A Eukaryote without Catalase-Containing Microbodies: *Neurospora crassa* Exhibits a Unique Cellular Distribution of Its Four Catalases†

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Microbodies usually house catalase to decompose hydrogen peroxide generated within the organelle by the action of various oxidases. Here we have analyzed whether peroxisomes (i.e., catalase-containing microbodies) exist in *Neurospora crassa*. Three distinct catalase isoforms were identified by native catalase activity gels under various peroxisome-inducing conditions. Subcellular fractionation by density gradient centrifugation revealed that most of the spectrophotometrically measured activity was present in the light upper fractions, with an additional small peak coinciding with the peak fractions of HEX-1, the marker protein for Woronin bodies, a compartment related to the microbody family. However, neither in-gel assays nor monospecific antibodies generated against the three purified catalases detected the enzymes in any dense organelar fraction. Furthermore, staining of an *N. crassa* wild-type strain with 3,3′-diaminobenzidine and H₂O₂ did not lead to catalase-dependent reaction products within microbodies. Nonetheless, *N. crassa* does possess a gene (cat-4) whose product is most similar to the peroxisomal type of monofunctional catalases. This novel protein indeed exhibited catalase activity, but was not localized to microbodies either. We conclude that *N. crassa* lacks catalase-containing peroxisomes, a characteristic that is probably restricted to a few filamentous fungi that produce little hydrogen peroxide within microbodies.

Microbodies are nearly ubiquitous organelles of the eukaryotic cell. They usually house a number of hydrogen peroxide-producing oxidases as well as catalase, which quickly removes the hydrogen peroxide generated within microbodies (10). Proteins are targeted to the lumen of microbodies by either of two peroxisomal targeting signals (PTS) (19, 48). The predominant signal is the PTS1, a tripeptide located at the C terminus and composed of the amino acids SKL or conservative variants thereof (16, 30). Depending on species, cell type, or developmental state, distinct types of microbodies can be prevalent, which emerge upon differential protein import. The various types are termed according to their marker enzyme content, such as peroxisomes, glyoxysomes, glyosomes, or Woronin bodies (4, 24). Remarkably, filamentous ascomycetes harbor at least two distinct types of microbodies within a single cell: (i) microbodies with a metabolic function (peroxisomes or glyoxysomes), which house the key enzymes of the glyoxylate cycle and a complete fatty acid β-oxidation system; and (ii) the Woronin body, which is required to seal septal pores after hyphal wounding. The Woronin body was identified as a microbody-like organelle because an anti-SKL antibody specifically recognized the dominant protein of this organelle (24). This protein was recently identified as HEX-1 (21, 49). HEX-1 indeed harbors the PTS1 sequence SRL, aggregates within the Woronin body, and gives rise to the typical hexagonal shape of this specialized organelle.

Interestingly, glyoxysomes of the filamentous fungus *Neurospora crassa* were reported to lack catalase activity. Instead, catalase activity was detected in organelles with higher density than glyoxysomes (25, 53). Further support for the existence of such an additional microbody-like compartment was provided by Wanner and Theimer (53), who subjected the *N. crassa* slime mutant, which lacks a rigid cell wall, to 3,3′-diaminobenzidine (DAB) staining. The DAB reaction product that is generated upon catalase-dependent hydrogen peroxide decomposition was absent from glyoxysomes but was found in crescent-shaped structures in close proximity to vacuoles. However, in the reports mentioned, the identity of this catalase-containing organelle remained elusive. Notably, in a more recent report, catalase activity was detected in Woronin body-enriched fractions (49). Since in sucrose density gradients the Woronin body sediments at a significantly higher density than glyoxysomes, the Woronin body might in fact represent the catalase-containing organelle described above. On the other hand, Woronin bodies are not associated with vacuoles and their hexagonal shape does not resemble the prolate structures seen by Wanner and Theimer (53).

Three catalases have been described in *N. crassa*: catalase 1 (CAT-1) and catalase 3 represent the typical large monofunctional catalases, whereas catalase 2 is a member of the catalase-peroxidase family and is possibly derived from a bacterial enzyme. All three isozymes are present throughout the *N. crassa* asexual life cycle, albeit to varying levels: CAT-1 is highly abundant in conidia, CAT-2 is mainly found in aerial hyphae and conidia (37), and CAT-3 activity increases during exponential growth and is induced under various stress condi-
tions (6, 33). Subcellular localization of the *N. crassa* catalases has not been thoroughly studied. Evidence exists that CAT-3 is processed and secreted; however, since only a little extracellular CAT-3 activity has been found, it has been suggested that most of the enzyme is either bound to the cell wall or remains within the cell (34). Completion of the *N. crassa* genome (14) revealed a fourth putative catalase that belongs to the family of small-subunit monofunctional catalases and is most similar to peroxisomal catalases of animals and yeasts (22). Thus, current knowledge is commensurate with the existence of a peroxisomal compartment in *N. crassa* that is distinct from glyoxysomes. To clarify whether or not peroxisomes exist in *N. crassa*, we have thoroughly analyzed catalase activities under peroxisome-inducing conditions. Neither cytochemistry nor catalase activity gels supported the existence of a microbody-associated catalase. Likewise, the application of antibodies against the three characterized catalase isozymes failed to detect a luminal catalase. Finally, characterization of the novel CAT-4 revealed that this protein is a bona fide catalase; however, this protein is not targeted to organelles. The impact of our finding of an eu-karyote devoid of peroxisomal catalase is discussed.

