of hydrogen peroxide by catalase was recorded as described earlier [20, 21]. It was registered at 240 nm using molar extinction coefficient 39.4 $M^{-1} \cdot cm^{-1}$. The reaction was started by addition of cell-free extract. One unit of activities of catalase and GR represents the amount of supernatant protein converting 1 μmol of substrate per minute. All activities were measured at 25°C and related to the amount of soluble supernatant protein.

Measurement of concentrations of total and oxidized glutathione. The levels of total and oxidized glutathione were measured with DTNB and GR [23], with minor modifications described earlier [21].

Evaluation of localization of Yap1 protein. The strain possessing the plasmid encoding chimeric protein Yap1–GFP was used. The localization of the chimeric protein was determined as distribution of green fluorescent protein using a Nikon 300 confocal microscope (excitation at 488 nm and emission at 540 nm). The photomicrographs were treated with IrfanView (Irfan Skiljan) and Corel Photo Paint X4 (Corel Corporation).

Measurement of protein concentration and statistics. The concentration of protein was measured with Coomassie Brilliant Blue G-250 [24] with the use bovine serum albumin as a standard. Experimental data represent mean ± its deviation, and data were statistically treated using MYNOVA followed Dunnett's test.

RESULTS

Activation of SOD and catalase. The treatment of wild type *YPH250* yeast with SNP enhanced the activities of SOD and catalase (Fig. 1) in concentration-dependent manner. Preincubation of the yeast with 250 μg/ml of cycloheximide, an inhibitor of protein biosynthesis in eukaryotes, prevented the increase in catalase activity but did not affect SOD activation (not shown). The isogenic to *YPH250* strain cells defective in the *YAP1* gene responded differently. First, in untreated cells of Δ*YAP1* strain SOD activity was 30% higher than that in the parental strain. Second, no increase in SOD and catalase

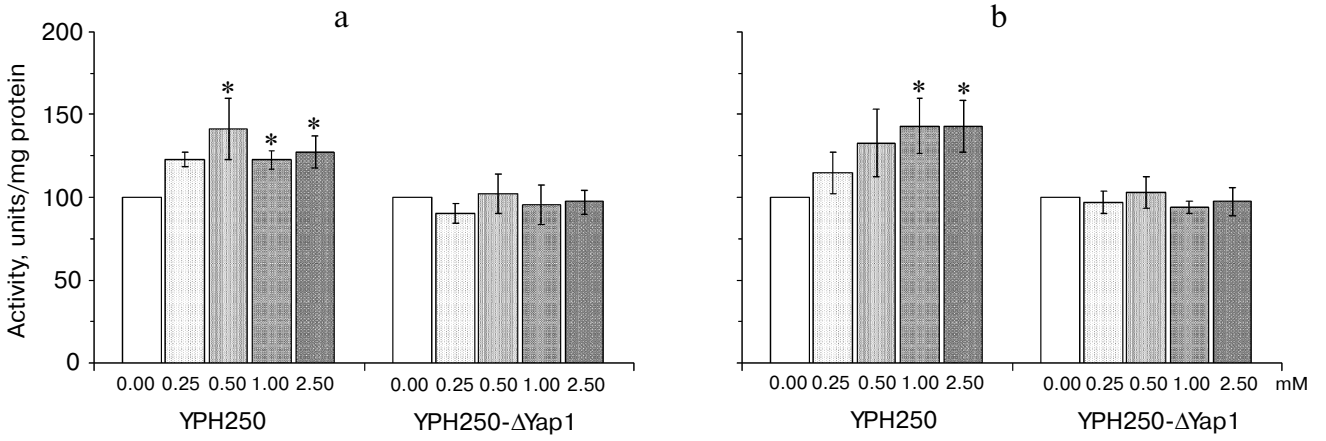

Fig. 1. Effect of SNP (0.25-2.5 mM) on activities of SOD (a) and catalase (b) in yeast cells of two strains ($n = 6$). * Here and in Figs. 2, 4, and 5, significantly different from control with $P < 0.05$.

