Redox regulation has been shown to be of increasing importance for many cellular processes. Here, redox homeostasis was addressed in *Aspergillus nidulans*, an important model organism for fundamental biological questions such as development, gene regulation or the regulation of the production of secondary metabolites. We describe the characterization of a thioredoxin system from the filamentous fungus *A. nidulans*. The *A. nidulans* thioredoxin A (AnTrxA) is an 11.6-kDa protein with a characteristic thioredoxin active site motif (WCGPC) encoded by the *trxA* gene. The corresponding thioredoxin reductase (AnTrxR), encoded by the *trxR* gene, represents a homodimeric flavoprotein with a native molecular mass of 72.2 kDa. When combined in vitro, the in *Escherichia coli* overproduced recombinant proteins AnTrxA and AnTrxR were able to reduce insulin and oxidized glutathione in a NADPH-dependent manner indicating that this in vitro redox system is functional. Moreover, we have created a thioredoxin A deletion strain that shows decreased growth, an increased catalase activity, and the inability to form reproductive structures like conidiophores or cleistothecia when cultivated under standard conditions. However, addition of GSH at low concentrations led to the development of sexual cleistothecia, whereas high GSH levels resulted in the formation of asexual conidiophores. Furthermore, by applying the principle of thioredoxin-affinity chromatography we identified several novel putative targets of thioredoxin A, including a hypothetical protein with peroxidase activity and an aldehyde dehydrogenase.

Due to the metabolism of molecular oxygen as the final electron acceptor of the respiratory chain, all aerobic organisms are exposed to reactive oxygen intermediates (ROIs). Whereas low concentrations of ROI are supposed to function as second-
Thioredoxin System of A. nidulans

| Strain     | Relevant phenotype and/or genotype | Source/reference |
|------------|------------------------------------|-----------------|
| E. coli    |                                    |                 |
| DH15a      | $r_{E. coli}$, lacZΔM15, Δ(lacZYA-argF)U169, recA1, endA1, hisG17, supE44, thi-1, gyrA96, relA1 | Invitrogen      |
| TOP10      | $r_{E. coli}$, lacZΔM15, Δ(lacZYA-argF)U169, recA1, endA1, hisG17, supE44, thi-1, gyrA96, relA1 | Invitrogen      |
| BL21(DE3)  | ompT, lacD(aadB), gal deh (DE3)     | Novagen, Darmstadt, Germany |

### Table 1

**Bacteria and fungi used in this study**

**A. nidulans**

| Strain       | Relevant phenotype and/or genotype | Source/reference |
|--------------|-----------------------------------|-----------------|
| TN02A7 (Δ interrupts) | pyrG89, pyroA4, nkua::argB, ribol2 | (26)            |
| AnTrxAKO     | pyrG89, pyroA4, nkua::argB, ribol2 | This work       |
| AXB4A2       | pyrG89, palua1, argB2, fwa1, bgaA1, auxB2 (per-uidA, rpm-A-lacZ), ArgB2 | (28)            |

The rapidly growing literature on thioredoxin reductases, thioredoxins, and redox-regulated proteins indicates the deep impact of oxidoreductase systems on cellular processes. In microbial eukaryotes, ROIs are involved in development, cell differentiation (10), and host-pathogen interaction (11). Also, a possible role of oxidoreductase systems in the penicillin biosynthesis has been discussed for Penicilium chrysogenum and Streptomyces clavuligerus (12, 13). In this report, we describe the isolation and characterization of a thioredoxin system from **A. nidulans**, which is an important model organism to study all kinds of biological questions, including development and the production of secondary metabolites (14). As shown here, the thioredoxin system is essential for development of **A. nidulans**, and novel target proteins of thioredoxin were identified. Furthermore, the *in vitro* and *in vivo* data indicate that this thioredoxin system possesses a key role in the redox regulation of **A. nidulans**, because correlations with other redox systems, such as catalases, the glutathione system, and a thioredoxin-dependent peroxidase seem to exist.

**Experimental Procedures**

**Strains and Molecular Genetic Techniques**—Bacterial and fungal strains used in this study are listed in Table 1. A detailed oligonucleotide description can be found in the supplemental data (Table S1). Standard techniques in the manipulation of DNA were carried out as described by Sambrook et al. (15). Genomic DNA from **A. nidulans** mycelia, grown for 24–48 h in Aspergillus minimal medium (AMM), was isolated by using the MasterPure™ Yeast DNA purification kit from Biozym Scientific (Oldendorf, Germany) according to a modified isolation protocol (16).

**Media and Cultivation of Strains**—AMM was prepared as previously described (17). If required, uridine (1 g/liter), p-aminobenzoic acid (3 mg/liter), riboflavin (2.5 mg/liter), pyridoxine-HCl (500 mg/liter), or reduced glutathione (307 mg/liter to 30.7 g/liter) was added to the medium.

**Isolation of Total RNA**—**A. nidulans** strain AXB4A2 was grown at 37°C in AMM supplemented with p-aminobenzoic acid and uridine. Mycelia were harvested, and cell extracts were obtained using liquid nitrogen as previously described (18). Total RNA was isolated by using the RNeasy kit from Qiagen following the “RNeasy Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi.” Aliquots (1–2 μg) of total RNA were used for the synthesis of AnTrxA-cDNA and AnTrxR-cDNA, as described in the following section.

**Synthesis of AnTrxA-cDNA and AnTrxR-cDNA—**AnTrxA-cDNA was synthesized with the gene-specific primers AnTrxAf and AnTrxARHisr by using the BioScript™ One-Step RT-PCR kit from Bioline (Luckenwalde, Germany) according to the manufacturer’s protocol. AnTrxR-cDNA was synthesized by using the gene-specific primers AnTrxAR-Hisr and AnTrxAf.

