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EXPERIMENTAL ARTICLES

Stress Factor-Induced Changes in the Activity of Antioxidant Protective Mechanisms in the Wild Type Strain of *Neurospora crassa* and in Its Photoreceptor Complex Mutants

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Abstract—The effect of stress factors (changes in oxygen content, temperature, and illumination) on superoxide dismutase (SOD) and catalase activity, as well as on the content of thiol and disulfide groups, in low-molecular-weight compounds and proteins of *Neurospora crassa* mycelium was studied in the wild type strain and *white collar-1* (*wc-1*) and *white collar-2* (*wc-2*) mutants. Environmental stress factors induced the activation of both SOD and catalase, as well as an increase in the thiol level in the wild type strain of *Neurospora crassa.* In the *wc-1* and *wc-2* mutants, an increase in catalase activity and in the total thiol level was revealed; however, activation of superoxide dismutase was not observed. A decrease in the formation of disulfide bonds in the proteins of *wc-1* and *wc-2* mutants (as compared with the wild type strain) was recorded. These results indicate disrupted transduction of stress factor signals that promotes reactive oxygen species (ROS) formation in the WCC mutants.

Key words: Neurospora crassa, antioxidant protection, stress factors.

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The presence of oxygen in the environment and its involvement in metabolic processes inevitably results in the intracellular generation of reactive oxygen species (ROS), primarily superoxide anion radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and oxygen in its excited singlet state ($^1O_2$). External factors (starvation, drying, illumination, ionizing radiation, wide temperature ranges, etc.) significantly increase ROS production in microbial cells and may induce oxidative stress [1]. The high resistance of fungi to external factors is provided by antioxidant protective systems (APS) and mechanisms which regulate the activity of APS components under various environmental conditions. Superoxide dismutase (SOD) and catalase, the APS components capable of decreasing the content of initially formed ROS, are of great importance. Glutathione plays a significant role in antioxidant protective systems, whereas the 2SH/SS ratio determines the redox status of cells [2]. The content of thiols may exert a significant effect on the ROS signal transduction and alter the profile of gene expression [3, 4].

In *N. crassa*, the reception and transduction of light signals (of the blue–violet spectral area) are mediated by the flavin-containing receptor protein White Collar-1 (WC-1) and White Collar-2 (WC-2), which form the heterodimeric photoreceptor White Collar Complex (WCC) [5]. In *N. crassa*, WC-1, a protein containing a LOV (Light, Oxygen, Voltage) domain, is a blue light photoreceptor, whereas WC-2 provides a stabilizing function in this photoreceptor complex [6]. WCC not only has complete control over some light-dependent reactions (carotenogenesis, production of conidia or protoperithecia and their tropism), but is also involved in the regulation of the dark processes, of which maintaining of the circadian clocks is the most significant [6]. It should be noted that enhanced WC-1 expression in the dark is not sufficient for the activation of light-dependent genes; however, the activation of some genes, such as those responsible for the synthesis of ribosomal proteins, was observed [7]. The presence of the LOV domain in the WC-1 protein allows us to imply that the photoreceptor complex is involved in the reception and transduction of ROS signals. Changes in the expression of light-dependent genes of carotenoid biosynthesis in response to the ROS content in cells support the assumption that ROS affect light signal transduction by the WCC [8, 9]. Changes in the redox state of WC-1 within the WCC complex which affects its DNA-binding activity may be one of the hypothetical mechanisms of the ROS signal transduction [8].

It is well-known that transition between the growth phases of *N. crassa* is accompanied by an increase in the intracellular ROS concentration [1]. The mecha-

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nism of reception and transduction of ROS signals is associated with changes in the conformation and functional properties of proteins directly interacting with ROS [10, 11]. The most frequent conformational changes in proteins involve oxidative modification of cysteine residues [12, 13].

We have previously demonstrated changes in the APS activity of the WCC mutants at various growth stages, clearly illustrated by an increase in catalase activity and total content of thiols as compared to the wild type strain of *N. crassa* [14].

