Iron starvation leads to increased expression of Cu/Zn-superoxide dismutase in *Aspergillus*

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Abstract In a search for iron-regulated proteins of *Aspergillus nidulans* and *Aspergillus fumigatus* a 16-kDa protein was identified which is about 5-fold upregulated during iron starvation in both species and which can be approximately 500-fold enriched by simple one-step chromatography on Amberlite XAD-16 resin. N-terminal protein sequence analysis and cloning of the respective *A. nidulans* cDNA identified this protein as a Cu/Zn-superoxide dismutase (SODA). Northern analysis revealed that upregulation of *sodA* expression occurs at the level of transcript accumulation. This seems to be a specific low iron response and not a general starvation answer since *sodA* transcript levels do not respond to carbon or nitrogen starvation. In contrast, copper depletion leads to transcriptional downregulation of *sodA*. Furthermore, *sodA* expression was found still to be subject to iron regulation in an *A. nidulans* mutant lacking SREA, a regulator of iron homeostasis, indicating that sodA expression is regulated by an SREA-independent mechanism. The data presented suggest that SODA plays a protective role under iron deplete conditions. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Iron regulation; Superoxide dismutase; *Aspergillus nidulans*; *Aspergillus fumigatus*

1. Introduction

Members of the genus *Aspergillus* are found ubiquitous in nature, typically as saprophytes on decaying organic material in soil. At the same time this genus comprises important opportunistic fungal pathogens that cause a wide spectrum of diseases in humans including allergic bronchopulmonary aspergillosis, aspergilloma and disseminated aspergillosis – the latter occurring frequently in immunocompromised patients. *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger* are the most common cause of aspergillosis; *Aspergillus nidulans* is a very much rarer cause of human disease but is genetically well characterized due to its sexual cycle and therefore represents a model ascomycete [1,2]. Various studies suggest that the virulence of an opportunistic fungal pathogen is probably related to its nutritional and physiological versality and that general metabolic pathways like folate biosynthesis can be essential for full pathogenicity of *A. fumigatus* [1,3]. Primarily in *A. fumigatus* a number of potential virulence determinants have now been investigated, e.g. proteases, gliotoxin, hemolysin and phospholipases [1,4,5]. In addition, recent evidence links melanin biosynthesis to the virulence of *A. fumigatus* conidia [6–8]. Loss of this conidial pigment increases the binding ability of complement component C3 to conidia, a process important for phagocytosis of inhaled conidia. Furthermore, melanin is a potent free radical scavenger which may be important in protecting the fungal cell from the direct killing mechanisms used by cells such as neutrophils [9]. Another important line of defense of cells against toxic effects of oxidants with a hypothetical role in virulence are superoxide dismutases (SOD). SOD disproportionate superoxide to hydrogen peroxide and dioxygen and this class of enzyme is found in almost all aerobic organisms [10]. Eukaryotes contain two classes of SOD: Mn-SOD located in the mitochondrial matrix, and Cu/Zn-SOD elsewhere in the cell, most notable in the cytoplasm, nucleus and lysosomes. It has been shown for *Saccharomyces cerevisiae* that Mn-SOD is the primary defense against superoxide anions produced as by-products of respiration [11]. Mutants of the yeast *S. cerevisiae* and the filamentous fungus *Neurospora crassa* lacking Cu/Zn-SOD are sensitive to oxygen and superoxide generating agents (e.g. paraquat) suggesting that this enzyme is responsible for the principal superoxide dismutating activity during oxidative stress [12,13]. Cu/Zn-SOD has been purified from *A. fumigatus, A. flavus, A. niger*, *Aspergillus terreus* and *A. nidulans*. Indirect fluorescent-antibody tests and immunoelectron microscopic studies demonstrated the presence of this enzyme in the cytoplasm, the cell wall of conidia and hyphae, as well as in filtrates of mid-logarithmic-phase *A. fumigatus* cultures [14,15]. Furthermore Cu/Zn-SOD of *A. fumigatus* has been shown to represent a valuable immunodiagnostic marker for *Aspergillus* infections – proving the expression of this enzyme during pathogenic growth [16] – but so far no studies on regulation of its synthesis have been performed.