**Generation of Recombinant Plasmids for AnTrxA and AnTrxR Overproduction**—For the overproduction of the C-terminal His-tagged AnTrxA(wt) fusion protein AnTrxA-cDNA was cloned into the Ndel-NcoI site of the pET-39b(+) vector (Novagen) to generate the plasmid pET39-AnTrxA(wt)-H6. For the overproduction of the C-terminally His-tagged AnTrxA(C39S) fusion protein a cysteine residue (Cys-39) in AnTrxA(wt) was replaced by a two-step PCR amplification technique, as described by Ho et al. (19) using pET39-AnTrxA(wt)-H6 as template and the primers AnTrxC39Sf and AnTrxC39Sr for mutagenesis. The resulting DNA fragment was cloned into the Ndel-NcoI site of the pET-39b(+) vector to generate the plasmid pET39-AnTrxA(C39S)-H6. For the overproduction of the N-terminally His-tagged AnTrxR fusion protein AnTrxR-cDNA was cloned into the Ndel-HindIII site of the pET-39b(+) vector to generate the plasmid pET39-AnTrxA(C39S)-H6. The DNA sequence of the inserts was verified by sequence analysis.

**Purification of Recombinant Proteins**—The recombinant soluble His$_{6-}$tagged proteins AnTrxA(wt), AnTrxA(C39S), and AnTrxR were overproduced and purified by Ni$_2$+-chelate and anion exchange chromatography, as described elsewhere (20). For storage at −20°C, the recombinant AnTrxA(wt), AnTrxA(C39S), and AnTrxR proteins were transferred into 50% (v/v) glycerol, 0.1 M potassium phosphate, pH 7.5, 2 mM EDTA, and 5 mM dithiothreitol (DTT), using a HiPrep desalting column (GE Healthcare, Freiberg, Germany). Protein concentrations were determined according to Bradford (21) using the Coomassie Plus™ protein assay reagent (Pierce).

**Purity and Molecular Weight Determination**—The purity and molecular weights of the recombinant proteins were determined by SDS-PAGE. In addition, the AnTrxA(wt) and AnTrxR proteins were subjected to gel filtration using a Superdex 200 HiLoad 16/60 column (GE Healthcare) equilibrated with a buffer containing 100 mM potassium phosphate, 150 mM NaCl, pH 7.0.
FAD Content and Reconstitution of the AnTrxR Holo-enzyme with FAD—The concentration of enzyme-bound FAD was determined by measuring the absorbance at 454 nm with a molar extinction coefficient of 11.3 mM⁻¹ cm⁻¹ for FAD (22). Due to the high production levels of AnTrxR in E. coli BL21(DE3) and the following purification procedures, the majority of the enzyme was present as an apo-enzyme. To reconstitute the AnTrxR holo-enzyme for further characterization, AnTrxR was incubated with a 60-fold molar excess of FAD for 20 min before adding on a NAP-5 column (GE Healthcare) to remove the excess of FAD.

Thioredoxin Reductase Activity—TrxR activity of the purified AnTrxR was determined by using two different methods. In the NBS₂ reduction assay AnTrxR activity was determined by the NADPH-dependent reduction of 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) (23). One enzyme unit is defined as the NADPH-dependent production of 2 μmol of 2-nitro-5-thiobenzoate (ε₁₃₂nm = 2 × 13.6 M⁻¹ cm⁻¹) per min. TrxR activity was also assayed based on the ability of AnTrxR to reduce AnTrxA(wt), which then reduces insulin disulfide bridges (24). AnTrxR activity was calculated from the decrease in absorbance at 340 nm using a molar extinction coefficient of 6.22 M⁻¹ cm⁻¹ for NADPH. One enzyme unit is defined as the amount of enzyme that leads to the consumption of 1 μmol of NADPH per minute.

Trx Activity—Trx activity was determined by using the TrxR-dependent insulin precipitation assay (24). After starting the reaction by the addition of NADPH, the NADPH consumption was followed by recording the decrease in absorbance at 340 nm, until turbidity appeared. The increase of turbidity was measured at 650 nm.

Trx-dependent GSSG Reduction Assay—The Trx-dependent GSSG-reduction assay was carried out as described elsewhere (25). After addition of NADPH, the activity was calculated from the decrease in absorbance at 340 nm.

Transformation of A. nidulans and Generation of trxA Deletion and Complemented Strains—As a parental strain for gene deletion, the uracil auxotrophic strain TN02A7 (ΔnkuA) was used (26). As a selectable marker, the pyr₄ gene, encoding orotidine-5’-monophosphate decarboxylase from Neurospora crassa, was applied. The trxA gene, including 1500-bp upstream and downstream flanking regions, was amplified from genomic DNA of the wild-type strain AXB4A₂ by the use of the oligonucleotides TrxA1500for and TrxA1500rev. The PCR product was cloned into the PCR2.1 vector (Invitrogen) to yield plasmid pAnTrxA-FLANK. Plasmid DNA of pAnTrxA-FLANK was cut with Clal and BmgBI (blunt end cutter) to release a 1606-bp fragment, including the complete trxA gene, 650 bp of the upstream region, and 552 bp of the downstream region. For the introduction of the pyr₄ gene, plasmid DNA of pKTB (27) was restricted with Clal and PvuII (blunt end cutter). The resulting pyr₄-containing DNA fragment was then ligated with the Clal- and BmgBI-restricted pAnTrxA-FLANK vector backbone to give plasmid pAnTrxAKO. Plasmid pAnTrxAKO was digested with NsiI and Acc65I to remove the PCR2.1 vector backbone. After gel purification (QIAquick gel extraction kit, Qiagen) the DNA fragment was directly used for transformation of A. nidulans TN02A7 (ΔnkuA) as previously described (28). Transformants were pre-screened for their ability to sporulate on AMM agar plates containing 20 mM reduced glutathione and their inability to sporulate on AMM-agar plates without reduced glutathione. Genomic DNA of putative trxA deletion strains was subjected to Southern blot analysis. Complementation experiments were carried out by transformation of strain AnTrxAKO with a trxA-encoding PCR product, including 1.5-kb upstream and downstream flanking regions. Genomic DNA of transformants that behaved like the wild type was subjected to Southern blot analysis. For detection of DNA fragments, the digoxigenin system (Roche Applied Science) was used.

