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Localization of Glucose Oxidase and Catalase Activities in *Aspergillus niger*

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The subcellular localization of glucose oxidase (EC 1.1.3.4) in *Aspergillus niger* N400 (CBS 120.49) was investigated by (immuno)cytochemical methods. By these methods, the bulk of the enzyme was found to be localized in the cell wall. In addition, four different catalases (EC 1.11.1.6) were demonstrated by nondenaturing polyacrylamide gel electrophoresis of crude extracts of induced and noninduced cells. Comparison of both protoplast and mycelial extracts indicated that, of two constitutive catalases, one is located outside the cell membrane whereas the other is intracellular. Parallel with the induction of glucose oxidase, two other catalases are also induced, one located intracellularly and one located extracellularly. Furthermore, lactonase (EC 3.1.1.17) activity, catalyzing the hydrolysis of glucono-δ-lactone to gluconic acid, was found to be exclusively located outside the cell membrane, indicating that gluconate formation in *A. niger* occurs extracellularly.

The subcellular localization of glucose oxidase in *Aspergillus niger* has been a point of discussion. For a long time, the enzyme was assumed to be located intracellularly (14). This was further supported by ultrastructural studies by van Dijken and Veenhuis, who concluded that the enzyme was located in peroxisomes (20). Mischak et al. (13) showed that, under manganese-deficient growth conditions, glucose oxidase is found almost quantitatively in the culture fluid. Their explanation for this was that a cell wall-localized glucose oxidase entered the culture fluid because of an altered cell wall composition resulting from manganese deficiency. Two other arguments are in favor of an extracellular localization of glucose oxidase: (i) glucose oxidase is strongly glycosylated (15, 18), a phenomenon which has never been observed for any peroxisomal protein; and (ii) the amino acid sequence derived from the glucose oxidase DNA sequence shows a typical secretion signal peptide (6). In this study, we reexamined the localization of glucose oxidase in *A. niger*.

An extracellular localization of glucose oxidase activity implies an extracellular production of hydrogen peroxide. The sequestration of this toxic process will prevent damage to the cell. However, there remains the necessity for an efficient removal of H_2O_2 since this compound is known to inactivate glucose oxidase (10) and can easily diffuse over the cell membrane. Mycelium containing high levels of glucose oxidase can produce 50 to 100 mmol of hydrogen peroxide per h per g of mycelium (dry weight) without large-scale cell lysis or inactivation of glucose oxidase; therefore, an effective protective system must exist. Both catalases and peroxidases might be involved in this process. The induction and localization of catalases under glucose oxidase-inducing conditions were therefore also investigated.

MATERIALS AND METHODS

Strains, media, and growth. All experiments were performed with *A. niger* N400 (CBS 120.49). Mycelium was grown in a 3-liter fermentor by using the same medium as described previously (26). To obtain mycelium which was induced for glucose oxidase, the oxygen level was kept above 30% air saturation (i.e., the dissolved oxygen concentration was 30% of the oxygen level in a solution saturated with air) during the whole growth period or glucose oxidase was induced by increasing the oxygen level to 50% air saturation for 4 to 6 h before harvesting the mycelium. The latter method gave somewhat lower activities of glucose oxidase. The noninduced mycelia for the catalase induction and localization experiments were grown at low oxygen levels (less than 7% air saturation), while the other conditions were identical to those under inducing conditions. Mycelium grown this way contains no detectable glucose oxidase activity (26). In all cases, the mycelium was grown at 30°C and harvested at approximately 24 h after inoculation.

Preparation of the protoplasts. Protoplasts were released from the mycelium by using 1 to 3 mg of Novozyme 234 ml^{-1} in protoplast-stabilizing buffer (PSB) containing 10 mM Tris HCl (pH 7.5), 50 mM $CaCl_2$, and 1.33 M sorbitol. The mycelium concentration was approximately 50 mg (wet weight) ml^{-1}. Protoplasts were separated from the mycelium by filtration over glass wool, washed twice with PSB, and subsequently lysed in 20 mM sodium phosphate buffer (pH 6.0). To complete cell lysis, the suspension was sonicated. The supernatant, obtained after 5 min of centrifugation at 10,000 × *g*, was used for enzyme measurements and analysis by polyacrylamide gel electrophoresis. Cell wall-bound catalase was released by partial degradation of the cell wall. This was achieved by incubation of the mycelium for 30 min in PSB with 0.1 mg of Novozyme 234 ml^{-1}. These levels of Novozyme did not cause detectable degradation of the catalase, and no catalase activity bands were detectable on gel when only the Novozyme solution was applied to the gel.