MATERIALS AND METHODS

Strains and culture conditions. The *Neurospora crassa* wild-type strains St. Lawrence 74-ORS-1a (FGSC#988) and 74-ORS23-1A (FGSC#987) were used for all biochemical experiments of this work. Strains NC15 and NC21 were generated by integrating the expression constructs MF272 (green fluorescent protein [GFP] expression) (13) and CW20 (GFP-CAT-4), respectively, into the his-3 locus of strain NC23 (FGSC#6103) by homologous recombination, followed by a screening of prototrophic His+ transformants for expression of GFP by immunoblotting. Strain NC23 was similarly generated by integrating plasmid pCW22 (CAT-4) into strain NC23 and screening for expression of CAT-4. Wild-type *Aspergillus tamarii* (DSM 825; ATCC 10836) was obtained from DSMZ, Braunschweig, Germany. Strains were maintained on Vogel's medium N supplemented with 2% sucrose or, for the induction of microbodies, 1 mM oleic acid plus 1% (vol/vol) Tergitol, 40 mM acetate, or 1% (vol/vol) ethanol. All manipulations were performed at 4°C. All procedures were carried out at 4°C. For subcellular fractionation, cultures were inoculated with conidia (10⁵/ml) in Vogel's minimal medium and were shaken (100 rpm) for 20 min. For the isolation of CAT-1 by immunoprecipitation, 30 mg of a postorganellar supernatant was collected and centrifuged for 20 min at 40,000 g and subjected to a SDS-PAGE analysis. For subcellular fractionation, cultures were incubated with conidia (10⁵/ml) in Vogel’s minimal medium and were shaken (100 rpm) for 20 h before shifting hyphae to the various peroxisome-inducing conditions. Hyphae were harvested by filtration, washed with water, mixed with 1 g wet weight quartz sand and 4 volumes of isolation buffer (150 mM Tricine, pH 7.4, 0.44 M sucrose, 10 mM KCl, 5 mM MgCl₂, 1 mM EDTA) and, ground with a pestle in a mortar at 4°C. The homogenate was squeezed through four layers of cheesecloth and subjected to centrifugation at 2,500 × g for 5 min. The resulting supernatant was taken as crude extract.

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For differential centrifugation, 10 ml of crude extract was separated in a pellet and supernatant fraction through centrifugation at 25,000 × g for 20 min. For density gradient centrifugation, 10 ml of crude extract was layered on top of a
Enzyme assays. Catalase (EC 1.11.1.6), urate oxidase (EC 1.7.3.3), isocitrate lyase (EC 4.1.3.1), enoyl-CoA hydratase (EC 4.2.1.17), and 3-hydroxyacyl-CoA dehydratase (EC 1.1.1.35) activities of MFP, fumarase (EC 4.2.1.2), and cytochrome c oxidase (1.9.3.1) were assayed by established procedures (25). The catalase activity of CAT-4 was similarly assayed, with the exception that various amounts of NADPH were added to the assay mixture. In-gel catalase activity assays were carried out as described by Woodbury et al. (55). In brief, samples were separated on native polyacrylamide gels, which were then incubated with 0.1% H2O2 for 10 min, washed twice with water, and treated with a solution containing 1% FeCl3 and 1% K3(Fe(CN)6). Upon emergence of the unstained catalase bands, reactions were stopped by washing the gels with water.

Fluorescence microscopy. Live yeast cells were analyzed for GFP and DsRed fluorescence as described previously (41). N. crassa mycelia were similarly prepared. After overnight growth in Vogel’s minimal medium, hyphae were harvested and grown overnight in induction medium (1× Vogel’s salt, 0.05% [wt/vol] Tween 40, 0.1% [wt/vol] oleic acid) at 30°C. For inspection, a suspension of mycelia was placed on a slide, mixed with an equal volume of 1% [wt/vol] low-melting-point agarose in H2O, and sealed with a coverslip. All micrographs were recorded on a Zeiss Axioplan 2 microscope with a Zeiss Plan-Apochromat x100/1.4 oil objective and an Axiosem MR digital camera and were processed with AxioVision 4.2 software (Zeiss, Jena, Germany).

Electron microscopy. For overall cell morphology, cells were fixed in 1.5% KMnO4, for 20 min, poststained in 0.5% uranyl acetate, subsequently dehydrated via an ethanol series, and embedded in Epon 812. For detection of catalase activity, cells were prefixed in 3% glutaraldehyde in 0.1 M cacodylate buffer. Catalase activity was detected with DAB and 0.086% hydrogen peroxide as described before (52). For immunocytochemistry, the glutaraldehyde-fixed cells were embedded in Unicryl; ultrathin sections were incubated with specific anti-CAT-1 antibodies and gold-conjugated goat anti-rabbit antiserum (54).