In this paper we report the identification of CU/Zn-SOD as an iron-regulated protein in *A. nidulans* and *A. fumigatus*. Iron metabolism plays a major role in virulence since microbial pathogens must compete with the iron-withholding defense systems of their host to acquire this essential nutrient [17].

2. Materials and methods

2.1. Strains and growth media

Generally, *A. nidulans* strain A4 (Glasgow wild type provided by the Fungal Genetic Stock Center), the *A. nidulans* SREA-deficient strain SRK01 [18], and *A. fumigatus* ATCC9197 were grown in shake

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culture at 37°C in minimal medium according to Pontecorvo et al. [19] containing 30 mM glutamine as nitrogen source, 2% glucose as carbon source, 10 μM $FeSO_4$ and 10 μM $CuSO_4$. After 24 h growth mycelia were harvested by filtration, washed with cold aqua dest. and frozen in liquid nitrogen. For nitrogen or carbon-starved cultures, mycelia were harvested after 16 h growth, washed and transferred to fresh medium lacking any nitrogen or carbon source, respectively, for 8 h. For preparation of low iron medium, $FeSO_4$ was omitted. Iron-starved growth was confirmed by detection of siderophores which occur in the culture filtrate after 24 h growth in low iron medium, but not under iron-repleted conditions [18]. For preparation of low copper medium, $CuSO_4$ was omitted.

2.2. Purification of Cu/Zn-SOD and N-terminal sequence determination

Cellular extracts were prepared by grinding the harvested mycelia under liquid nitrogen and resuspending the proteins in 50 mM potassium phosphate buffer (pH 7.5). After vigorous vortexing, cell debris was pelleted and the supernatant saved for analysis. The protein content was measured according to Bradford [20] and 3 mg of soluble cellular proteins were loaded onto 1 ml of Amberlite XAD-16 (CWG, Mannheim, Germany) in a Pasteur pipet, previously equilibrated with 50 mM potassium phosphate buffer. Subsequently, the XAD-16 material was washed with 3 ml of 50 mM potassium phosphate buffer and the proteins bound were eluted with 1.5 ml methanol. The methanol was removed by evaporation, proteins were resuspended in 0.3 ml of 50 mM potassium phosphate buffer, and 20 μl were subjected to SDS–PAGE analysis and Coomassie blue staining [21]. N-terminal protein sequencing was performed from proteins blotted onto PVDF membrane as described previously [22].

2.3. Molecular techniques

Isolation of RNA, cDNA synthesis and Northern analysis were performed as described previously [18]. Standard molecular techniques were performed as described by Sambrook et al. [23].

The 5′- and 3′-ends of the *sodA* cDNA were cloned employing the rapid amplification of cDNA ends (RACE) protocol according to Frohman [24]. For the amplification of the *sodA* 3′-cDNA end, the degenerated primer 5′-ATGGTNAARGCNGTNGC was used. For cloning of the 5′-end, total RNA was reverse-transcribed using primer 5′-CTTGGAGCCCTTGGAGTT. Subsequently, PCR amplification was performed using the primer 5′-CTTGGAGCCCTTGGAGTT. The obtained PCR fragments were subcloned into the pGEM-T vector system (Promega, Madison, WI, USA) according to the manufacturer's recommendations. The nucleotide sequence was determined on both strands. The *sodA* cDNA and deduced protein sequences were deposited in GenBank under accession number AF305546.

Hybridization probes labeled with digoxigenin (Boehringer, Mannheim, Germany) for Northern analysis were generated by PCR using oligonucleotides 5′-GCACCGTCACCTTTGAAC and 5′-CTTGGAGCCCTTGGAGTT for *sodA*, 5′-TTTCGAGTCGCTAGGCT and 5′-TCCGTCCTCTCCCCTTT for *sreA*, and 5′-CGGTGATGAGGCACAGT and 5′-CGGACGTCGACATCACA for *acnA* (M22869).