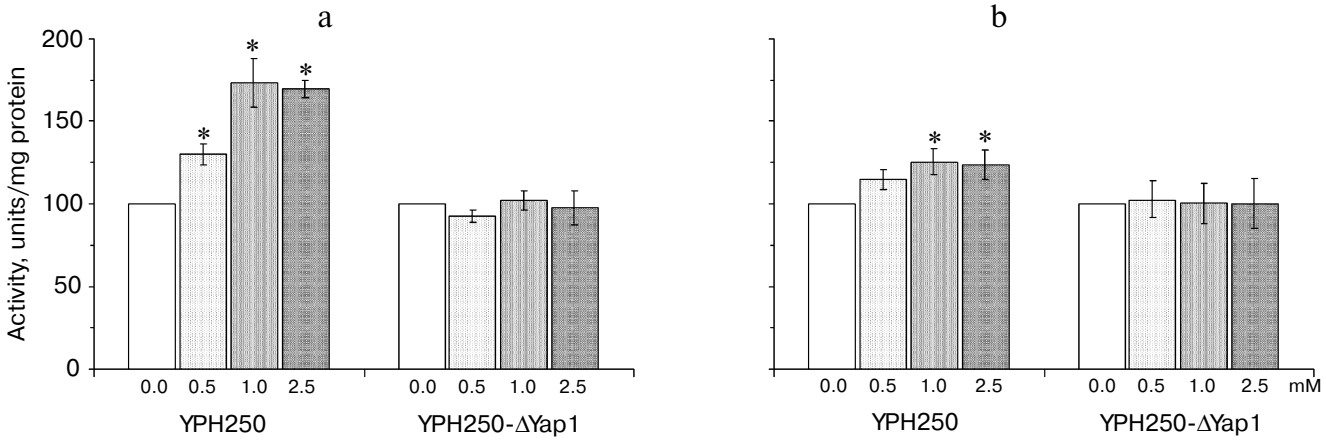

Fig. 2. Effect of GSNO (0.5-2.5 mM) on activities of SOD (a) and catalase (b) in yeast cells of two strains ($n = 6$).

activities in response to nitrosative stress was found in Δ*YAP1* strain (Fig. 1). This suggests the involvement of Yap1p in coordination of yeast response to nitrosative stress.

To exclude the possibility that SOD and catalase are activated by SNP specifically, but not by ˙NO release, a second NO-donor, GSNO, was used at concentrations of 0.5, 1, and 2.5 mM. It was found that GSNO activated SOD and catalase similarly to SNP (Fig. 2). Again, the GSNO activation effect was not found in the strain defective in the *YAP1* gene. Catalase activation was blocked by preincubation with cycloheximide, whereas the latter did not affect SOD activation (not shown).

The yeast strain carrying the plasmid encoding chimeric protein Yap1–GFP was used to gain additional information on involvement of regulatory protein Yap1 in response to the studied stress. The cellular localization of the chimeric protein was visualized using confocal microscopy. Under control conditions, the fluorescence was equally distributed in the cell (Fig. 3a). Yeast cells treated with hydrogen peroxide were used as a positive control. Under hydrogen peroxide-induced stress, Yap1–GFP protein was concentrated in the nucleus, demonstrating its redistribution in the cell (Fig. 3b). The stress induced by both SNP and GSNO resulted in concentration of Yap1–GFP protein in the nucleus (Figs. 3c and 3d).

Yeast cells were treated with decomposed solutions of SNP and GSNO to determine whether activation of SOD and catalase was induced by released ˙NO or by formed stable products of decomposition. The latter were prepared by the incubation of solutions of the donors for 24-48 h at room temperature. The decomposed solutions of SNP and GSNO did not affect SOD and catalase activities (not shown).