Trx- affinity Chromatography—5 mg of AnTrxA(C39S) were coupled to a Hi-Trap NHS-activated 1-ml affinity column (GE Healthcare) according to the manufacturer’s instructions. A. nidulans mycelia of the wild-type strain TN02A7 and the trxA deletion strain AnTrxAKO were grown in liquid nitrogen using mortars and pestles. The powder was resuspended in 100 mM potassium phosphate, pH 7.5, and 150 mM NaCl. After centrifugation (10,000 × g, 30 min) the soluble protein-containing supernatants were applied to the prepared thioredoxin-affinity column by injection at a flow rate of 1 ml/min. The column was washed with 100 mM potassium phosphate and 250 mM NaCl, pH 7.5, at 1 ml/min. Elution was carried out with 100 mM potassium phosphate containing 10 mM DTT, pH 7.5, and 150 mM NaCl. Aliquots of the supernatants, flow-through, wash, and elution fractions were analyzed by SDS-PAGE.

Identification of AnTrxA Targets—Protein bands of the elution fraction were excised manually and digested with trypsin (Promega, Madison, WI). Peptides were extracted as described (29) and peptide mass fingerprint and fragmentation data were collected on a Bruker ultraflex TOF/TOF using Bruker Compass 1.2 software (FlexControl/FlexAnalysis 3.0). Obtained peak lists were sent to a Mascot in-house server (version 2.1.03) with the current NCBI nr data base for protein identification. Search parameters were set as follows: mass tolerance of 200 ppm for peptide mass fingerprint and 0.5 Da for fragmentation, maximum of one missed cleavage by trypsin, taxonomy “fungi,” fixed carbamidomethyl modification, and optional methionine oxidation. The most significant hits were verified by comparison with the combined peptide mass fingerprint/fragmentation spectrum. With the chosen settings protein score of >67 are significant (p < 0.05).

Trx-dependent Peroxidase Activity—The elution fractions of AnTrx(A(C39S)) affinity-purified protein solutions were applied to a NAP-10 column to remove the excess of DTT. Then aliquots of the DTT-free protein solution in 0.1 M potassium phosphate, 150 mM NaCl, pH 7.5, were incubated with or without the recombinant A. nidulans thioredoxin system and 200 μM NADPH. After addition of H₂O₂, the activity was calculated from the decrease in absorbance at 340 nm.

Hydrogen Peroxide Sensitivity Assay—1.5 × 10⁸ spores of the strains TN02A7 and AnTrxAKO were inoculated in 30 ml of liquid AMM agar (2% w/v) containing 0 mM, 1 mM, and 20 mM of GSH. After the agar became solidified a hole of 1 cm in diameter in the center of the agar plate was created and filled with 150 μl of a 4.5% (v/v) H₂O₂ solution. The agar plates were incubated at 37 °C, and the zone of growth inhibition was measured after 48 h.
Catalase Activity—A. nidulans mycelia and freshly harvested spores of the strains TN02A7 and AnTrxAKO were ground in liquid nitrogen using mortar and pestle. The obtained powder was resuspended in 100 mM potassium phosphate, pH 6.5. After centrifugation (10,000 × g, 30 min) the soluble protein extracts were diluted in 50 mM potassium phosphate, pH 6.5, to a final concentration of 5–50 μg of protein ml⁻¹. After adding H₂O₂ (20 mM) the decrease in absorbance at 240 nm was measured. Catalase activity was calculated from the decrease in absorbance at 240 nm using a molar extinction coefficient of 0.0436 mM⁻¹ cm⁻¹ for H₂O₂ (30). Catalase activity was also investigated by zymography (31).

RESULTS

Cloning and Sequence Analysis of the trxA and trxR Genes from A. nidulans—Two genes with the accession numbers XM_652682 and XM_656093 have been annotated to encode a classic cytoplasmatic thioredoxin (TrxA) and a hypothetical protein similar to thioredoxin reductase (TrxR), respectively. By using gene-specific primers for the reverse transcription and cDNA synthesis of the gene encoded by XM_652682, a DNA fragment was synthesized encoding a sequence identical to the deposited trxA cDNA. The deduced AnTrxA protein contains the thioredoxin-specific active site motif WCGPC and further highly conserved amino acids (see sequence alignment with other thioredoxins in supplemental Fig. S1A). AnTrxA exhibits all the characteristics of thioredoxins and represents the A. nidulans thioredoxin sequence (accession number AAB24444) described earlier (32).

Here, by reverse transcription of the gene designated with accession number XM_656093, we identified a shorter cDNA version for the A. nidulans thioredoxin reductase (accession number AM396558). This coding sequence is identical to an updated version of the trxR coding sequence deposited in the A. nidulans data base (AN3581.3). It contains a putative FAD-binding domain formed by the GXXXG(A/G) motif in the N-terminal region and the TXXXXVFAAGD motif at the C terminus of the protein (33, 34). An NADPH-binding domain was also identified near the middle of the protein encoded by the motif GGGXXA (33, 34). Furthermore, it contains the pyridine-nucleotide-disulfide oxidoreductases class-II active site motif, including a redox-active cysteine pair (CAVC). This motif was found by a pattern search using the “PROSITE data base of protein families and domains” (http://www.expasy.org/prosite/). The motif is characteristic of prokaryotic and eukaryotic thioredoxin reductases (8, 9, 35, 36), bacterial alkyl hydroperoxide reductases (37), bacterial NADH:dehydrogenases (38), and a probable oxidoreductase encoded by the Clostridium pasteurianum rubredoxin operon (39). An alignment of AnTrxR with other low molecular weight thioredoxin reductases can be found in the supplemental data (Fig. S1B).