Biochemical analyses. Crude cell extracts were prepared as described previously (24), except that 20 mM sodium phosphate (pH 6.0) was used as the extraction buffer.

Separation and visualization of catalase was performed by using a modification of the procedure described by Gregory and Fridovich (7). Crude extracts were separated on a

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1.5-mm-thick, 7.5% polyacrylamide gel containing 20% glycerol. For visualizing catalase activities, the gel was incubated for 30 min in 50 ml of 50 mM sodium phosphate (pH 7.0) containing 0.2 mg of horseradish peroxidase ml^{-1}. Twelve microliters of 30% H_2O_2 was then added, and the mixture was incubated for 10 min. Subsequently, the liquid was washed away quickly, and an *o*-dianisidine solution (0.5 mg ml^{-1}) was added to visualize the presence of H_2O_2. At positions where catalase was present, no staining occurred.

Glucose oxidase was measured spectrophotometrically as described previously (26). Catalase activity was measured by monitoring the decrease in A_{240} caused by H_2O_2 degradation (1). Glucose-6-phosphate dehydrogenase and $NADP^+$-dependent glutamate dehydrogenase were assayed by the method of Bruinenberg et al. (4). Dihydroxyacetone reductase was assayed by the method of Witteveen et al. (25). Citrate synthase was assayed as described by Stitt (17). Lactonase activity was determined by titration with 50 mM sodium hydroxide of a 50-ml 10 mM sodium phosphate buffer (pH 6.0) with an appropriate amount of extract or culture filtrate to which 0.45 g of glucono-δ-lactone was added at the start of the assay. The acidification was followed for 5 min. Corrections were made for spontaneous hydrolysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (12) by using the Pharmacia-LKB midget electrophoresis system. For detection of glucose oxidase on Western blot (immunoblot), an alkaline phosphatase assay (Bio-Rad) was used.

Protein concentrations were estimated, after denaturation and precipitation of protein with sodium deoxycholate and trichloroacetic acid (2), by a microbiuret method (8) with bovine serum albumin as the standard.

Generation of antisera. Rabbit polyclonal antibodies against glucose oxidase were raised by standard procedures by using purchased glucose oxidase (Boehringer, grade I) which, prior to injection, was further purified as follows. The enzyme was dissolved in 20 mM Tris HCl (pH 7.4), containing 0.5 M NaCl, 0.25 mM $MnCl_2$, and 0.25 mM $CaCl_2$, and loaded onto a concanavalin A-Sepharose 4B column (Pharmacia). The enzyme was eluted with 20 mM sodium acetate buffer (pH 4.5) containing 0.5 M NaCl and 1 M α-methyl mannoside. The eluted protein was dialyzed extensively against 0.1 M sodium phosphate buffer (pH 7.5). The glucose oxidase solution thus obtained was denatured and deglycosylated by using *N*-glycanase (Genzyme) according to the procedure described by Tarentino et al. (19) and subsequently used for immunization. The protein prepared this way showed a single, sharp band on an SDS-polyacrylamide gel. The antisera obtained were further purified by incubation with D-xylose- and acetate-grown *A. niger* mycelia, which lack glucose oxidase, to remove nonspecific antibodies directed against antigens other than glucose oxidase. Finally, the immunoglobulin G fraction was purified on a Bio-Rad Affi-Gel Blue column according to the manufacturer's procedure. The antibodies thus obtained were used in the experiments described below.

Immunocytochemical and cytochemical experiments. For immunocytochemical experiments, the mycelium was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 90 min at 0°C, dehydrated in a graded ethanol series, and embedded in Lowicryl K4M (27). Immunolabeling was performed on ultrathin sections by the protein A–gold method described by Slot and Geuze (16).

Cytochemical staining experiments were performed on glutaraldehyde-fixed cells. Catalase activity was demonstrated by the 3,3′-diaminobenzidine-based method; glucose oxidase activity was demonstrated by using $CeCl_3$ as the H_2O_2-capturing agent (21).

RESULTS

The overall cell morphology of *A. niger*, grown on glucose, is shown in Fig. 1. The cells contain the usual cell organelles such as nucleus, mitochondrial and vacuolar profiles, and strands of endoplasmic reticulum. Remarkably, the proliferation of microbodies (both in size and number) is low in these cells. Cytochemically, catalase activity could be demonstrated inside these microbodies. In addition, the outer layer of the cell wall was densely stained (Fig. 2). In cytochemical experiments using $CeCl_3$ for the detection of glucose oxidase activity, the bulk of the reaction products were confined to extracellular regions. Apart from the periplasmic space, staining was also observed in the cell wall and associated with the outer cell wall layer (Fig. 3). It is, however, unclear to what extent the staining of this outer layer is specific. During incubation of cells with $CeCl_3$ and glucose, the incubation mixture assumed a yellow-brownish color, most probably due to precipitation of cerium perhydroxide formed from H_2O_2 that had leaked from the site of its generation. Therefore, staining of the outer cell wall layer may, at least partly, be due to adsorption of cerium precipitate formed in the incubation mixture.