The question which of two catalases possessed by *S. cerevisiae* was activated by ˙NO remained open. It was answered by the use of yeast strains isogenic to *YPH250*

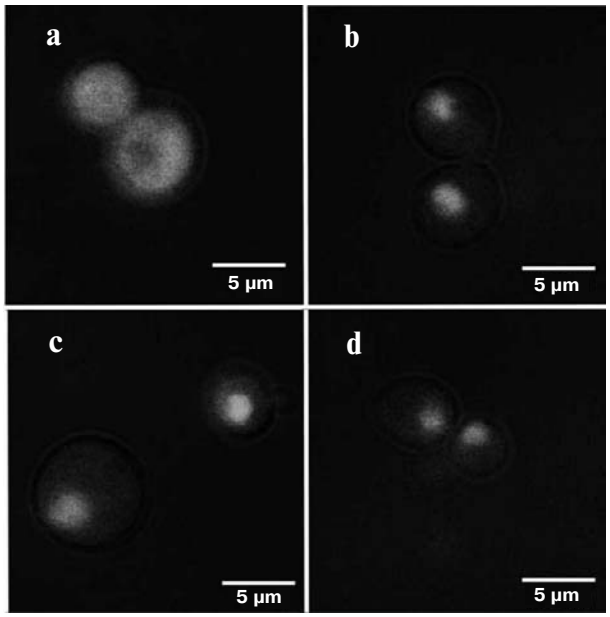

Fig. 3. Distribution of Yap1 protein in response to nitrosative stress. Chimeric protein Yap1–GFP is freely distributed in the cell under control conditions (a) and is concentrated in the nucleus in response to the stress induced by H_2O_2 (b), SNP (c), and GSNO (d).

but defective in one or the other catalase. Incubation of yeast with SNP activated peroxisomal catalase A, while it did not affect cytosolic catalase T (data not shown).

Markers of oxidative stress: glutathione and aconitase. Under control conditions, the concentration of reduced cysteine-containing tripeptide GSH was 29.0 ± 2.5 and 30.3 ± 2.8 μmol/OD_{600} in wild type *YPH250* strain and its isogenic but defective in *YAP1* gene, respectively. Sodium nitroprusside did not affect the concentration of reduced glutathione in yeast strains *YPH250* and Δ*YAP1* (Fig. 4a).

The concentration of oxidized glutathione (GSSG) in the cells of *YPH250* and Δ*YAP1* strains was 0.10 ± 0.01 and 0.20 ± 0.03 μmol/OD_{600}, respectively. Although treatment of the yeast with nitric oxide donor enhanced GSSG level in concentration-dependent manner (Fig. 4b), in the Δ*YAP1* strain it did not affect this parameter. Consequently, in the *YPH250* strain the incubation with SNP increased the [GSSG]/[GSH] ratio, but it did not have a similar effect in the Δ*YAP1* strain (not shown).

Aconitase containing in its active site cubic [4Fe-4S] cluster is rather sensitive to oxidation, leading to its inactivation. Therefore, its activity is used as a marker of oxidative stress. Incubation of yeast *YPH250* strain with SNP decreased the activity by ~50% (Fig. 5). Aconitase activity in yeast cells of Δ*YAP1* strain under control conditions consisted of approximately 50% of that in the parental strain, but it was not affected by treatment with SNP (Fig. 5).

Activities of glutathione reductase and dehydrogenases of glucose-6-phosphate, isocitrate, and malate. GR and G6PDH are known to be inactivated by free radicals [12, 25], and the contents of other enzymes are known to increase in response to oxidative stress. The activity of G6PDH under control conditions was 25% higher in cells of parental *YPH250* strain than in the defective in *YAP1* strain (Table 1). The incubation of yeast strains *YPH250* and Δ*YAP1* with SNP affected neither GR nor G6PDH activities (Table 1).

Isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH) are known to be inactivated by free radicals [26]. The incubation of yeast *YPH250* and isogenic Δ*YAP1* strains with SNP did not influence the activities of IDH and MDH (Table 1).