Both AnTrxA(wt) and AnTrxR were overproduced as His-tagged proteins in E. coli BL21(DE3) and purified to homogeneity. Additionally, an AnTrxA mutant version (AnTrxA(C39S)) was created, which had the second cysteine of the AnTrxA active site substituted by serine (Cys-39 → Ser-39). SDS-PAGE analysis of the purified proteins showed molecular masses of 12.7 and 37.6 kDa for AnTrxA and AnTrxR, respectively (Fig. 1A). After subtracting the molec-
ular mass due to the His tag, the molecular masses of both proteins are in agreement with the values deduced from the respective cDNA sequences. The data obtained by gel filtration revealed apparent native molecular masses of 12.9 kDa for the AnTrxA(wt) and 88.0 kDa for the AnTrxR protein (Fig. 1B). These data indicate that, without the His tag, the native AnTrxA is a monomer of 11.6 kDa, whereas the native AnTrxR is a homodimer of 72.2 kDa. Consequently, the concentrations of AnTrxR given in the following refer to the homodimer.

**AnTrxA Is a Flavoenzyme**—Both the sequence analysis and the yellow color of the purified AnTrxA led to the assumption that the enzyme is a flavoenzyme. Consistently, the UV-visible absorbance spectrum of the reconstituted AnTrxA holo-enzyme with absorbance maxima at 280, 380, and 460 nm and an absorbance ratio $A_{280}/A_{460}$ of 7.6 (Fig. 1C) is characteristic of a pure thioredoxin reductase with one FAD molecule per subunit (33, 40). The creation of the reduced form of AnTrxA by adding a 12 M excess of NADPH resulted in a decreased absorbance at 460 nm (Fig. 1D).

**TrxR Substrate Specificity**—For the determination of the kinetic parameters of the AnTrxA protein, we used the NBS$_z$ insuline, and GSSG reduction assays, as described under “Experimental Procedures.” The Cys-39 $\rightarrow$ Ser-39 substitution in the active site led to an AnTrxA mutant protein (AnTrxA(C39S)), which was unable to cycle between its oxidized disulfide (Trx-S$_2$) and reduced dithiol [Trx-(SH)$_2$] form. Thus, this mutant protein did not serve as a substrate for AnTrxR, which does not allow kinetic parameter determination. AnTrxR was also able to catalyze the NADPH-dependent reduction of DTNB, but the protein was unable to use GSSG and insulin as substrates directly. The kinetic parameters of AnTrxR for various substrates are summarized in Table 2.

**TrxA Activity**—Thioredoxins are known for their ability to catalyze the reduction of insulin disulfide bonds in an NADPH and thioredoxin reductase-dependent manner. The cleavage of two disulfide bridges between the insulin $\alpha$- and $\beta$-chains results in the precipitation of the free $\beta$-chain, which can be measured by an increase in turbidity at 650 nm. The NADPH-dependent reduction of bovine insulin by either AnTrxA(wt) or AnTrxA(C39S) and AnTrxR was carried out as described under “Experimental Procedures.” When the coupled insulin reduction assay was employed, in contrast to the wild-type form AnTrxA(wt), for the AnTrxA(C39S) mutant protein no NADPH consumption (Fig. 1E) and no increase in turbidity (Fig. 1F) were measured. The kinetic parameters of AnTrxA(wt) for insulin are summarized in Table 3.

**GSSG Reduction by the A. nidulans Thioredoxin System**—Because AnTrxA does not accept GSSG as a substrate, it is likely that AnTrxA(wt) acts as a redox mediator between AnTrxR and GSSG, as it has been described for the *P. falciparum* thioredoxin (25). The kinetic parameters of AnTrxA(wt) for GSSG are summarized in Table 3. Cellular GSSG levels of 1 mM and above were reported for glutathione reductase-deficient yeast mutants (41). In experiments simulating high GSSG concentrations by using 1 mM GSSG and physiological concentrations of AnTrxA(wt) (10 $\mu$M) and AnTrxR (125 nm) (40, 42), the *A. nidulans* thioredoxin system gave GSSG fluxes up to 90 $\mu$M s$^{-1}$ (Table 3). At higher GSSG levels (5–10 mM) and under steady-state conditions 10 $\mu$M AnTrxA(wt) could reduce GSSG with higher turnover rates as high as 340 $\mu$M s$^{-1}$.

**Southern Blot Analysis of ΔtrxA and Complemented Strains**—*A. nidulans* was transformed using the plasmid pAnTrxAKO as described under “Experimental Procedures.” For the PstI digestion of genomic DNA from a trxA deletion strain a shift from 5.9 to 1.0 kb was expected in the case of a homologous integration of the deletion construct into the trxA locus. The EcoRV digestion should result in a shift from 8.0 to 2.3 kb (Fig. 2A). Transformants 3 (designated AnTrxAKO and used for further studies), 8, 10, and 11 showed the expected bands, whereas transformant 5 seemed to possess either tandem and/or ectopic integrations (Fig. 2B).