The goal of the present work was to study the changes in activity of the APS components (SOD, catalase, and the total thiol content) of the ascomycete *N. crassa* (wild type strain and *wc-1* and *wc-2* mutants) in response to various stress factors (oxygen, light, temperature) which increase the ROS content in cells. In addition, we studied the changes in the thiol content in low-molecular-weight compounds and proteins) and protein disulfides as marker compounds of oxidative stress induced by stress factors.

MATERIALS AND METHODS

In our study, we used *Neurospora crassa* strains (wild type strain RL3-8a [FGSC 2218] and WCC mutants, *wc-1* (P829) [FGSC 143] and *wc-2* (ER33) [FGSC 4408]) which were kindly provided by the Fungal Genetics Stock Center (FGSC; University of Missouri–Kansas City, United States).

Cultivation. *N. crassa* cultures were maintained on the agarized Vogel's medium. The medium was inoculated with a spore suspension of *N. crassa* (10^6 spores/ml). The mycelium was grown to the stationary phase in 750-ml flasks containing 200 ml of liquid Vogel's medium on a shaker (200 rpm) at 28°C.

The effect of stress factors on the mycelium. The stationary-phase mycelium of *N. crassa* (21 h for the wild type strain and *wc-1* mutant and 24 h for the *wc-2* mutant) was washed with cold water. The excess water was removed by suction filtration on a Buchner funnel, and the obtained mycelium specimens were placed on wet filter paper in petri dishes [15]. We changed the oxygen concentrations in the environment by incubation of mycelium grown in the liquid medium under air or argon. Illumination by a fluorescent lamp (15 W/m^2 mounted at a distance of 30 cm), as well as high temperature (37°C), were the additional stress factors.

After the end of the exposure (30 min, 60 min, and 3 h), the excess water was removed from the mycelium, which was then frozen in liquid nitrogen. The frozen mycelium was ground with liquid nitrogen, supplemented with a fivefold amount of buffer (0.015 M potassium phosphate supplemented with 0.1 mM EDTA and 0.5 mM phenyl methane sulfonyl fluoride; pH 7.8), and centrifuged at 4500 *g* for 20 min. The obtained supernatant was used to determine the catalase and SOD activities.

Assessment of the SOD and catalase activities was performed according to the previously described procedure [14].

Determination of the content of thiol and disulfide groups in proteins and low-molecular-weight compounds in homogenized mycelium was carried out according to the previously described technique [16]. This technique is based on fractionation of thiols into protein and low-molecular-weight compounds with trichloroacetic acid, as well as on determination of disulfide concentrations in proteins after treatment with $NaBH_4$. Determination of SH groups was performed using Ellman's reagent. In the course of analysis, SH groups of proteins (PSH) and low-molecular-weight compounds (NPSH), as well as protein disulfides (PSSP) and mixed disulfides produced by proteins and low-molecular-weight thiols (PSSR), were determined.

Obtaining the homogenate for fractionating of thiol/disulfide groups of the mycelium. The mycelium frozen in liquid nitrogen was ground into a homogeneous powder and supplemented with seven volumes of 10 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM butylhydroxytoluene (dissolved in the minimum amount of ethanol). The homogenate was sonicated for 1 min and used for further experiments.

Determination of free thiol (SH) groups in the homogenate (total thiol content of low-molecular-weight compounds and proteins, fraction A). A 150-μl portion of the homogenate was supplemented with 150 μl of 10 mM potassium phosphate buffer (pH 7.2); 300 μl of 0.3 M Tris–HCl (pH 8.2); 1.5 ml of the buffer containing 0.1 M Tris–HCl, 0.1 M glycine, 0.6 mM EDTA, and 10.2 M urea; pH 8.6; 850 μl of ethanol; and 30 μl of 10 mM Ellman's reagent. The mixture was incubated at room temperature for 30 min and centrifuged for 5 min to separate the sediment fractions from the mixture. The absorption of the supernatant was measured with an SF-46 spectrophotometer (Russia) at 412 nm.