3. Results and discussion

3.1. Cu/Zn-SOD is upregulated during iron starvation at the protein level in A. nidulans and A. fumigatus

In a search for iron-regulated proteins of *A. nidulans* a 16-kDa protein was identified in cellular extracts which is significantly upregulated during iron starvation. This protein can be about 500-fold enriched and purified to more than 90% homogeneity, as judged by SDS–PAGE and Coomassie blue staining, by simple one-step chromatography on Amberlite XAD-16 resin. This material was previously shown to adsorb a variety of lipophilic compounds, e.g. siderophores [25]. After 24 h growth under iron deplete conditions, this 16-kDa protein, is approximately 5-fold upregulated, as judged by densitometric analysis (Fig. 1). This ratio was confirmed by N-terminal amino acid sequence determination via calculation of the initial yield; the amino acid sequence determined was VKAVAVLRGDSKVSGTVTFE.

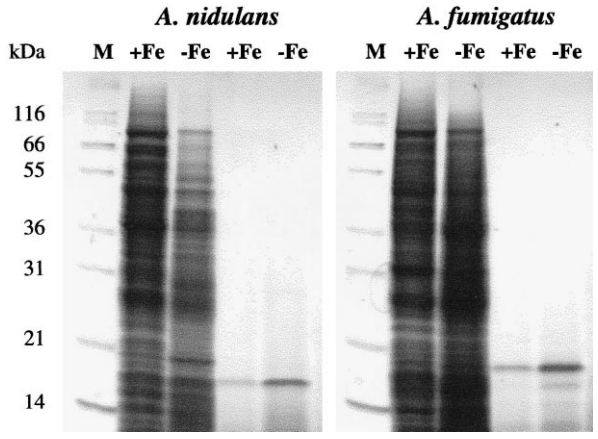

Fig. 1. Purification of SODA from *A. nidulans* and *A. fumigatus* mycelia grown under iron replete (+Fe) and iron deplete (−Fe) conditions. Samples contained 40 μg of total soluble cellular protein extract and 6.7% of Amberlite XAD-16-bound protein loaded with 3 mg extract. Proteins were fractionated by SDS–PAGE and visualized by Coomassie blue staining. Lane M is a molecular mass marker. Results are representative for at least three different experiments.

Searches in several databases applying the BLAST alignment computer program displayed significant similarity to the N-termini of various Cu/Zn-SOD. Application of the same purification protocol to extracts of *A. fumigatus* confirmed the same iron-dependent regulation of Cu/Zn-SOD suggesting that this is a general mode of control in *Aspergillus* and not specific for *A. nidulans* (Fig. 1). The identity of the *A. fumigatus* 16-kDa protein with Cu/Zn-SOD was again confirmed by N-terminal sequencing yielding the published amino acid sequence [15]. Consistently, the Cu/Zn-SOD of all fungal species analyzed so far lack the start methionine, making valine the mature N-terminus [15]. According to the 'N-end rule' these enzymes are therefore highly stable proteins [26].

In contrast to the time consuming purification process commonly used [15,16], the described chromatography on XAD-16 represents a novel and simple protocol for Cu/Zn-SOD enrichment from *Aspergillus* and due to the high degree of structural conservation of this enzyme probably also from other species.

3.2. Cloning and analysis of the A. nidulans Cu/Zn-SOD cDNA (sodA)

To confirm that this 16-kDa protein is indeed the Cu/Zn-SOD of *A. nidulans*, the respective cDNA was PCR-amplified employing the 5′- and 3′-RACE protocol according to Frohman [24]. The deduced amino acid sequence with a predicted molecular mass of 16 kDa, termed SODA, displays significant similarity to Cu/Zn-SOD of various species, e.g. 90% identity to *A. fumigatus* (AF128886), 76% to *Candida albicans* (AF046872), 74% to *N. crassa* (M58687), and 73% to *S. cerevisiae* (J03279). Interestingly, despite the high degree of identity of SODA from *A. nidulans* and *A. fumigatus* (Fig. 2), the *A. fumigatus* enzyme was shown to be more resistant to heat inactivation [15].