The homologous integration of an AnTrxA encoding PCR fragment into the former trxA locus (replaced by ppyr-4) should lead to the complementation of the wild-type phenotype due to the restoration of the wild-type locus organization in a complemented knock-out strain (see Fig. 2A). Transformant strains C1 and C5 showed the expected hybridization pattern, whereas strains C3, C6, and C7 neither showed the ΔtrxA nor the wild-type situation (Fig. 2C), indicating that the complementation construct was integrated into the former trxA locus in an inaccurate way.
Deletion of trxA Affects Development and Resistance against H₂O₂—On AMM agar plates, AnTrxAKO was characterized by reduced hyphal growth and almost no sporulation, even after incubation for more than 6 days (see Figs. 3B, 3D, and 4A). Cultivation of AnTrxAKO in liquid AMM without reduced glutathione resulted in no growth at all (data not shown). However, addition of 15–25 mM of reduced GSH to the media led to the restoration of the wild-type phenotype, i.e., AnTrxAKO grew, sporulated, and produced colored conidia as the wild type (see Figs. 3A, 3C, and 4B). The data obtained here suggest that a reducing environment within the cell is essential for the initiation of growth and sporulation. The phenotype observed for the trxA deletion strain was fully complemented in strain AnTrxAKO transformed with the trxA gene, indicating that the observed deletion phenotype was due to a deletion of the trxA gene and not caused by unrelated secondary effects (see Fig. 3). When AnTrxAKO was cultivated with low concentrations of reduced glutathione, at levels ranging from 0.5 to 1 mM GSH, the ΔtrxA strain started to produce cleistothecial initials and Hülle cells already after 72 h. Fully developed cleistothecia were detected after 144 h of incubation (Fig. 4C). This was unexpected because the parental strain used for generation of AnTrxAKO exhibits a mutated veA gene. In A. nidulans veA⁺ strains, light reduces and delays the formation of cleistothecia. Consequently, the fungus develops asexual conidia, whereas in the dark fungal development is directed toward the formation of cleistothecia (43). Thus, mutation of veA blocks cleistothecial production in A. nidulans in the dark (44). Although AnTrxAKO should be restricted in formation of cleistothecia in the dark (incubator conditions) due to the veA mutation, there was a premature and highly increased formation of cleistothecia, when compared with the wild-type cultivated under the same conditions (Fig. 4D). This finding indicates that oxidative stress or at least an imbalanced intracellular redox environment leads to sexual development.

To study the importance of the thioredoxin system for defense against ROIs, AnTrxAKO and the wild-type strain TN02A7 were challenged with H₂O₂ and cultivated for 48 h in AMM agar containing 0, 1, or 20 mM GSH. The treatment of AnTrxAKO with H₂O₂ by filling a hole in the center of agar plates containing none or 1 mM GSH resulted in an increased gas bubble formation around the H₂O₂ solution, already visible after 15–30 min (Fig. 5A). After 48 h, the whole inhibition zone was filled with gas bubbles (Fig. 5B), which most likely resulted from the decomposition of H₂O₂ to oxygen and water. Furthermore, the inhibition zones at these GSH concentrations were slightly increased for AnTrxAKO when compared with that of the wild-type strain (Table 4), that only showed a slight gas bubble formation (Fig. 5, C and D). However, at higher GSH concentration (20 mM) the inhibition zone of AnTrxAKO was 1.4-fold larger than that of the wild type (Table 4).

Catalase Activity—It is likely that the increased gas bubble formation of strain AnTrxAKO is due to an increased catalase activity. A. nidulans contains at least three catalases designated CatA, CatB, and CatC (45, 46). Recently, a fourth protein, designated CatD, was shown to be a catalase-peroxidase (47). By using A. nidulans protein extracts from different physiological stages, we were able to detect all four different catalase activities in zymograms. Due to the early and highly increased gas bubble formation of strain AnTrxAKO exhibits a mutated veA gene. In A. nidulans veA⁺ strains, light reduces and delays the formation of cleistothecia. Consequently, the fungus develops asexual conidia, whereas in the dark fungal development is directed toward the formation of cleistothecia (43). Thus, mutation of veA blocks cleistothecial production in A. nidulans in the dark (44). Although AnTrxAKO should be restricted in formation of cleistothecia in the dark (incubator conditions) due to the veA mutation, there was a premature and highly increased formation of cleistothecia, when compared with the wild-type cultivated under the same conditions (Fig. 4D). This finding indicates that oxidative stress or at least an imbalanced intracellular redox environment leads to sexual development.

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To study the importance of the thioredoxin system for defense against ROIs, AnTrxAKO and the wild-type strain TN02A7 were challenged with H₂O₂ and cultivated for 48 h in AMM agar containing 0, 1, or 20 mM GSH. The treatment of AnTrxAKO with H₂O₂ by filling a hole in the center of agar plates containing none or 1 mM GSH resulted in an increased gas bubble formation around the H₂O₂ solution, already visible after 15–30 min (Fig. 5A). After 48 h, the whole inhibition zone was filled with gas bubbles (Fig. 5B), which most likely resulted from the decomposition of H₂O₂ to oxygen and water. Furthermore, the inhibition zones at these GSH concentrations were slightly increased for AnTrxAKO when compared with that of the wild-type strain (Table 4), that only showed a slight gas bubble formation (Fig. 5, C and D). However, at higher GSH concentration (20 mM) the inhibition zone of AnTrxAKO was 1.4-fold larger than that of the wild type (Table 4).