Determination of the total content of thiols/disulfides in proteins and low-molecular-weight compounds (thiols and disulfides after the recovery of disulfide bonds with $NaBH_4$, fraction B). A 120-μl portion of homogenate was supplemented with 120 μl of 10 mM potassium phosphate buffer (pH 7.2), 45 μl of 0.43 M Tris–HCl (containing 0.46 M glycine, 2.7 mM EDTA; pH 8.6), 600 μl of 10.2 M urea buffer (pH 8.6), and 0.5 ml of freshly prepared 1% $NaBH_4$. The mixture was then incubated at 40°C for 40 min. To terminate the reaction, 270 μl of 1 N HCl and 900 μl of acetone were added. Then the mixture was stirred, supplemented with 900 μl of 1 M Tris–HCl containing 1.3 mM EDTA (pH 8.5) and 90 μl of 10 mM Ellman's reagent, and incubated for 30 min. The sediment fractions were separated by centrifugation. The absorption of the supernatant was measured with an SF-46 spectrophotometer at 412 nm.

Determination of thiols (SH groups) in low-molecular-weight compounds (fraction C). A 600-µl portion of homogenate was supplemented with 480 µl of 10 mM potassium phosphate buffer (pH 7.2) and 120 µl of 50% trichloroacetic acid for protein precipitation. The mixture was then cooled for 10 min and centrifuged in order to separate sediment fractions from the mixture. The supernatant (1 ml) was supplemented with 2 ml of 0.4 M Tris–HCl (pH 8.9) and 50 µl of 10 mM Ellman's reagent. The mixture was incubated for 30 min; the optical density was measured with an SF-46 spectrophotometer at 412 nm.

Determination of protein disulfides (fraction D). A 300-µl portion of homogenate was supplemented with 240 µl of 10 mM potassium phosphate buffer (pH 7.2) and 60 µl of 50% trichloroacetic acid. The mixture was then cooled, incubated for 10 min, and centrifuged in order to harvest sediment fractions. The pellet was washed with 3 ml of 10% trichloroacetic acid and centrifuged again. Sediment fractions were supplemented with 130 µl of 10 mM potassium phosphate buffer (pH 7.2), 20 µl of 0.43 M Tris–HCl (pH 8.6), 300 µl of 10.2 M urea buffer (pH 8.6) (see above), and 220 µl of 1% $NaBH_4$ and incubated at 40°C for 40 min. To terminate the reaction, 130 µl of 1 N HCl and 450 µl of acetone were added. The mixture was then stirred, supplemented with 1.45 ml of 1 M Tris–HCl (pH 8.5) and 50 µl of 10 mM Ellman's reagent, incubated for 30 min, and centrifuged in order to separate sediment fractions. The supernatant absorption was measured with an SF-46 spectrophotometer at 412 nm.

Determination of mixed disulfides (fraction E). A 300-µl portion of homogenate was supplemented with 240 µl of 10 mM potassium phosphate buffer (pH 7.2) and 60 µl of 50% trichloroacetic acid. The mixture was then cooled, incubated for 10 min, and centrifuged in order to separate precipitated protein; the pellet was washed with 3 ml of 10% trichloroacetic acid and centrifuged again. The sediment was supplemented with 130 µl of 10 mM potassium phosphate buffer (pH 7.2), 20 µl of 0.43 M Tris–HCl (pH 8.6), 300 µl of 10.2 M urea buffer (pH 8.6), and 230 µl of 1% $NaBH_4$ and incubated at 40°C for 40 min. After incubation, the mixture was supplemented with 170 µl of 75% trichloroacetic acid and centrifuged. To terminate the reaction, the supernatant was supplemented with 140 µl of 1 N HCl and 450 µl of acetone. The mixture was then stirred and supplemented with 1.6 ml of 2.5 M Tris–HCl (pH 11.2) and 50 µl of 10 mM Ellman's reagent and incubated for 30 min. The optical density of the solution was measured with an SF-46 spectrophotometer at 412 nm.