RESULTS

Expression of catalases under peroxisome-inducing conditions. To analyze whether catalase activity is induced under conditions that require microbody-borne metabolism, wild-type cells were grown for 12 h in media containing ethanol (Fig. 1, lane 1), acetate (lane 2), or oleic acid (lane 3) as the sole carbon source. Under these conditions, the glyoxylate cycle enzymes and, in the last case, the β-oxidation enzymes are induced (25). In addition, media with β-methionine (lane 10), which induces the expression of β-amino acid oxidase (46), as well as media with uric acid (lane 9), which increases the levels of urate oxidase (36), were tested for coinduction of catalase. Total catalase activity did not increase significantly under any of these conditions relative to that in control cells that were grown for 24 h in medium with sucrose (lane 5) as the sole carbon source (Fig. 1A). In contrast, stationary-phase cells that had been grown for 96 h in sucrose medium (lane 8) exhibited a sevenfold increase in activity, as observed previously (6, 33). Since three differentially regulated catalase isozymes are described in N. crassa, the contribution of each enzyme to the total catalase activity was visualized on native acrylamide gels that had been loaded with equal amounts of enzyme activities. All three enzymes were clearly discernible under all conditions (Fig. 1B). More CAT-2 was observed in ethanol- and acetate-grown cells, and as expected, CAT-3 was predominant in cells grown to the stationary phase (33). Beyond that we did not obtain evidence for an additional catalase isozyme under all conditions tested.

Intracellular distribution of catalase activity. To determine the intracellular distribution of catalase activities in an oleic acid-induced N. crassa wild-type strain, a postnuclear supernatant devoid of cell debris and nuclei was separated on a continuous sucrose density gradient (30 to 60%). The resulting fractions were assayed for the mitochondrial marker enzymes fumarase and cytochrome c oxidase as well as for the glyoxysomal marker enzymes isocitrate lyase and the multifunctional β-oxidation enzyme (Fig. 2A). Peak Woronin body fractions were identified immunologically by using an anti-HEX-1 antibody (Fig. 2B). The organelles were separated well, with mitochondria being identified at a density of 1.19 g/cm3, glyoxysomes at 1.21 g/cm3, and Woronin bodies at 1.26 g/cm3. During cell breakage, a fraction of organelles always gets ruptured, particularly the mechanically and osmotically fragile microbodies (25). Portions of the glyoxysomal and mitochondrial matrix enzymes were therefore also found in the lighter fractions of the gradient where soluble proteins and possibly organelar remnants are recovered. Most of the measurable catalase activity was found in the soluble fractions and was entirely absent from mitochondria and glyoxysomes. Nonetheless, a small but significant fraction of catalase, estimated to account for 5% of total activity, cosedimented with the Woronin body fractions. A similar catalase distribution was obtained when cells were
grown on acetate or sucrose as the sole carbon source (not shown). At the same time, no isocitrate lyase or fumarase activity could be measured in these fractions. We also tested the distribution of urate oxidase activity as this enzyme was reported to cosediment with catalase in the slime mutant (53). However, urate oxidase was exclusively found in the upper soluble fractions, indicating that urate oxidase is a soluble enzyme in *N. crassa*.

To analyze which of the catalase isozymes caused the observed catalase activity in Woronin bodies, fractions of a similar sucrose gradient were subjected to an in-gel catalase activity assay. Surprisingly, all three isozymes were only detected in the soluble fractions, although activity was clearly measurable also in the dense fractions (Fig. 3). Loading samples from the dense and soluble fractions that contained equal catalase activities (as measured with the liquid assay) also failed to reveal any catalase activity with the in-gel assay. These data were interpreted to mean that the measured consumption of
hydrogen peroxide in the Woronin body fractions was not due to a catalase activity, albeit we could not entirely rule out that the sensitivity of the in-gel assay was too low to detect organelar catalase.

In an alternative approach, eventual organellar catalase activity was assayed cytochemically. Prefixed cells that had been induced by oleic acid were treated with \( \text{H}_2\text{O}_2 \) and DAB, and ultrathin sections thereof were subjected to electron microscopy. Neither glyoxysomes nor Woronin bodies contained a clear DAB reaction product that is formed in the presence of catalase activity (Fig. 4). Significant reaction products were only obtained for mitochondrial cristae, most likely caused by an intramitochondrial peroxidase reaction (39). Notably, the DAB-positive organelles adjoined to vacuoles as described for the \( \text{N. crassa} \) slime mutant were never observed, even when serial ultrathin sections of a cell were inspected (see Fig. S1 in the supplemental material). Thus, cytochemistry did not provide conclusive evidence for a compartmentalized catalase.