Catalase Activity—It is likely that the increased gas bubble formation of strain AnTrxAKO is due to an increased catalase activity. A. nidulans contains at least three catalases designated CatA, CatB, and CatC (45, 46). Recently, a fourth protein, designated CatD, was shown to be a catalase-peroxidase (47). By using A. nidulans protein extracts from different physiological stages, we were able to detect all four different catalase activities in zymograms. Due to the early and highly increased gas bubble formation of strain AnTrxAKO exhibits a mutated veA gene. In A. nidulans veA⁺ strains, light reduces and delays the formation of cleistothecia. Consequently, the fungus develops asexual conidia, whereas in the dark fungal development is directed toward the formation of cleistothecia (43). Thus, mutation of veA blocks cleistothecial production in A. nidulans in the dark (44). Although AnTrxAKO should be restricted in formation of cleistothecia in the dark (incubator conditions) due to the veA mutation, there was a premature and highly increased formation of cleistothecia, when compared with the wild-type cultivated under the same conditions (Fig. 4D). This finding indicates that oxidative stress or at least an imbalanced intracellular redox environment leads to sexual development.
formation observed for AnTrxAKO in the hydrogen peroxide diffusion assay, we were interested in which of the one or more catalases were predominantly responsible for the detoxification of \( \text{H}_2\text{O}_2 \) in AnTrxAKO. Therefore, the specific catalase activities were measured and compared with the corresponding zymographic results. The intracellular catalase activity of AnTrxAKO conidia was only 1.6-fold increased when compared with the wild-type strain grown under the same conditions (B). Consistently, no significant changes for the intracellular activities of CatA and CatB from both the wild-type strain and AnTrxAKO were detectable in zymograms (Fig. 6A). However, when using supernatants of conidia for activity measurements, the specific catalase activity was \( 4-5 \times \) increased for AnTrxAKO compared with the wild type. This finding led us to conclude that the one or more catalases that are responsible for the rapid stress response in AnTrxAKO are either excreted to the medium or located on the conidial surface. Based on the known catalase expression patterns (45–47) and a predicted secretory signal peptide in the N terminus of CatB (48), the increased early catalase activity of AnTrxAKO is due to extracellular CatB. After 18-h cultivation only residual CatA activity was determined for both strains, whereas the CatB activity was still elevated and CatC activity became detectable in AnTrxAKO (Fig. 6B). When comparing the specific catalase activities of intracellular protein extracts from AnTrxAKO and TN027A at this time point, a 30-fold increase in catalase activity was measured for AnTrxAKO (Fig. 6B). This increase can be predominantly assigned to an increased CatB activity as can be seen from the zymographic results (Fig. 6B). After 48 h, CatB and CatD activities appeared to be the major ones. Although the zymogram did not show significant differences of any catalase activity between the two strains, the direct measurement revealed a four times higher intracellular total catalase activity for AnTrxAKO (Fig. 6B).

**TABLE 4**

| Strain   | 0 mM GSH | 1 mM GSH | 20 mM GSH |
|----------|----------|----------|-----------|
| TN02A7   | 3.9 ± 0.1| 4.2 ± 0.1| 4.6 ± 0.1 |
| AnTrxAKO | 4.6 ± 0.1| 4.5 ± 0.1| 6.2 ± 0.1 |

Novel Target Proteins of Thioredoxin A—To identify thioredoxin A target proteins, we applied thioredoxin-affinity chromatography (49) using the AnTrxA(C39S) mutant protein as bait to catch proteins from soluble protein extracts of both \( A. \text{nidulans} \) strains TN02A7 and AnTrxAKO. Strain TN02A7 was
cultivated in AMM without addition of GSH. AnTrxA KO is not
to grow in AMM without GSH and was therefore grown
in AMM supplemented with the minimal dose of 1 mM GSH
to allow initiation of growth. After immobilization of the
AnTrxA(C39S) protein on an NHS-activated column, protein
extracts from the respective strains were loaded, and proteins
that were specifically retained via formation of a disulfide bond
to AnTrxA(C39S) were eluted using DTT. Samples represent-
itive of each chromatography step (Fig. 7, A and B) were sepa-
ated by SDS-PAGE (Fig. 7C). Furthermore, proteins of the elu-
tion fractions were identified by a database-assisted mass
spectrometry analysis as described under “Experimental Pro-
cedures.” For both protein extracts derived from TN02A7 and
AnTrxA KO, two proteins were recovered by elution with DTT.
A detailed spectrum analysis report for both proteins can be
found in the supplemental data (Table S2). The 18-kDa protein,
representing the major band of the two proteins, is a hypothet-
ical protein (accession number XP_681961) with high similar-
ity to AspF3 proteins from other related fungal species contain-
ing a PRX5-like domain. The 55-kDa protein was identified as
an aldehyde dehydrogenase (accession number XP_658158).
The elution profiles and SDS-PAGE band intensities indicate
that the amounts of oxidized aldehyde dehydrogenase and the
PRX5-like protein were increased in the protein extract
obtained from AnTrxA KO. The total amounts of DTT-eluted
proteins were 2.1 μg/mg of protein extract for the wild-type
strain and 7.5 μg/mg of protein extract for AnTrxA KO. Thus,
in AnTrxA KO the levels of disulfide forms of AnTrxA targets
were increased. Because many proteins need to be reduced to
maintain their biological function, the inability of AnTrxA KO
to recycle the target proteins into the active thiol form might
contribute to the deletion phenotype.

**Discussion**

Here, we describe the characterization of a thioredoxin sys-
tem from the filamentous fungus *A. nidulans*. The sequence
and *in vitro* data of the recombinant proteins, e.g., molecular
masses, specific activities, and absorbance spectra are consist-
ent with data obtained for thioredoxins and low molecular
weight thioredoxin reductases from other organisms, indicat-
ing that we have identified a functional *A. nidulans* thioredoxin
system. Although there are several other genes in the *A. nidu-
lan*s genome encoding hypothetical proteins with a thioredoxin
domain (e.g., XP_681840.1 and XP_659243.1), the genes/pro-
teins characterized here, which were already annotated to
encode a classic cytoplasmic thioredoxin and a hypothetical
protein similar to thioredoxin reductase (accession number
AAB24444 and CAL36645), respectively, represent the major
thioredoxin system, as indicated by the phenotype of the *trxA*
deletion mutant. Moreover, we generated a recombinant
mutant version of thioredoxin A, which still binds target pro-
teins but does not reduce them. By applying the principle
of thioredoxin-affinity chromatography with this thioredoxin
mutant protein AnTrxA(C39S), we were able to identify two
putative thioredoxin target proteins. The major protein, iso-
lated from both the wild-type and the *trxA* deletion strain
AnTrxA KO represents a hypothetical protein with a PRX5-like
domain. Proteins of the PRX5-like subfamily belong to the Prx