Some correlations. Table 2 demonstrates some correlations between the activities of the enzymes and ratio of oxidized to reduced glutathione in *YPH250* strain under treatment with SNP. A strong positive correlation was

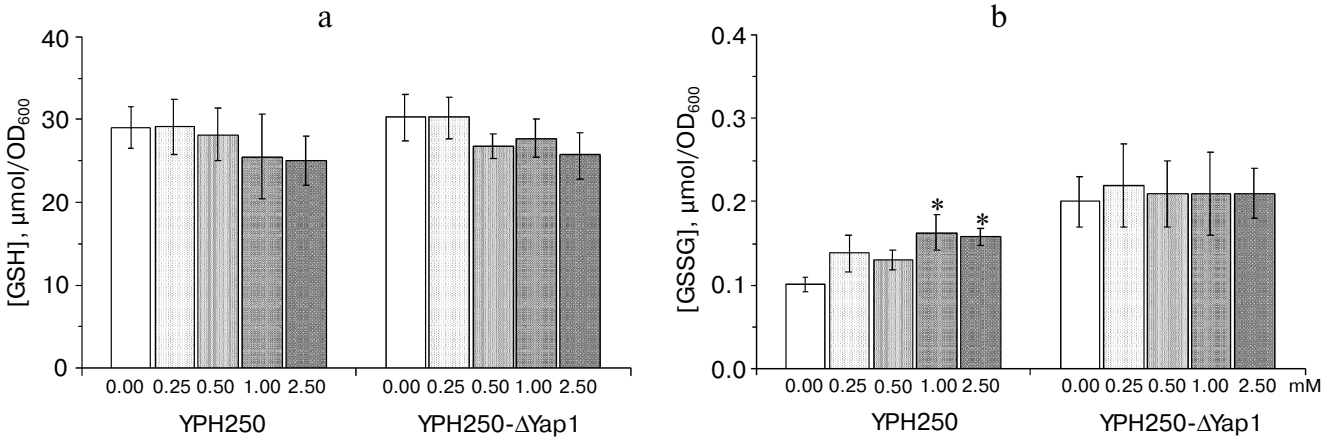

Fig. 4. Content of reduced (a) and oxidized (b) glutathione in cells of parental and isogenic defective in Yap1 protein strains after treatment with sodium nitroprusside (n = 6).

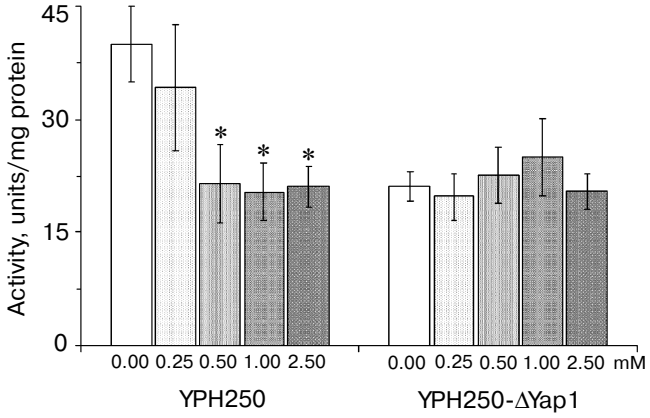

Fig. 5. Effect of nitrosative stress induced by SNP (0.25-2.5 mM) on aconitase activity in yeast cells of parental and defective in Yap1 protein strains ($n = 6$).

found between the activities of SOD and catalase. The activities of SOD and catalase strongly positively correlated with [GSSG]/[GSH] ratio, the first being stronger than the second. The activity of aconitase negatively correlated with SOD and catalase activities and with [GSSG]/[GSH] ratio.

DISCUSSION

Earlier we found that SNP at concentrations up to 10 mM had virtually no effect on survival of *S. cerevisiae* strain *YPH250* [19]. Therefore, the SNP concentrations used in this work were clearly sublethal. In addition, we used yeast cultures in stationary phase, where this as well as other microorganisms is very resistant to different stresses [27]. The second NO-donor used here, GSNO, also had virtually no effect of yeast survival up to concentration of 5 mM [20].