**Purification and immunological detection of the catalase isozymes.** To detect the catalase proteins in situ, we set out to produce antibodies against the three isozymes. To that end, the three catalase activities were purified, starting with extracts from acetate (CAT-2)- or sucrose (CAT-1, CAT-3)-grown wild-type cells. For CAT-1, a previously established protocol was used (20). The various purification steps for CAT-2 and CAT-3 are summarized in Table 1. The purity of the enzymes was analyzed by denaturing SDS-PAGE (Fig. 5A) as well as by an in-gel catalase assay (Fig. 5B). One major band was seen for catalases 1 and 3 in the Coomassie-stained gel; the purified catalase 2 sample contained one additional protein of approximately 46 kDa. Nonetheless, since the activity assay revealed that each catalase gave rise to only one \( \text{H}_2\text{O}_2 \)-reactive band, the samples were considered sufficiently pure to immunize rabbits. Tests for the specificity of the resulting antisera showed that all antibodies were monospecific. They did not significantly cross-react with the other purified catalases (Fig. 5C), and the antisera against CAT-2 and CAT-3 each gave rise to a single band at the expected sizes of 83 kDa and 79 kDa, respectively, in a Western blot performed with crude extract from oleic acid-induced wild-type mycelium (Fig. 5D). The specificity of the CAT-1 antiserum was further proved by an immunoprecipitation experiment. Incubation of crude wild-type extract with increasing amounts of antiserum led to depletion of isoform 1, but not of CAT-2 and CAT-3, in the unbound fraction (Fig. 5E).

There is some ambiguity in the literature regarding the nomenclature of the three catalase isozymes. Originally described by Chary and Natvig (6), the catalase running most slowly in the native polyacrylamide gel was designated as CAT-3, the slightly faster running catalase as CAT-1, and the one with the highest mobility as CAT-2, the latter of which clearly represents the catalase-peroxidase family member (NCU05770.2).

![FIG. 4. In situ catalase staining. Ultrathin sections of fixed oleic acid-grown \( \text{N. crassa} \) hyphae were stained with DAB and \( \text{H}_2\text{O}_2 \) for catalase activity. Visualization by electron microscopy revealed that only mitochondrial cristae showed an electron-dense reaction product.](image)

**TABLE 1. Purification of three \( \text{N. crassa} \) catalases**

| Fraction                        | Amt of protein (mg) | Total activity (mkat) | Sp act (mkat/mg) | Yield (%) | Fold purified | In-gel catalase activity* |
|--------------------------------|---------------------|-----------------------|------------------|-----------|--------------|--------------------------|
| Purification of CAT-3 and CAT-2 |                     |                       |                  |           |              |                          |
| Crude extract                   | 780                 | 1.32                  | 1.7              | 100       | 1            | CAT-1, CAT-2, CAT-3      |
| P11/Blue-Sepharose flowthrough  | 191                 | 1.14                  | 6                | 86.5      | 3.5          | CAT-1, CAT-2, CAT-3      |
| DE52 pH 6.7 eluate              | 24.3                | 0.35                  | 14.4             | 26.5      | 8.5          | CAT-3                   |
| HA eluate                       | 1.03                | 0.26                  | 252              | 19.7      | 148          | CAT-3                   |
| S300 peak fraction pool         | 0.3                 | 0.17                  | 567              | 12.9      | 334          | CAT-3                   |
| DE52 pH 6.7 flowthrough         | 80.6                | 0.62                  | 7.7              | 47        | 4.5          | CAT-1, CAT-2 (CAT-3)    |
| DE52 pH 7.5 eluate              | 5                   | 0.03                  | 5.6              | 2.1       | 3.3          | CAT-1, CAT-2            |
| S300 peak fraction pool         | 1                   | 0.02                  | 21               | 1.6       | 12.4         | CAT-2                   |
| Purification of CAT-1           |                     |                       |                  |           |              |                          |
| Crude extract                   | 2,037               | 0.84                  | 0.4              | 100       | 1            | CAT-1, CAT-2, CAT-3      |
| Ammonium sulfate fractionation  | 180                 | 0.30                  | 1.7              | 35        | 4.2          | CAT-1 (CAT-3)           |
| DE52 eluate                     | 6.8                 | 0.27                  | 39.7             | 32        | 101.2        | CAT-1                   |
| S300 peak fraction pool         | 2.0                 | 0.18                  | 91.7             | 22        | 229.2        | CAT-1                   |

* Parentheses indicate residual activity (presence in low amounts).
More recently, a **cat-3** knockout strain (NCU00355.2) was described by the Hansberg laboratory that surprisingly lacked the catalase band of intermediate mobility (34), which would be referred to as CAT-1 according to the Chary and Natvig nomenclature (6). To therefore find out at the molecular level which protein is represented by the most slowly migrating catalase, the dominant immunoprecipitated protein of approximately 85 kDa that remained bound to the antibody after stringent washing was digested with trypsin and analyzed by ESI-MS. Gene product NCU08791.2 (**N. crassa** genome release 7 at the Broad Institute) could be clearly assigned to this sample (data not shown). Since this reading frame is identical to catalase 1 (GenBank accession no. AY027545), it became obvious that the catalase that was formerly known as isozyme 3 (6) is now termed CAT-1 and vice versa. We therefore also chose the nomenclature that was in line with the GenBank entries for CAT-1 and CAT-3.