**Thioredoxin System of A. nidulans**

![Graph A](Image 60x447 to 288x733)

**FIGURE 6. Catalase activity.** Protein extracts prepared from strain AnTrxA KO and its recipient strain TN02A7 grown for 0 h (spores), 18 h, and 48 h were analyzed for catalase activity. Cultivation of the wild-type strain with reduced glutathione was carried out to exclude possible effects of GSH on catalase activity when compared with the ΔtrxA strain. A, total catalase activity from freshly harvested spores compared with the respective protein extracts (20 μg) that were separated in a native polyacrylamide gel, which was stained to detect catalase activity (I = catalase activity of spores from the wild-type strain obtained from agar plates with 20 mM GSH; 2 = catalase activity of spores from the wild-type strain obtained from agar plates with 1 mM GSH; 3 = catalase activity of spores from the ΔtrxA strain obtained from agar plates with 20 mM GSH). B, total catalase activities of 18- and 48-h-old mycelia compared with the respective protein extracts (20 μg) that were separated in a native polyacrylamide gel, which was stained to detect catalase activity (I = catalase activity of mycelia from the wild-type strain obtained without GSH; 2 = catalase activity of mycelia from the wild-type strain obtained with 1 mM GSH; 3 = catalase activity of mycelia from the ΔtrxA strain obtained with 1 mM GSH).
family, a ubiquitous family of antioxidant enzymes that also controls cytokine-induced peroxide levels, which mediate signal transduction in mammalian cells (50). As shown for other organisms, thioredoxin-dependent peroxidases (peroxiredoxins) are prominent targets of thioredoxin (reviewed in Ref. 49). All Prx classes share the same basic catalytic reaction, in which an active site cysteine (the peroxidatic cysteine) is oxidized to a sulfenic acid by a peroxide substrate (50). However, the recycling mechanism of the sulfenic acid to a thiol is different for the Prx classes. Human PRX5 is able to resolve this intermediate by forming an intramolecular disulfide bond with its C-terminal cysteine (the resolving cysteine), which can then be reduced by Trx, just like an atypical 2-Cys Prx (51). With the thioredoxin-dependent peroxidase activity assay we showed that the PRX5-like protein reduces \( \text{H}_2\text{O}_2 \) only in the presence of a functional thioredoxin system. Furthermore, the kinetic parameters of this enzyme for \( \text{H}_2\text{O}_2 \) are in agreement with activities of a human thioredoxin peroxidase, a thioredoxin peroxidase from \( \text{Plasmodium falciparum} \), and a thioredoxin peroxidase-1 from \( \text{Drosophila melanogaster} \) (52–54). This led us to conclude that the identified PRX5-like protein is a functional thioredoxin-dependent peroxidase in \( \text{A. nidulans} \). Interestingly, the PRX5-like protein identified here has \( \approx 90\% \) amino acid identity to allergen AspB from \( \text{Aspergillus fumigatus} \). This opportunistic human pathogen has to cope with high oxidative stress during infection of the human host (55, 56). Therefore, it is very likely that \( \text{A. fumigatus} \) recruits enzymes like peroxidases to detoxify ROI. In agreement with this assumption is the observation that sera from conidium-exposed mice contain antibodies predominantly against allergen AspB (57). The second enzyme identified here by using the thioredoxin-affinity technique was an aldehyde dehydrogenase (ALDH), which contains 5 cysteine residues in its amino acid sequence. This enzyme was shown to be involved in the catabolism of ethanol, by converting the toxic byproduct acetaldehyde into acetate, which then enters the mainstream metabolism in its activated form, acetyl-CoA (58). However, ALDHs were already identified as thioredoxin targets from plant mitochondria (59). Although the redox regulation of ALDHs remains to be shown, there is evidence that ALDHs can be inactivated by thiol-modifying agents, such as the alcohol aversion therapy drug disulfiram, as demonstrated for the rat liver ALDH (60). It was suggested that disulfiram inhibits rat liver ALDH by forming an intramolecular disulfide between two of the three adjacent cysteines in the active site, possibly via a fast intramolecular disulfiram-interchange reaction. This assumption was confirmed by the fact that addition of DTT led to a partial restoration of the enzyme activity (60).

A possible role of thioredoxins in maintaining a high GSH:GSSG ratio has been discussed for \( \text{S. cerevisiae} \) (41). \( \text{S. cerevisiae} \) glr1\( \Delta \) mutants lacking glutathione reductase, accumulate high levels of oxidized glutathione, i.e. the disulfide form of glutathione (GSSG) represented 63\% of the total glutathione in a glr1\( \Delta \) mutant compared with only 6\% in the wild type. Also, an increase of GSSG from 6 to 22\% of the total glutathione was observed in a thioredoxin double mutant (\( \text{trx1}\Delta \) and \( \text{trx2}\Delta \)). Other organisms like \( \text{D. melanogaster} \) or \( \text{Anopheles gambiae} \), an important vector of \( \text{P. falciparum} \) causing malaria, do not possess typical glutathione reductases (61, 62). In these organisms, the capacity of a thioredoxin system to reduce oxidized glutathione is sufficient for maintaining a high GSH:GSSG ratio. Here, it was shown that the recombinant \( \text{A. nidulans} \) thioredoxin system is able to reduce oxidized glutathione in vitro under in situ conditions with fluxes of 90 \( \mu \text{M} \text{min}^{-1} \) and up to 340 \( \mu \text{M} \text{min}^{-1} \) when measured under saturated conditions (reduced AnTrxA not limiting). Interestingly, these fluxes are in the range of thioredoxin-dependent GSSG turnover rates, described for the DmTrx-2 protein from \( \text{D. melanogaster} \), an organism that lacks a classic glutathione reductase (54). Although \( \text{A. nidulans} \) encodes a hypothetical protein (accession number XP_658536) with \( \approx 81\% \) identity to a glutathione reductase from \( \text{Aspergillus terreus} \) (accession number XP_001214364), it remains to be elucidated whether this enzyme is functional and/or whether AnTrxA and AnTrxR can
repeated the glutathione reductase function. However, both the in vitro data and the fact that the wild-type phenotype can be restored in the ΔtrxA mutant by the addition of 15–25 mM GSH to the media indicate that there is a link between the thioredoxin and glutathione system. This leads to the conclusion that the A. nidulans thioredoxin system contributes to keep glutathione in the reduced form thereby ensuring high GSH:GSSG ratios, which are required for a reducing environment in the cell.