The polyadenylation site is identical to the ones found in the EST clones n3e06a1.f1 and e9d06a1.f1 [27] revealing a 3′ untranslated region of 159 nucleotides. The 5′ untranslated region is 53 nucleotides in length.

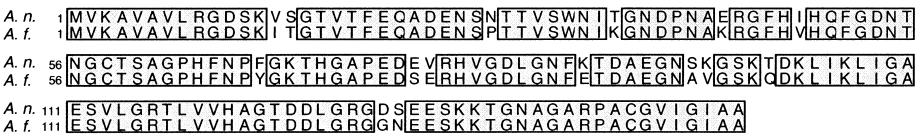

Fig. 2. Comparison of the amino acid sequences of SODA from *A. nidulans* and *A. fumigatus*. Common residues are boxed. *A.n.*, *A. nidulans*; *A.f.*, *A. fumigatus*.

3.3. *sodA is upregulated during iron depletion and downregulated during copper starvation at the level of transcript accumulation*

Northern analysis displayed that upregulation of the SODA protein level during iron starvation is due to increased *sodA* steady-state transcript levels (Fig. 3A). Since *sodA* transcript levels do not respond to carbon or nitrogen starvation (Fig. 3A), the upregulation in response to low iron conditions seems to be a specific effect and not a general starvation response. In contrast, copper depletion leads to transcriptional downregulation of *sodA* (Fig. 3A). Decrease of Cu/Zn-SOD at the protein and transcript level in copper-starved cells has previously been observed in *S. cerevisiae* [28]. Addition of copper to the culture medium of such cells results in transcriptional induction of Cu/Zn-SOD and this regulation is mediated by ACE1, a copper-sensing transcription factor [29]. The obvious rationale for copper-dependent control of Cu/Zn-SOD expression is that the apoprotein lacks its enzymatic activity without its metal cofactors. Generally, copper-mediated regulation of sodA appears to function similarly in *A. nidulans* and yeast.

To our knowledge this is the first description of upregulation of Cu/Zn-SOD in response to iron starvation in any eukaryote. Iron is thought to exert a toxic effect on cells by catalyzing the formation of hydroxyl radicals from hydrogen peroxide via Fenton chemistry [10]. In vitro, and possibly in vivo as well, superoxide can be involved in this reaction as the reducing agent for the iron. Consequently, SOD action would be of higher importance under iron replete conditions which contradicts our results of Cu/Zn-SOD upregulation during iron starvation. Recently, a more likely mechanism of superoxide toxicity was proposed based on the observation that superoxide can very specifically oxidize exposed [4Fe–4S] clusters of dehydratases like aconitase or fumarase [10]. Subsequently, this leads to inactivation of the enzymes and release of the iron. In this model superoxide is (i) directly involved in enzyme inactivation and (ii) indirectly involved in oxidative damage via Fenton chemistry through the released iron. This model is supported by a vast body of evidence, e.g. if Fenton chemistry was primarily responsible for superoxide toxicity addition of iron to SOD-deficient cells would be expected to be harmful – but on the contrary it was found to be beneficial to *S. cerevisiae* and *E. coli* strains probably via reconstitution of the [4Fe–4S] clusters [30,31]. The latter model also better explains our observation: iron starvation endangers the integrity of [4Fe–4S] clusters and the activity of the respective enzymes since their reactivation is slow. Consequently, the rationale for upregulation of Cu/Zn-SOD under this condition is the protection of this class of prosthetic groups and the respective enzyme activities.