The procedure described for the preparation of antigen and antibody turned out to be essential to obtain reproducible results. Polyclonal antibodies against nondeglycosylated glucose oxidase turned out to be nonspecific when tested by Western blot after SDS-PAGE of mycelial extracts. However, antibodies raised against deglycosylated and denatured glucose oxidase revealed only the broad protein band characteristic for this enzyme. When used in immunocytochemical experiments, these antisera showed a minor reactivity against cell walls of *A. niger* cells which lacked glucose oxidase. This nonspecific labeling could be removed by incubation of the antisera with intact D-xylose- and acetate-grown mycelia and subsequent purification of the immunoglobulin G fraction by using an Affi-Gel Blue column. When applied to glucose oxidase-containing mycelium, the purified antibodies resulted in an intense labeling of the cell wall (Fig. 4).

The possibility of an exclusive localization of glucose oxidase in the cell wall of *A. niger* was further studied biochemically. We measured the specific activities in lysates from protoplasts of glucose oxidase-induced mycelium and compared these with the specific activities in extracts of intact mycelium. This method also gave information on the distribution of catalase activities. Besides catalase and glucose oxidase, three cytoplasmic enzymes were measured as a control together with citrate synthase which is known to be localized in mitochondria (Table 1). The results convincingly indicate the localization outside the cell membrane of a catalase activity and of a glucose oxidase activity.

The intermediary value for the protoplast/mycelium specific activity ratio of catalase as compared to that of glucose oxidase and the intracellularly located enzymes can be explained by the presence of more than one catalase. To clarify this, we separated mycelial and protoplast extracts on a nondenaturing polyacrylamide gel and visualized the catalases by activity staining. These experiments revealed the presence of at least four different catalases, two of which were induced parallel with glucose oxidase. Under nonin-