Application of anti-CAT-1 antibodies to thin sections of oleic acid-induced **N. crassa** cells revealed that subcellular compartments were not significantly labeled. CAT-1 was rather concentrated at the cell wall (Fig. 6), suggesting that a portion of CAT-1 is secreted. This observation is reminiscent of CAT-3, which is also secreted, yet only small percentage of CAT-3 is lost to the medium (34). The antibodies were also used to determine the distribution of the catalases within a sucrose density gradient. CAT-2 and CAT-3 did not enter the gradient.

**FIG. 5.** Immunologic analysis of three purified catalase isoenzymes from **N. crassa**. (A and B) Determination of purity. For each isoenzyme, the gel filtration fraction with the highest specific catalase activity was analyzed by denaturing (A) and non-denaturing (B) PAGE. Catalases were visualized by Coomassie (A) and in-gel activity (B) staining, respectively. The sizes as predicted by the genome sequence are as follows: CAT-1, 85.5 kDa; CAT-2, 83.4 kDa; CAT-3, 79.2 kDa. (C to E) Specificity of antisera directed against the individual purified catalases. (C) Equal amounts (5 μg) of purified catalase isoenzymes were subjected to Western blot analysis. Primary rabbit antisera were used at dilutions of 1:1,000 and in combination with alkaline phosphatase-conjugated goat anti-rabbit antibodies (α-CAT-1, α-CAT-2, and α-CAT-3). (D) Crude extract (10 μg) from oleic acid-induced wild-type mycelium was analyzed by Western blotting using anti-CAT-2 and anti-CAT-3 antibodies (dilution of 1:20,000). (E) Immunoprecipitation of CAT-1. Increasing amounts of CAT-1 antiserum or preimmune serum were added to crude extract (200 μkat catalase activity), incubated for 1 h, and subjected to centrifugation. The soluble fraction was analyzed for the presence of catalase isoenzymes by an in-gel activity assay.

**FIG. 6.** Immunocytochemical localization of CAT-1. Ultrathin sections of glutaraldehyde-fixed mycelia of **N. crassa** that had been grown on sucrose-containing medium were decorated with anti-CAT-1 antibodies and gold-conjugated goat anti-rabbit antiserum. G, glyoxysomes; Lb, lipid bodies; M, mitochondria; V, vacuole; Wb, Woronin bodies.
Characterization of a novel \textit{N. crassa} catalase. We and others (22) have searched the \textit{N. crassa} genome sequence for the presence of catalase isozymes. Interestingly, there is indeed a fourth open reading frame with strong similarity to that for the catalases, NCU05169.2, hereafter referred to as \textit{cat-4} (Fig. 8A). Since under various growth conditions and developmental states only three distinct activities were detectable in native gels (Fig. 1), there was no evidence for a fourth catalase being produced in \textit{N. crassa}. To determine experimentally whether this reading frame encodes a true catalase, we set out to amplify \textit{cat-4} from a cDNA library (FGSC, Kansas City, MO). However, this approach failed, probably due to a (very) low expression rate of this reading frame. We therefore amplified \textit{cat-4} from genomic DNA by using a primer pair that allowed synthesis of the intron-less gene (see Materials and Methods for details). \textit{cat-4} was then cloned into an expression vector designed to heterologously express \textit{N. crassa} \textit{CAT-4} in \textit{S. cerevisiae}. Since the endogenous yeast catalases are completely repressed in the presence of 2% glucose (8, 40), catalase activity eventually measured under such conditions in the transformants must stem from the expressed \textit{cat-4} gene.

A high catalase activity (461 ± 41 U/mg protein) was indeed measured in the wild-type \textit{S. cerevisiae} strain expressing \textit{CAT-4}, whereas the wild-type strain transformed with the empty vector control exhibited only very low catalase activity (8 ± 0.7 U/mg protein). Endogenous catalase activity (218 ± 11 U/mg protein) was measured in the latter strain upon induction by oleic acid (Fig. 8B). Expression of the \textit{cat-4} gene in a yeast strain devoid of Cta1p and Ctt1p, its endogenous two catalases, gave rise to similar catalase activities (578 ± 85 U/mg protein), thereby demonstrating that \textit{CAT-4} is a bona fide catalase (Fig. 8B). The observed rapid decrease in \textit{CAT-4} activity was prevented by the addition of NADPH (Fig. 8C), indicating that NADPH effectively protected \textit{CAT-4} against inactivation by its substrate, H$_2$O$_2$, as has been reported for other catalases (26). To see whether our highly specific antibody against \textit{S. cerevisiae} peroxisomal catalase A (Cta1p) recognizes \textit{CAT-4}, the same protein extracts were analyzed by immunoblotting. A band of the expected size for \textit{CAT-4} was clearly visible in strains harboring the \textit{cat-4} expression construct, but not in the empty vector control strains (Fig. 8D). At the same time, antibodies against the \textit{N. crassa} catalases \textit{CAT-1}, \textit{CAT-2}, and \textit{CAT-3} failed to detect \textit{CAT-4} (not shown). Crude protein extracts from \textit{N. crassa} wild-type mycelia grown on Vogel’s sucrose medium or oleic acid-containing medium were also analyzed for the presence of \textit{CAT-4}. The yeast Cta1p antibody did not recognize any protein in these samples (Fig. 8D) or in the gradient fractions (Fig. 7B), indicating that \textit{CAT-4} is not expressed or is only very weakly expressed in \textit{N. crassa} under the tested conditions.