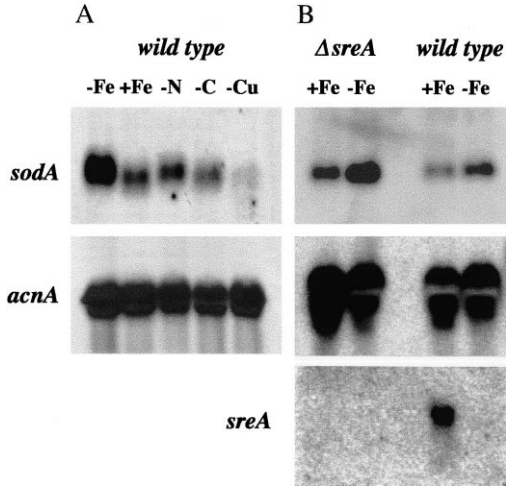

Fig. 3. Northern analysis of *sodA* expression in *A. nidulans* under different growth conditions (A) and comparison between the *A. nidulans* wild type and the Δ*sreA* strain (B). Each sample contained 20 μg total RNA isolated from *A. nidulans* mycelia grown under different growth conditions: standard minimal medium (+Fe), iron starvation (−Fe), nitrogen starvation (−N) carbon starvation (−C), and copper starvation (−Cu). After fractionation in agarose-formaldehyde gels and transfer to nylon membrane, hybridizations were performed with a *sodA* specific probe and a γ-actin (*acnA*) probe as a control for loading and RNA quality. The hybridization with the *sreA* probe serves for confirmation of the strains and the iron supply – *sreA* transcription is confined to iron replete conditions [18]. Results are representative for at least three different experiments.

Coupling of SOD induction to low iron conditions is not completely new. Many prokaryotes, like *E. coli*, contain three types of SOD: a periplasmic Cu/Zn-SOD, the cytosolic Fe-SOD, and Mn-SOD. Fe-SOD is downregulated during iron starvation whereas Mn-SOD is upregulated during iron starvation in a FUR-dependent manner [10,32]. Upregulation of Mn-SOD under low iron conditions again seems to counteract inactivation of [4Fe–4S]-cluster-containing enzymes. In addition it was suggested that Mn-SOD induction upon iron starvation occurs in order to prevent effects of iron overload upon return to high iron concentrations [28].

3.4. *Regulation of sodA expression in an A. nidulans sreA-deletion strain*

In *E. coli* iron regulation of Mn-SOD expression is mediated by FUR which is a negative regulator of genes involved in high affinity iron uptake. FUR-deficiency leads to derepression of siderophore biosynthesis, iron uptake and Mn-SOD expression under sufficient iron supply [33,34]. Recently, a regulatory protein, with a similar mode of action was identified in *A. nidulans*: loss of the GATA factor SREA leads to derepression of siderophore biosynthesis as well as iron uptake in *Aspergillus* under iron replete conditions [18]. Northern analysis revealed that the *sodA* transcript level is still subject to iron availability in the Δ*sreA*-mutant indicating that another regulatory mechanism must exist. In this respect it is noteworthy that not all iron regulatory effects can be ascribed to SREA in *A. nidulans*, e.g. derepression of siderophore biosynthesis is only partial in the SREA loss of function

strain [18]. In contrast, FUR represents the general control element of iron metabolism in *E. coli* [33]. Nevertheless, according to densitometric analysis of the Northern blot and comparison to γ-actin expression, *sodA* transcript levels are increased approximately 4-fold during iron deplete conditions and 3-fold during growth in iron replete conditions in the *sreA*-deletion mutant. (Fig. 3B). These data are consistent with SREA representing a repressor for *sodA* expression, albeit it is not clear if SREA affects *sodA* expression directly or indirectly. Deletion of *sreA* results in increased iron uptake [18] and consequently the general disturbance of iron metabolism could be the reason for upregulation of *sodA*.

sodA represents the first identified *Aspergillus* gene induced by iron starvation. Since iron is tightly sequestered by high-affinity iron-binding proteins in mammalian hosts, e.g. transferrin, ferritin or lactoferrin, resulting in low concentrations of free iron, it is anticipated that SodA plays an important role during *Aspergillus* infections and is highly expressed. This is consistent with the occurrence of specific antibodies against Cu/Zn-SOD in the sera of patients suffering from different types of diseases caused by *Aspergillus* [16].

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