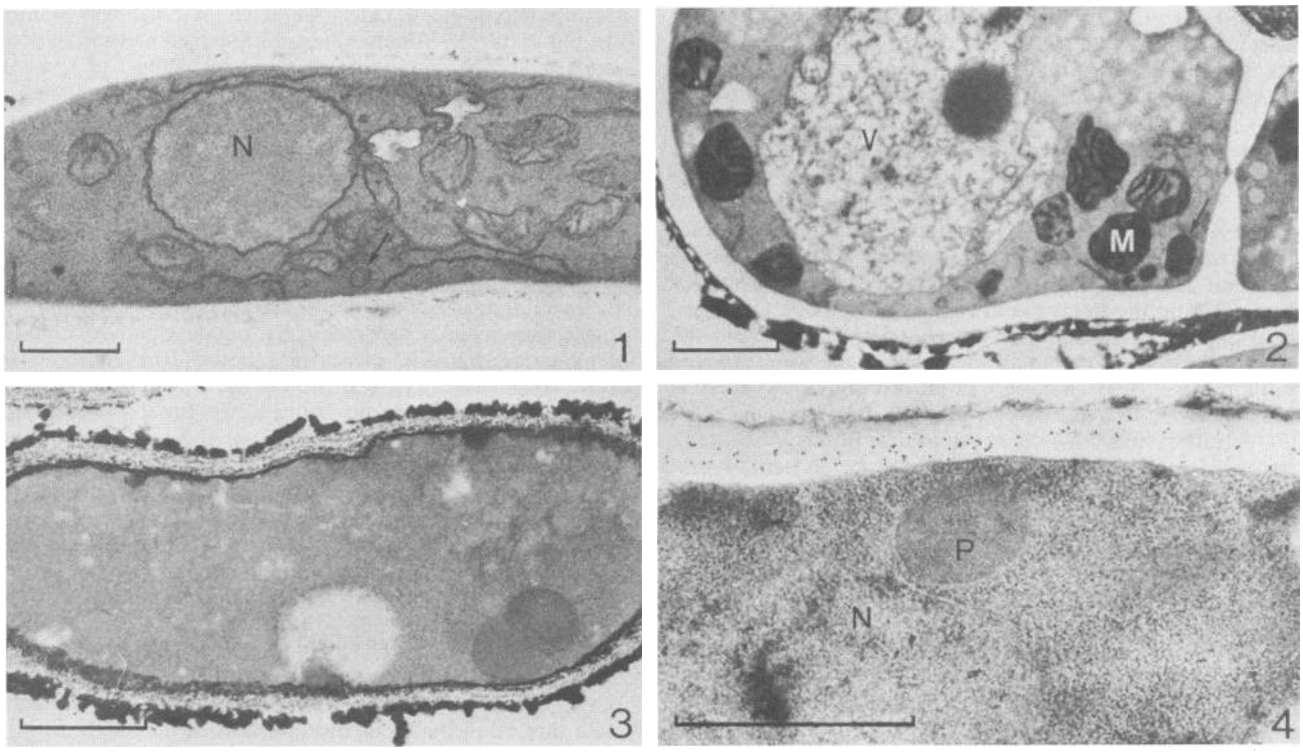

FIG. 1. Thin section of a $KMnO_4$-fixed cell of glucose-grown *A. niger* to show overall cell morphology. The small microbody is indicated by an arrow. N, nucleus. Bar, 0.5 μm.

FIG. 2. Cytochemical staining of catalase activity (3,3'-diaminobenzidine-H_2O_2) in a glucose-induced cell of *A. niger*. In addition to the peroxisomes (arrow), the outer cell wall layer is densely stained. Mitochondrial staining is due to cytochrome *c* oxidase or peroxidase activity. V, vacuole; M, mitochondria. Bar, 0.5 μm.

FIG. 3. Cytochemical staining of glucose oxidase activity after aerobic incubations of cells with $CeCl_3$ and glucose. Specific reaction products are confined to the periplasmic space and the cell wall. Bar, 0.5 μm.

FIG. 4. Immunocytochemical staining using specific antibodies against glucose oxidase and protein A-gold. Labeling is confined to the cell wall. Peroxisomes are not labeled. N, nucleus; P, peroxisome. Bar, 0.5 μm.

ducing conditions, a weak band and a stronger catalase activity band were visible (Fig. 5, lane A). Protoplast extracts of this mycelium showed only the weaker upper band (Fig. 5, lane B). From this, we conclude that this particular catalase is intracellularly located (catalase I), whereas the more rapidly migrating form is apparently localized in the cell wall (catalase II). The presence of a catalase localized outside the cell membrane under noninducing conditions was also indicated by the difference in relative specific activities in protoplast and mycelial extracts (Table 1). Under conditions where glucose oxidase was induced, an additional catalase band of high mobility was found (catalase III), whereas at the level of catalase I a strong band became visible (Fig. 5, lanes C and E). In protoplasts of an induced mycelium, only the high-mobility band was visible, whereas at the position of catalase I only a weak activity was found (Fig. 5, lane D). From this we conclude that the high-mobility band, which becomes visible under inducing conditions (catalase III), is intracellular and that the low-mobility band appearing under inducing conditions is not the intracellular catalase I but a different extracellular catalase (cata-

TABLE 1. Enzyme activities in extracts of mycelium and protoplasts of glucose oxidase-induced and noninduced cells[a]

Extract	Enzyme activity (μmol min^{-1} mg of $protein^{-1}$)							
	Induced						Noninduced	
	Gdh	DHAred	G6Pdh	CS	cat	gox	G6Pdh	cat
Protoplast	0.68	0.62	0.80	0.06	21	0.11	0.46	3.9
Mycelium	0.42	0.62	0.89	0.04	178	5.4	0.6	38.6

[a] Abbreviations: GDH, $NADP^+$-dependent glutamate dehydrogenase; DHAred, NADPH-dependent dihydroxyacetone reductase; G6Pdh, glucose-6-phosphate dehydrogenase; CS, citrate dehydrogenase; cat, catalase; gox, glucose oxidase.

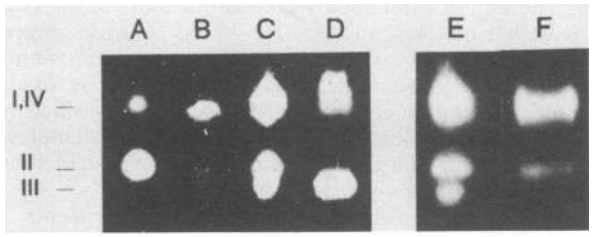

FIG. 5. Catalases in mycelial and protoplast extracts of induced and noninduced mycelia visualized on nondenaturing polyacrylamide gels. Lanes: A and B, extracts of mycelium and protoplasts of noninduced mycelium; C and D, extracts of mycelium and protoplasts of induced mycelium; E, same as that described for lane C; F, material released from induced mycelium when low concentrations of Novozyme 234 were used.

lase IV). To obtain further confirmation on the cell wall localization, we treated induced mycelium with low concentrations of Novozyme 234 to see whether eventual cell wall-localized catalases could be released from the mycelium. Analysis of catalase activity on a gel showed that, at the position of the two catalases which we assumed to be cell wall localized, activity bands were visible (Fig. 5, lane F). This was not the case at the position of catalase III.