A phylogenetic analysis of catalases conducted by Johnson et al. (22) revealed that \textit{CAT-4} belongs to the family of small monofunctional catalases that are found in bacteria, animals, and fungi. Since this clade includes all known peroxisomal catalases, it was tempting to assume that \textit{CAT-4} is also a peroxisomal protein. Most peroxisomal catalases harbor a C-terminal peroxisomal targeting signal (PTS1) (15, 28, 38). However, the three C-terminal amino acid residues of \textit{CAT-4} are all acidic (DDE) and drop out of representing a peroxisomal protein. Most peroxisomal catalases harbor a C-terminal peroxisomal targeting signal (PTS1) (15, 28, 38). However, the three C-terminal amino acid residues of \textit{CAT-4} are all acidic (DDE) and drop out of representing a peroxisomal protein. Since this clade includes all known peroxisomal catalases, it was tempting to assume that \textit{CAT-4} is also a peroxisomal protein. Most peroxisomal catalases harbor a C-terminal peroxisomal targeting signal (PTS1) (15, 28, 38). However, the three C-terminal amino acid residues of \textit{CAT-4} are all acidic (DDE) and drop out of representing a peroxisomal protein. Since this clade includes all known peroxisomal catalases, it was tempting to assume that \textit{CAT-4} is also a peroxisomal protein. Most peroxisomal catalases harbor a C-terminal peroxisomal targeting signal (PTS1) (15, 28, 38). However, the three C-terminal amino acid residues of \textit{CAT-4} are all acidic (DDE) and drop out of representing a peroxisomal protein.
FIG. 8. CAT-4 is a novel catalase from *N. crassa*. (A) Sequence alignment of NcCAT-4 (NCU05169.2) with *S. cerevisiae* Cta1p (ScCta1p). The amino acid sequences were aligned using CLUSTALW with its default parameters (http://www.ebi.ac.uk/clustalw/). Asterisks denote identical residues, double dots (:) and single dots denote positions with conserved and semiconserved substitutions, respectively (7). The consensus signatures for protoheme binding and the proximal active site (PROSITE; http://ca.expasy.org/prosite/) as well as the seven-element fingerprint of the catalase protein family (http://umber.sbs.man.ac.uk/dbbrowser/sprint/) are highlighted. The targeting signal of Cta1p according to Kragler et al. (28) is also denoted (PTS1). (B) Catalase enzyme activity assays. *S. cerevisiae* wild-type strain UTL7-A and the catalase-less mutant strain GA1-7D ctt1/H9004 cta1/H9004 were transformed with a plasmid designed to heterologously express CAT-4 from the constitutive PGK1 promoter (CAT-4) or as control with the empty vector (vector). Strains were grown in the presence of 2% glucose or 0.1% oleic acid as indicated in the legend and assayed for catalase activity. Endogenous yeast catalase activity was only measurable in oleic acid-induced cells (18), whereas CAT-4 was active in glucose-grown wild-type (~) and cta1/H9004 ctt1/H9004 mutant (E) cells. (C) Protection of CAT-4 by NADPH. Catalase activity was determined as described for panel B, but increasing amounts of NADPH were added to the assay mixture. (D) Immunological detection and expression of CAT-4. The same yeast strains as well as *N. crassa* wild-type mycelia grown on sucrose or oleic acid were processed for SDS-PAGE and Western blot analysis. Anti-Cta1p antibodies were used at a dilution of 1:10,000 in combination with the ECL detection system.
acids is also missing. Notably, though, *S. cerevisiae* catalase Cta1p is imported into peroxisomes in the absence of its C-terminal targeting signal, since it additionally possesses an internal targeting signal (28). Thus, despite lacking an eye-catching PTS, it remained entirely possible that CAT-4 is targeted to microbodies.

We therefore determined the subcellular localization of a GFP-CAT-4 fusion protein in an *S. cerevisiae* strain that expressed PTS2-DsRed, a synthetic peroxisomal marker protein (47). As expected, synthesis of GFP-SKL gave rise to a punctate staining pattern that is congruent with that of PTS2-DsRed, showing that the appended PTS1 directed GFP to peroxisomes. In contrast, diffuse fluorescence was observed when GFP-CAT-4 or GFP alone was expressed (Fig. 9A). The diffuse staining obtained with the latter protein could not have been caused by a degradation product of GFP-CAT-4, since Western blotting demonstrated the appropriate expression of the full-length fusion protein (Fig. 9B). Notably, though, the GFP-CAT-4 fusion protein was enzymatically inactive. To therefore exclude that the GFP tag compromised the localization of CAT-4, the untagged enzyme was expressed in the *cta1*/*H9004 ctt1*/*H9004* mutant and its distribution between a 25,000 g organelar pellet and the corresponding supernatant was determined. All catalase activity was recovered from the supernatant, whereas the peroxisomal matrix enzyme Pcs60p was detected in the pellet fraction (Fig. 9D). As control, yeast Cta1p was recovered from the organellar pellet fraction of a wild-type strain (Fig. 9C). Thus, CAT-4 did not possess any peroxisomal targeting information; rather it was located in the cytosol.