Incubation of yeast with SNP for 1 h increased the activities of SOD and catalase. However, although catalase activation was blocked by preincubation of the yeast with cycloheximide, it did not prevent SOD activation. This means that protein biosynthesis is required for catalase activation, but not for SOD activation. The use of the strains defective in catalases demonstrated that peroxiso-

Table 1. Activities (10^{-3} units/mg protein) of glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH), and malate dehydrogenase (MDH) in yeast strains *YPH250* and *ΔYAP1* treated with SNP at concentrations of 0.25-2.5 mM ($n = 6$)

Strain	SNP, mM	GR	G6PDH	IDH	MDH
YPH250	0	48.3 ± 4.5	233 ± 17	133 ± 7	9.55 ± 0.52
YAP1Δ	0	36.4 ± 5.9	186 ± 12	148 ± 16	10.4 ± 0.6
YPH250	0.25	50.1 ± 2.4	219 ± 17	132 ± 11	10.7 ± 1.3
YAP1Δ	0.25	39.3 ± 6.1	169 ± 10	198 ± 34	10.5 ± 2.3
YPH250	0.5	47.3 ± 2.8	211 ± 16	122 ± 12	9.27 ± 1.22
YAP1Δ	0.5	39.4 ± 5.6	208 ± 10	187 ± 35	10.2 ± 1.0
YPH250	1	47.4 ± 4.3	207 ± 15	135 ± 16	12.5 ± 1.52
YAP1Δ	1	35.8 ± 5.2	200 ± 7	192 ± 37	11.3 ± 0.73
YPH250	2.5	46.2 ± 2.3	185 ± 10	124 ± 11	10.2 ± 0.9
YAP1Δ	2.5	34.4 ± 5.8	194 ± 7	166 ± 26	8.91 ± 0.80

Table 2. Correlations (R^2) between enzyme activities and contents of reduced and oxidized glutathione in yeast strains *YPH250* and *ΔYAP1* treated with SNP at concentrations of 0.25-2.5 mM ($n = 5$)

	SOD	Catalase	Aconitase	[GSH]	[GSSG]
Catalase	0.50				
Aconitase	–0.62	–0.96			
[GSH]	–0.08	–0.75	0.61		
[GSSG]	0.28	0.80	–0.64	–0.67	
[GSSG]/[GSH]	0.21	0.84	–0.67	–0.84	0.96

mal catalase A was activated, while the cytosolic isoenzyme, catalase T, was not. Therefore, it can be concluded that SNP increased the activity of peroxisomal catalase A via synthesis of new molecules of the enzyme, and this process is regulated by Yap1p.

The activity of SOD was also enhanced by incubation of yeast cells with SNP and GSNO, but cycloheximide did not block the process. Therefore, it can be suggested that SOD might be activated at the posttranslational level, which was discussed by us earlier in connection with activation of the enzyme on treatment of yeast cells with hydrogen peroxide [28]. It is known that in yeasts both SODs – SOD1 (Cu,Zn-containing) and SOD2 (Mn-containing) – have metal ions in active sites (copper/zinc or manganese, respectively). Therefore, SOD maturation requires incorporation of the metal ions in their active centers. The process proceeds by special mechanisms. For example, it is known that copper ions are incorporated in SOD1 apoenzyme by a special chaperone, Ccs1 [29]. The NO-donors SNP and GSNO might stimulate maturation of SOD1 molecules. On analyzing effects of SNP and GSNO on SOD activity in Δ*YAP1* strain, it seems that this process is not sensitive to NO-donors. This might be, but comparison of SOD activities in parental and Δ*YAP1* strains reveals that in the defective strain it is as high as in the NO-stimulated parental strain. The possibility of activation of antioxidant enzymes via Yap1p in *S. cerevisiae* is supported by data obtained with *Schizosaccharomyces pombe*, where nitrosative stress operated via Pap1 protein, a functional homolog of Yap1p in this species [22]. The involvement of Yap1p in increase in mRNA of 15 antioxidant enzymes was suggested on induction of nitrosative stress in *S. cerevisiae* [30].