Besides glucose oxidase and catalase, we also measured lactonase. Activities of this enzyme were found both in mycelial extracts and in the culture fluid (approximately 50%). We were not able to measure this enzyme in the protoplast extracts. These data indicate that lactonase is also transported across the cell membrane and, therefore, that all enzyme activities involved in gluconate biosynthesis reside extracellularly.

DISCUSSION

The results described in this paper confirm the conclusion of Mischak et al. (13) that, in *A. niger*, glucose oxidase is located in the cell wall. The (immuno)cytochemical data (Fig. 3 and 4) show this very clearly, and the localization outside the cell membrane is confirmed by the ratio of the specific glucose oxidase activities in protoplast and mycelial extracts (Table 1). The results of van Dijken and Veenhuis (20), who described a peroxisomal localization of glucose oxidase, are in contradiction with these results. Their cytochemical data may be explained as an artifact of the method used. Within this view, the observed positive staining of microbodies, caused by catalase, may result from H_2O_2 generated by glucose oxidase located in the cell wall after the addition of glucose to the incubation sample, which subsequently diffuses inside the cell. The high glucose oxidase activities in the cell wall might produce sufficient H_2O_2 for the peroxisomal staining observed. Peroxisomal staining has been observed by us after H_2O_2 was added to the cell suspension. The observation of van Dijken and Veenhuis that the size and number of peroxisomes increases with glucose oxidase synthesis is more difficult to explain and might be an indication of strain differences. The extracellular localization of glucose oxidase is in line with the finding that glucose oxidase is glycosylated (15) and that the amino acid sequence derived from the DNA sequence (6) clearly shows a characteristic secretion signal peptide. Similarly, in *Penicillium* strains, glucose oxidase is also excreted in the culture fluid (11). The peroxisomal localization of glucose oxidase in other *A. niger* strains cannot be ruled out but is considered unlikely by us.

The differences in localization and induction patterns of catalase permits the conclusion that at least four different catalase proteins are formed by *A. niger* and that the bands visible on the gels are not degradation products of a single catalase. The catalases I and II represent more or less constitutive catalases, whereas the catalases III and IV are induced under conditions when glucose oxidase is formed. Chary and Natvig (5) showed the presence of three catalase genes in *Neurospora crassa*. However, the information available is insufficient for a comparison of the individual enzymes to be made.

The extracellular localization of a catalase, as concluded from the cytochemical experiments (Fig. 2), the patterns on a native gel of mycelial and protoplast extracts stained for catalase activity (Fig. 5), and the relative specific activities of catalase in mycelial and protoplast extracts, is further supported by the observation that a catalase isolated from *A. niger* is glycosylated (9, 23).

The protection of glucose oxidase in the cell wall against inactivation by H_2O_2 might very well be achieved by catalase, despite its relative low affinity for H_2O_2, because inactivation as observed by Kleppe (10) plays only a role at H_2O_2 concentrations up to at least a few millimolar. The very strong induction of a catalase which is localized at the site of H_2O_2 formation, in the cell wall, makes it very likely that this enzyme plays a major role in the breakdown of H_2O_2 formed by glucose oxidase. The role of catalase in the protection of the cell interior is less clear. Studies in the methylotrophic yeast *Hansenula polymorpha* showed that cytochrome *c* peroxidase may play an essential role in the detoxification of H_2O_2. In this organism, catalase could only effectively compete with cytochrome *c* peroxidase when it was located at the site of H_2O_2 generation in the peroxisomes (22). The induction of an intracellular catalase is an indication that, in *A. niger*, catalase plays a role in the intracellular detoxification. However, it is very well possible that other enzymes are involved.

Lactonase activity has been demonstrated before in *A. niger*. It has been purified from Novozyme 188, a commercial enzyme preparation from *A. niger* (3). The presence of a lactonase is not obligatory since the hydrolysis of the lactone also occurs spontaneously, although at a lower rate. The extracellular localization of lactonase implies that the complete glucose oxidation system is localized outside the cell membrane.

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