Quantitative calculation of the thiol/disulfide concentrations in various fractions. The calibration curve for determination of the content of SH groups in the specimens was built using the reaction of a prefabricated reduced glutathione preparation with Ellman's reagent and expressed in nmol of glutathione equivalents/mg protein. The disulfide bond splitting efficiency was determined after treatment of the prefabricated oxidized glutathione and bovine serum albumin (BSA) preparations with a $NaBH_4$ solution, assuming one thiol and 17 disulfide groups in a BSA molecule. The disulfide bond splitting efficiency factor obtained by the fraction B analysis (see above) and by protein precipitation with trichloroacetic acid (fractions D and E) were $k = 0.18$ and $l = 0.15$, respectively, which coincides with the factors ($k = 0.2$ and $l = 0.15$) obtained by the authors of this method [16]. The completeness of recovery of the prefabricated disulfide preparations added to the specimens (fractions B and D) were $m = 0.48$ and $r = 0.75$ (for oxidized glutathione and BSA, respectively).

The results obtained by the fraction B analysis directly account for the content of low-molecular-weight thiols (NPSH), mainly represented by glutathione. The content of thiol groups in proteins (PSH) was determined from the difference between the total content of thiols (fraction A) and low-molecular-weight thiols (fraction C), i.e., PSH = A – C.

The content of disulfides in proteins (PSSP) and mixed disulfides (PSSR) was calculated using the following formulas proposed by the authors of the method [16], taking into account the experimentally obtained values of disulfide bond splitting efficiency factors (k and l), as well as the recovery factors m and r:

$$PSSP = [D - m(A - C) - 2E]/2lm$$
$$= [D - 0.48(A - C) - 2E]/0.144,$$
$$PSSR = E/lm = E/0.07,$$

where k is the disulfide bond splitting efficiency factor obtained by the fraction B analysis ($k = 0.18$);

l is the disulfide bond splitting efficiency factor obtained by protein precipitation with trichloroacetic acid (fractions D and E) ($l = 0.15$);

r is the coefficient of total determination of the SH group concentration obtained by the disulfide bond splitting in low-molecular-weight compounds ($r = 0.75$);

m is the coefficient of total determination of the SH group concentration obtained by the disulfide bond splitting in proteins after their precipitation with trichloroacetic acid ($m = 0.48$);

and A, B, C, D, and E are the SH group concentrations in nmol/mg protein determined by analysis of the relevant fractions.

The protein concentration was determined using the Bradford method.

RESULTS

Figure 1 shows the changes in SOD activity in the mycelium of the *N. crassa* wild type strain and the WCC mutants *wc-1* and *wc-2*. SOD activity in the mycelium of the wild type strain transferred from liquid medium to air increased by 35% at the end of the first