Although for a long time ROIs have been regarded as harmful by-products of aerobic metabolism, there is growing evidence that at certain concentrations ROIs play an important role in processes such as differentiation, growth, and signaling (10). In response to different signals A. nidulans is able to propagate via two different developmental pathways. The asexual development or conidiation is induced by nutrient starvation or exposure to air (63), whereas the sexual development, which leads to the formation of cleistothecia, is induced by oxygen limitation (64) and the absence of light (65). Recently, it was shown that the deletion of the noxA gene in A. nidulans, which encodes the ROI-generating enzyme NADPH oxidase, leads to mutants with a developmental defect in production of sexual cleistothecia, whereas hyphal growth and asexual development were unaffected (66). On the other hand, deletion of the A. nidulans SakA MAP kinase, which is activated in response to osmotic and oxidative stress, led to mutants that developed cleistothecia prematurely and in higher numbers than the wild type (67). As shown here for the ΔtrxA strain AnTrxAKO, similar results were obtained compared with the ΔsakA mutant. The ΔtrxA mutant developed cleistothecia at low glutathione levels already after 72–144 h. This led us to conclude that an interfer- ence with the redox balance of the cell affects the differentiation of A. nidulans. Consequently, asexual development and conidiation are not only induced by nutrient starvation or exposure to air but also by a reducing environment of the cell. On the other hand, sexual development is not only induced by oxygen limitation and the absence of light but also by oxidative stress, which occurs when genes encoding for key enzymes involved in oxidative stress response, such as trxA or sakA, were deleted. The fact that various catalases are differently expressed and regulated during the life cycle of A. nidulans also supports the correlation of redox regulation and developmental processes. Consistently, different catalase activity patterns were identified and verified here at different developmental stages of A. nidulans. These results confirm the catalase expression and activity patterns described elsewhere (45–47). Moreover, we could clearly show that the deletion of trxA has an inducing effect on the total catalase activity of A. nidulans and in particular on CatB. The mechanism behind this induction in the trxA deletion strain remains to be elucidated. However, it was shown for other fungi, that genes encoding enzymes for oxidative stress response are under the control of a transcription factor homologous to the human AP-1 (reviewed in Ref. 68). Such AP-1-like transcription factors include Yap1 in Saccharomyces cerevisiae, Ppa1 in Schizosaccharomyces pombe, Cap1 in Candida albicans, Kap1 in Kluyveromyces lactis (reviewed in Ref. 69), and Af Yap1 in A. fumigatus. Yap1 and Af Yap1 are located in the cytoplasm of unstressed cells but quickly accumulate in the nucleus after challenge with H2O2 or diamide due to the oxidation of conserved cysteine residues within the C-terminal cysteine-rich domain. Thereby, the formation of disulfide bridges between certain cysteine residues leads to a protein structure that masks the nuclear export signal of Yap1. The subsequent export from the nucleus is thus abolished. Recent evidence indicates that deactivation (reduction) of oxidized Yap1 is mediated by the thioredoxin system. Mutations that affect thioredoxin or thioredoxin reductase activity result in nuclear localization of Yap1 under non-stressed conditions. Oxidative stress-induced nuclear accumulation of Yap1 also leads to the activation of thioredoxin and thioredoxin reductase-encoding genes, suggesting that the nuclear localization of Yap1 is regulated by a negative feedback loop. A homologue of Af Yap1 could also be identified for A. nidulans (accession number XP_680782). Based on the assumption that trxA is under transcriptional control of this putative An Yap1, it is reasonable to assume that in the ΔtrxA strain reduction of An Yap1 is abol- ished. Consequently, An Yap1 accumulated in the nucleus also under non-stressed conditions, resulting in a permanent activation of target genes, such as catalase-encoding genes. This model would explain the increased catalase activity, which is responsible for the elevated H2O2 decomposition by AnTrxAKO. At higher glutathione concentrations within the medium also the GSH content of the cell increases. The result- ing reducing environment keeps An Yap1 reduced, which leads to an increased accumulation of An Yap1 in the cytoplasm, and therefore to a decreased transcription of catalase-encoding genes. Consequently, a decreased catalase activity increases the sensitivity of A. nidulans against H2O2, resulting in an increased inhibition zone with or less H2O2 decomposition. In summary, this work demonstrates the impact of the thioredoxin system from A. nidulans on differentiation, sexual development, and oxidative stress response. Although the regulatory networks between the A. nidulans thioredoxin system and the different redox-regulating systems described here remain to be elucidated, it is obvious that there is a link between the A. nidulans thioredoxin system and other redox-regulating mecha- nisms, such as catalases, a thioredoxin-dependent peroxidase, and the glutathione system.

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