The increased concentration of oxidized glutathione, and consequently its ratio [GSSG]/[GSH], demonstrates the development of oxidative stress in yeast on treatment with ·NO. Although the concentrations of reduced glutathione demonstrated only a tendency to decrease, approximately twice higher level of oxidized glutathione in Δ*YAP1* cells demonstrates the shift of intracellular redox potential to the more oxidized state. Interestingly, GSSG concentration in cells of Δ*YAP1* strain was 2-fold higher than that in the parental strain, which might reflect that cells of the defective strain are under constant oxidative stress. Glutathione is involved in regulation of redox-sensitive sensor proteins. For example, in *E. coli* GSH is used for reduction of oxidized protein OxyR, a key regulator of bacterial response to hydrogen peroxide-induced stress [12]. In *S. cerevisiae* cells glutathione is supposed to be involved in reduction of Yap1p [31]. However, there is no direct evidence of this process *in vivo* as it was well documented *in vitro*.

The inactivation of aconitase in *YPH250* strain and SNP-induced stress also demonstrate the induction of oxidative stress. Again, it is interesting to note that in the cells of the parental strain SNP inactivated the enzyme by ~50%, i.e. to the level found in the strain defective in *YAP1*. It is possible that in the defective strain cells aconitase under normal conditions is inactivated to the extent, which in *YPH250* strain is reached on treatment with the NO-donor. Anyway, yeast aconitase *in vivo* is sensitive to oxidative stress and, therefore, can be used as its marker. A similar situation has been found many times in mammals, for example, in rat heart [26].

The activities of other investigated enzymes (GR, G6PDH, IDH, and MDH) were not affected by nitrosative stress (Table 1). It is known that the level of these enzymes in yeast cells is increased under hydrogen peroxide-induced oxidative stress. These enzymes were inactivated by free radicals [25]. Apparently the unchanged activities under nitrosative stress might result from the balance between inactivation by free radicals and synthesis of new molecules.

The coordinated response of the activities of SOD and catalase and SNP treatment (R^2 = 0.81) demonstrates that it is critically important to maintain a certain ratio between the activities of these enzymes. Earlier we found a similar correlation between SOD and catalase activities on induction of oxidative stress in *S. cerevisiae* by hydrogen peroxide [28], on study of strains defective in SODs [32, 33], and on SOD inhibition by diethyldithiocarbamate [34]. This could be associated with the fact that SOD substrate (superoxide anion) inactivates catalase [35], and catalase substrate (hydrogen peroxide) inactivates SOD [36].

The [GSSG]/[GSH] ratio might be an efficient regulator of redox processes as it was described for OxyR in *E. coli* [37]. The positive correlation between this index and the activities of SOD (R^2 = 0.50) and catalase (R^2 = 0.83) might mirror its involvement in activation of these enzymes. The higher coefficient correlation between [GSSG]/[GSH] and catalase activity than with SOD demonstrates a stronger relationship in the first case. The aconitase activity also demonstrates several interesting correlations. First, negative correlation with [GSSG]/[GSH] ratio is well understood from known facts: under oxidative stress, both glutathione and aconitase are oxidized, which leads to increase in GSSG level and aconitase inactivation. The strong negative correlation between the activities of aconitase from one side, and catalase and SOD from the other (R^2 = 0.96 and R^2 = 0.86, respectively), might demonstrate not only involvement of oxidative stress in increase in the activities of the enzymes, but also the possible role of the antioxidant enzymes in protection of aconitase against inactivation by free radicals.

In conclusion, it should be said that NO-donors, SNP and GSNO, in yeast cells lead to oxidation of glutathione and inactivation of aconitase, demonstrating the induction of oxidative stress. This process leads to enhancement of SOD and catalase activities. Since cyclo-

heximide, an inhibitor of protein biosynthesis, prevents the increase in catalase activity, it seems that the activity increase is connected with synthesis of new molecules. On the other hand, cycloheximide did not prevent the increase in SOD activity, which might demonstrate the stimulation by ˙NO the maturation of the apoenzyme. Because the increase in the activities of SOD and catalase was not found in the strain defective in *YAP1*, we conclude that Yap1 protein is also involved in the process. That means that nitrosative stress induces the enzymes in Yap1p-related pathway(s).

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