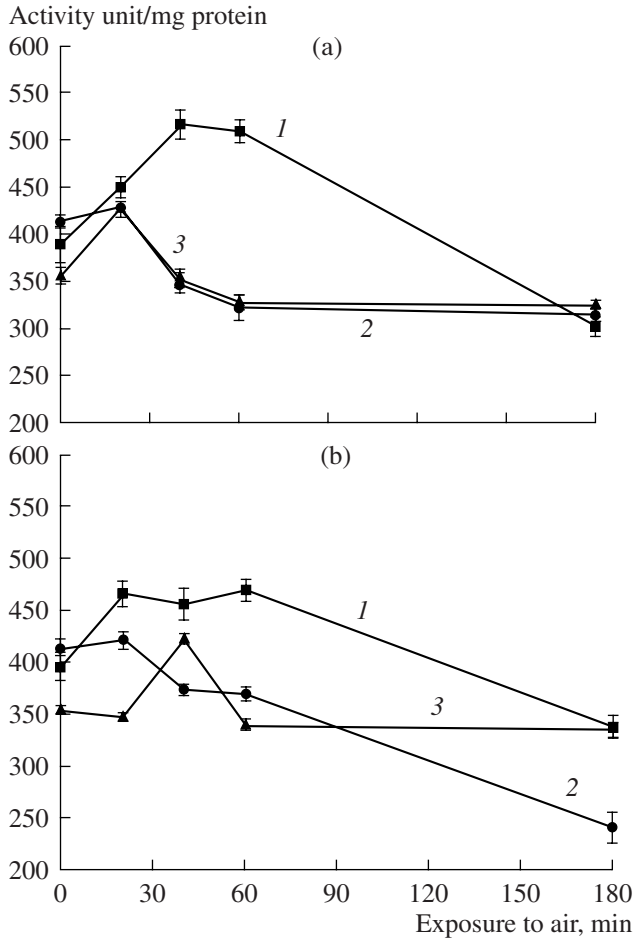

Fig. 1. SOD activity in the mycelium of *N. crassa* exposed under air (a) in the dark and (b) under illumination: (*1*) wild type strain, (*2*) *wc-1* mutant, and (*3*) *wc-2* mutant.

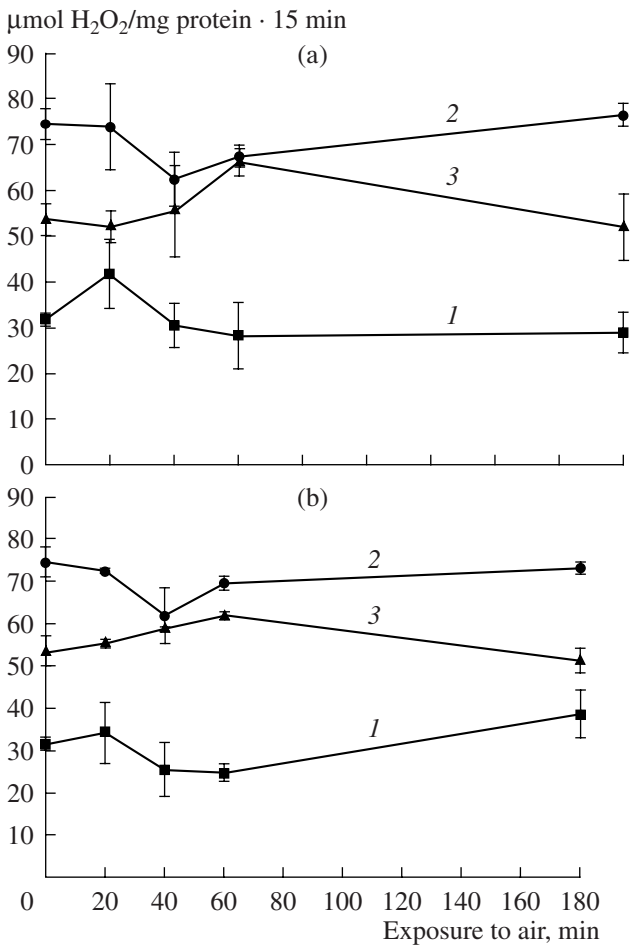

Fig. 2. Catalase activity in the mycelium of *N. crassa* exposed under air (a) in the dark and (b) under illumination: (*1*) wild type strain, (*2*) *wc-1* mutant, and (*3*) *wc-2* mutant.

hour; after three hours of incubation, it was restored to the initial level (Fig. 1). Under illumination, the transitive increase in SOD activity was less pronounced. SOD activity in *wc-1* and *wc-2* mutants exposed to air decreased immediately (*wc-1*) or after a short-term increase (*wc-2*) irrespective of the light intensity. Hence, a pronounced transitive increase in SOD activity was observed only in the wild type strain at the beginning of its exposure to air. Additional illumination of the mycelium for three hours was not accompanied by an increase in SOD activity of both the wild type strain and *wc-2* mutant, whereas the SOD activity of the *wc-1* mutant slightly decreased.