The presented localization data strongly support the notion that CAT-4 is a cytosolic enzyme; nonetheless, a remote possibility was that *N. crassa* maintains a distinct and unique peroxisomal import mechanism for CAT-4. A similar GFP-CAT-4 fusion protein was therefore also expressed in *N. crassa* (Fig. 10B). Not only GFP but also GFP-CAT-4 was uniformly distributed throughout hyphae, and Woronin bodies did not contain GFP-CAT-4 (Fig. 10A). Finally, a wild-type strain overexpressing untagged CAT-4 was generated. Its total catalase activity (70 U/mg protein), measured

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**FIG. 9.** CAT-4 is localized to the cytosol when expressed in *S. cerevisiae*. (A) Fluorescence microscopy of GFP-CAT-4-expressing *S. cerevisiae* cells. Yeast strain yHRP251 expressing the synthetic peroxisomal marker protein PTS2-DsRed was transformed with plasmids designed to express GFP-CAT-4, GFP-SKL or GFP. Strains were grown on solid oleic acid-containing medium for 2 days and were subsequently examined for GFP and DsRed fluorescence. Colocalization with PTS2-DsRed indicates peroxisomal targeting of the GFP fusion proteins. Nomarski images demonstrate the structural integrity of the cells. (B) Expression of GFP-CAT-4. Correct expression of GFP and the full-length CAT-4 fusion protein in yeast was determined by Western blotting using anti-GFP antibodies. (C and D) Differential centrifugation. Postnuclear supernatants (PNS) of an oleic acid-induced wild-type strain (C) and the *cta1Δ ctt1Δ* mutant expressing CAT-4 (D) were separated into a 25,000 × g organelar pellet (OP) and a supernatant (S) fraction. Fractions were assayed for catalase activity (upper panels) and for the presence of cytosolic Pgk1p, peroxisomal Pcs60p, and Cta1p (C) or CAT-4 (D) by Western blot analysis. Activities measured in the PNS fractions were taken as 100%. 

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in a postnuclear supernatant, exceeded that of the wild-type strain (18 U/mg protein) by a factor of 4, demonstrating that expression of CAT-4 in *N. crassa* gave rise to an active enzyme. Subcellular fractionation of these extracts showed that CAT-4 was exclusively present in the supernatant, while a significant portion of the glyoxysomal marker protein MFP was recovered from the organellar pellet fraction (Fig. 10D). A similar distribution of MFP was obtained in the wild-type control strain (Fig. 10C). The combined results therefore demonstrated that CAT-4 is localized to the cytosol and not imported into microbodies.

**Appearance of peroxisomal catalase in other filamentous fungi.** To analyze whether microbodies of other *Euascomycetes* also lack catalase activity, potential catalases from fungi with an available genome sequence were scrutinized for the presence of peroxisomal targeting signals. Close relatives of *N. crassa* from the Sordariomycete family including *Fusarium graminearum* and *Podospora anserina* do possess potential catalases of the peroxisomal family (i.e., small monofunctional catalases) with high similarity to CAT-4, and these proteins also lack obvious C-terminal PTS sequences (see Fig. S2 in the supplemental material). In contrast, one of the four catalases of the Eurotiomycete *Aspergillus* is a small-subunit catalase (23) with potential PTS1 sequences in all sequenced *Aspergillus* species (ARL in *A. nidulans*, *A. oryzae*, and *A. terreus* and SRL in *A. fumigatus*). We therefore tested whether *Aspergillus* indeed contains peroxisomal catalase. To that end, cell lysate of oleic acid-induced *A. tamarii* mycelia was separated by sucrose density gradient centrifugation and the resulting fractions were assayed for catalase activity by a spectrophotometric assay. When compared with the distribution of marker enzymes, it became evident that catalase cosedimented with the multifunctional protein (Fig. 11, upper panel). An in-gel activity assay showed that one isoform of catalase was indeed present in the peak microbody fractions (Fig. 11, lower panel). Furthermore, the genome sequences of several other Eurotiomycetes, such as *Histoplasma capsulatum*, *Coccidioides immitis*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea* also revealed the presence of putative peroxisomal catalases with a PTS1 (see Fig. S2 in the supplemental material). Thus, the absence of catalase-containing microbodies is probably restricted to a few filamentous fungi of the Sordariomycetes.

**DISCUSSION**

The filamentous *Euascomycete* *N. crassa* is thought to possess three types of microbodies within a single cell: glyoxysomes,
Catalase is clearly discernible in the peroxisomal fractions from the indicated fractions had been separated by native PAGE. Woronin bodies, and a distinct catalase-containing compartment. Here we have shown that \textit{N. crassa} lacks particulate catalase. As a consequence, the existence of a peroxisomal compartment in \textit{N. crassa} can be disregarded. The observation made was rather surprising in light of the fact that catalase was actually the first established marker enzyme for microbodies (10). Indeed, all catalase-containing eukaryotes examined so far also contained a peroxisomal isoform of this enzyme.