During the first 30–60 min of the mycelium exposure to air, catalase activity of the wild strain of *N. crassa* increased; it then gradually decreased to its initial level (Fig. 2). Under illumination, the transitive increase in catalase activity in the beginning of incubation was similar to that in the mycelium exposed to air in the dark. During the first 30–60 min of incubation under air, the high constitutive catalase activity of the *wc-1* and *w-2* mutants underwent insignificant multidirectional transitive fluctuations; however, by the third hour, the constitutive catalase activity of these mutants was restored to its initial level.

When the mycelium was incubated in argon for three hours, SOD activity of all the studied strains decreased twofold, catalase activity decreased slightly, and the content of SH groups in mycelium extracts was the same as in the control specimen (exposed to air in the dark at 20°C) (Fig. 3). The decrease in the SOD activity in the absence of oxygen confirms the close association of this enzyme with the process of respiration.

An increase in temperature (up to 37°C) induced a steady 1.5- to 2-fold increase in the SOD and catalase activities in the wild type strain exposed to air for three hours; the content of extractable thiol compounds increased by 40% (Fig. 3). The catalase activity and the content of extractable thiol compounds in the WCC mutants increased in a similar manner; however, the SOD activity did not change.

Figure 4 shows changes in the thiol (SH group) content in low-molecular-weight compounds and proteins,

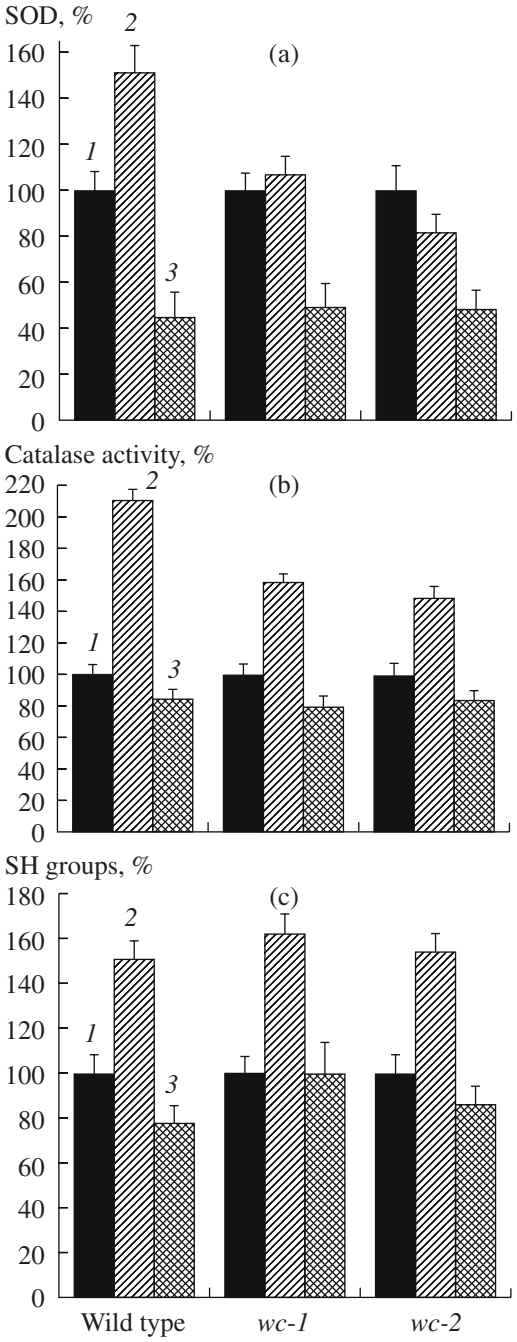

Fig. 3. Comparison of (a) SOD activity, (b) catalase activity, and (c) total content of SH groups in the mycelium of *N. crassa* exposed in the dark under air at (*1*) 20°C, (*2*) 37°C, and (*3*) under argon at 20°C.

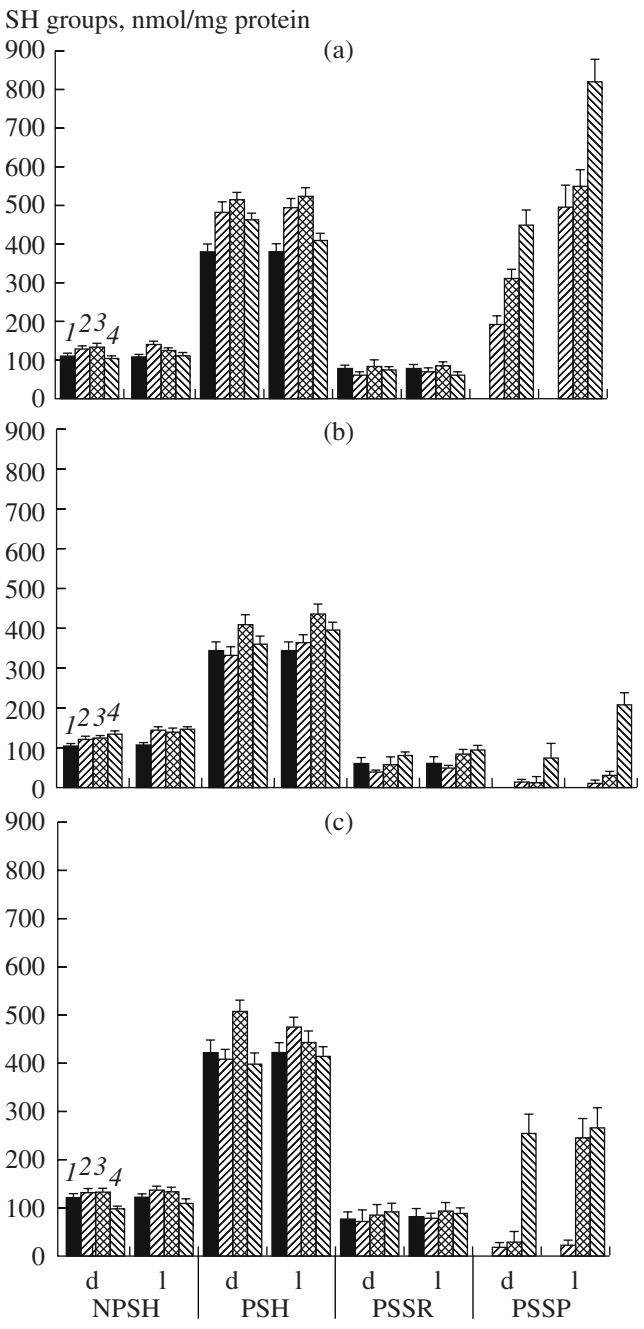

Fig. 4. Changes in the content of SH groups in low-molecular-weight compounds (NPSH) and proteins (PSH), as well as production of mixed disulfides (PSSR) and formation of disulfide bonds in proteins (PSSP) (a) in the wild type mycelium and in (b) *wc-1* and (c) *wc-2* mutants exposed under air (d) in the dark and (l) under illumination: (*1*) initial content, (*2*) 30 min, (*3*) 60 min, (*4*) 180 min.

as well as in the disulfide bond content in proteins of *N. crassa* mycelium exposed to air. In all the studied strains grown in the dark and under illumination, the content of low-molecular-weight thiols increased by 22–25% during the first hour. After three hours of exposure to air, the concentration of thiols of low-molecular-weight compounds in the wild type strain and *wc-2* mutant decreased to the initial level irrespective of the illumination, whereas it remained high in the *wc-1* mutant (Fig. 4). In all the studied strains, during the first hour of exposure to air both in the dark and under illumination, a 25–35% transitive increase of thiol content in proteins was observed. Thiol content in proteins decreased both in the wild type strain and in the mutant

strains after three hours of exposure to air, irrespective of illumination (Fig. 4). In all the studied strains, the concentration of protein/nonprotein mixed disulfides was significantly lower than the total concentration of free thiols (SH groups) in low-molecular-weight compounds and proteins; after three hours of exposure to air, no changes were observed (Fig. 4). In all the studied strains, the initial content of protein disulfide bonds was very low. In the wild type strain exposed to air, the content of disulfide bonds in proteins increased rapidly, especially under illumination (Fig. 4). In the *wc-1* and *wc-2* mutants exposed to air, the concentrations of protein disulfides increased as well; however, it was significantly lower than in the wild type strain (Fig. 4).