The catalase activities that were detected in oleic acid-induced \textit{N. crassa} mycelia were identical to the three described catalase isoforms, CAT-1, CAT-2, and CAT-3 (33, 37). To address their subcellular localization, all three catalases were purified and used for the preparation of monospecific antibodies. These immunoglobulins did not detect any of the three catalases in the organellar fractions of sucrose density gradients; instead, they were localized to the cytosol. Electron microscopy revealed that CAT-1 additionally associated with the cell wall, as was also reported for CAT-3 (34). Thus, it appears advantageous for filamentous fungi to furnish the extracellular medium with catalase so as to detoxify peroxide already outside the mycelia.

Interestingly, also \textit{N. crassa} contains one gene (cat-4) that does encode a peroxisomal type of catalase. The deduced amino acid sequence of cat-4 features consensus signatures for both protoheme binding as well as the catalase active site. We showed that CAT-4 indeed represents a bona fide catalase, as it was able to decompose hydrogen peroxide in a heterologous environment. Notably, the enzyme quickly lost its activity upon addition of its substrate, hydrogen peroxide, an effect that was not observed for yeast catalase. Several catalases are susceptible to inactivation by their own substrate, \textit{H}_2\textit{O}_2, but this can be largely prevented by bound NADPH (26). Addition of NADPH indeed stimulated the activity of CAT-4, probably by preventing it from oxidative damage. The CAT-4 sequence did not reveal any obvious peroxisomal targeting signals, suggesting that this catalase is not targeted to peroxisomes. However, a few peroxisomal matrix proteins such as yeast acyl-CoA oxidase (27, 44) lack either of the two prevalent targeting signals, PTS1 and PTS2. Also \textit{N. crassa} glyoxysomes contain at least one such protein, the multifunctional enzyme (MFP) encoded by the fox-2 gene (12). It is worth noting that the orthologous catalase from \textit{S. cerevisiae} can use an ill-defined internal targeting signal in lieu of its C-terminal PTS1 (28). These alternative avenues of import notwithstanding, ectopic expression of CAT-4 in \textit{N. crassa} did not result in targeting to any intracellular membrane-bound structure. Furthermore, CAT-4 was also not localized to peroxisomes upon heterologous expression in \textit{S. cerevisiae}.

Although it proved possible to demonstrate catalase activity for CAT-4 in both \textit{N. crassa} and \textit{S. cerevisiae}, we did not obtain evidence for endogenous CAT-4 being expressed in \textit{N. crassa}: anti-yeast Cta1p antibodies, which did recognize CAT-4 when expressed heterologously in yeast or ectopically in \textit{N. crassa}, failed to yield a signal when applied to protein extracts from wild-type mycelia grown in sucrose or oleic acid-containing medium. Furthermore, under various peroxisome-inducing conditions only CAT-1, CAT-2, and CAT-3 were discernible in native gels stained for catalase activity. Obviously, our studies still leave room for environmental conditions under which CAT-4 will be expressed. However, important for our core statement is the fact that ectopic expression of CAT-4 was feasible and this led to a cytosolic localization of the protein.

The previously reported microbody-like organelle of high density with apparent catalase activity (25, 53) is likely to coincide with the Woronin body, since spectrophotometric assays at 240 nm conducted by us and others (49) indeed measured a weak activity associated with CAT-4 in \textit{N. crassa} mycelia. Also native gel assays could not confirm this activity. Specific immunologic detection of the three catalase isoforms CAT-1, CAT-2, and CAT-3 showed that these proteins are not associated with any intracellular compartment. Consistent with this result, the coding sequences of CAT-1, -2, and -3 lack PTS1 or PTS2 sequences. Finally, a GFP fusion of the novel CAT-4 was also shown to be localized to the cytosol. One plausible explanation for the spectrophotometric hydrogen peroxide breakdown could be the presence of an unidentified peroxidase associated with the Woronin body fraction. Little is known about the protein composition of this organelle. Its dominant protein, HEX-1, possessed neither catalase nor per-
oxidase activity when enriched by cation-exchange/gel filtration chromatography (data not shown).

Why is catalase dispensable for *Neurospora* microbodies? This is likely to be a direct consequence of the organellar enzyme content of this organism. The glyoxysomal β-oxidation system of *N. crassa* is unusual in that the first step is catalyzed by an acyl-CoA dehydrogenase instead of an acyl-CoA oxidase (25). As a consequence, hydrogen peroxide is not formed within glyoxysomes during that process. Furthermore, the H₂O₂-generating urate oxidase activity was localized to the cytosol. In all mammals that do express urate oxidase, this enzyme involved in purine metabolism is localized to peroxisomes (18). Even urate oxidases from *Aspergillus* are peroxisomal and bear PTS1 sequences, while the probable *Neurospora* ortholog, NCU06558.2, harbors the canonical PTS1 tripeptide SKL at its D-amino acid oxidase, since its putative reading frame, chromatography (data not shown).

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