DISCUSSION

It has been previously demonstrated that the effect of ROS on light signal transduction is mediated by the White Collar Complex [8, 9]. In this work, we have demonstrated that the ROS signal transduction may be controlled by the WCC.

The superoxide radical $O_2^{-\cdot}$ produced in the course of respiration is known to undergo dismutation accompanied by the production of H_2O_2 and molecular oxygen [1]; in the presence of SOD, which catalyzes this reaction, the reaction rate is several orders of magnitude higher. The data obtained demonstrate that SOD activation in the wild type strain was observed under increased values of oxygen content and ambient temperature. The exposure of *N. crassa* to light did not result in an increase in SOD activity; however, in the wild type strain, light induced the biosynthesis of carotenoids that efficiently bind the $^1O_2$ produced under illumination. In the WCC strains, carotenogenesis did not occur in the presence of light; increased SOD activity at elevated oxygen concentrations and temperature (stimulating cell respiration) was also not observed. These findings indicate certain abnormalities in reception and transduction of the $O_2^{-\cdot}$. signal in these mutants.

H_2O_2 produced within the cells during $O_2^{-\cdot}$ dismutation, as well as under the influence of various stress factors, is decomposed by catalase into water and molecular oxygen [1, 3]. The initial catalase activity in the WCC mutants was significantly higher than that in the wild type strain; it increased with increasing temperature, which stimulated the production of intracellular ROS. The results obtained suggest that *N. crassa* possibly possesses H_2O_2 signal transduction pathways not associated with the White Collar Complex. It should be noted that although the WCC mutants have no circadian rhythms and do not respond to light, an increase in temperature up to 30–37°C leads to the emergence of the temperature rhythms of conidia formation. These data apparently confirm that the signal transduction of ROS under elevated temperature occurs independently of the White Collar Complex [17].

The redox status, which depends significantly on the ratio between oxidized and reduced glutathione, changes according to the organism condition [2–4]. The presence of oxidants induces the reversible (with the formation of a disulfide bond) or irreversible (to a sulfenic or sulfonic group) oxidation of protein SH groups and thiols in low-molecular-weight compounds, primarily in glutathione [18]. Reversible production of mixed disulfides in the presence of oxidants prevents the irreversible oxidation of proteins [12]. It has been shown that, in the presence of ROS, disulfide production occurs in certain proteins with thiol groups sensitive to oxidation; these proteins are therefore potential ROS targets and participants in the ROS signal transduction [11–13]. The study of the changes in the content of low-molecular-weight thiol compounds and of thiol groups in proteins, as well as of the dynamics of the disulfide bond formation in proteins, showed that the greatest differences between the WCC mutants and the wild type strain were found in the formation of protein disulfide bonds. In both mutants exposed to air, the formation of disulfide bonds in proteins was less intense than that in the wild type strain and did not depend on illumination. Enhanced catalase activity in both mutants possibly represents a compensatory reaction to the oxidative stress induced by some abnormalities in the $O_2^{-\cdot}$. signal transduction. Under experimental conditions, the content of mixed disulfides in all the studied strains was low. The decreased disulfide production in the proteins of the WCC mutants against the background of low content of mixed disulfides may be due to more intense irreversible oxidation of protein SH groups to sulfenic or sulfonic groups [13].

Thus, the obtained results demonstrate that the WCC mutants exhibit abnormalities in the transduction of light signals associated with the $^1O_2$ production, as well as of other stress signals associated with the ROS production, probably resulting from $O_2^{-\cdot}$ production during respiration. The increase in catalase activity in the WCC mutants induced by stress factors indicates that, apart from the White Collar Complex, the mutants may have distinct pathways of H_2O_2 signal transduction. Impaired ROS signal transduction is accompanied by a low intensity of the disulfide bond formation in proteins.

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