JOURNAL OF BACTERIOLOGY, Nov. 1980, p. 509-517
0021-9193/80/11-0509/09$02.00/0

Vol. 144, No. 2

Developmental Regulation of Laccase Levels in *Aspergillus nidulans*

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Asexual spores (conidia) of *Aspergillus nidulans* contain a dark green pigment which is not present in other cell types. Synthesis of this pigment is catalyzed, in part, by a developmentally controlled *p*-diphenol oxidase, or laccase, encoded at the *yA* genetic locus (A. J. Clutterbuck, J. Gen. Microbiol. **70**:423-435, 1972). We have investigated the mechanisms regulating expression of the *yA* gene of *A. nidulans*. Vegetative hyphae grown in submerged culture lacked detectable laccase enzyme activity and neither contained nor synthesized immunoprecipitable laccase protein. When such cultures were induced to conidiate by harvesting the cells onto filter papers and aerating them, laccase levels began to increase after 10 to 16 h, reached a peak at 30 to 36 h, and then declined slowly. Immunological assays showed that increases in laccase enzyme activity were (i) preceded by a transient rise in the relative rate of laccase protein synthesis and (ii) closely paralleled by increases in the amount of laccase protein. Addition of cycloheximide to cultures at any time after inducing conidiation inhibited further accumulation of laccase enzyme activity. These data are most consistent with increases in laccase levels being due to regulated, de novo synthesis of laccase protein. Addition of inhibitors of ribonucleic acid synthesis to conidiating cultures also inhibited further accumulation of laccase, suggesting that laccase expression is regulated by alterations in the transcriptional activity of the *yA* locus.

Asexual reproduction in the ascomycete fungus *Aspergillus nidulans* involves the differentiation of complex, multicellular, spore-producing structures called conidiophores and spherical, uninucleate spores called conidia (4, 20, 22, 24). The conidia of this species contain in their walls a dark green pigment which is produced as the spores mature (16). Formation of the spore pigment requires the expression of at least two known genetic loci, designated *wA* and *yA* (20). The product of the *wA* gene mediates the synthesis of a yellow pigment intermediate from a colorless precursor. This yellow intermediate is subsequently converted to the mature green form in a reaction catalyzed by an enzyme encoded at the *yA* locus. Thus, *wA* mutants fail to synthesize any pigment and produce white conidia, *yA* mutants accumulate the pigment intermediate and form yellow conidia, and *wA* is epistatic to *yA*.

The protein product of one of these genes, *yA*, has been identified and partially characterized. Clutterbuck (5) demonstrated that wild-type strains of *Aspergillus* produced a *p*-diphenol oxidase, or laccase, activity (EC 1.10.32) during conidiation, whereas univariant, *yA* mutants did not. He also investigated a conditional mutation (*yA31*) which caused the formation of yellow spores at 37°C but allowed some green pigment to be produced at 22°C. Laccase extracted from strains carrying the temperature-sensitive *yA31* allele was temperature sensitive in vitro, thus confirming that the *yA* locus encodes all or part of the *p*-diphenol oxidase enzyme. In addition, Clutterbuck (5) found that whereas laccase was present in conidiating wild-type cultures, somatic hyphae lacked detectable enzyme activity. Thus, the *yA* gene is preferentially expressed when vegetative hyphae begin to differentiate conidiophores and to produce conidia.

The genetic characteristics of the *yA* locus and the biochemical properties of its protein product make the processes regulating laccase expression particularly accessible to experimental analysis. We have begun to investigate the molecular basis for developmental regulation of the *yA* gene of *A. nidulans*. In this paper, we show that accumulation of laccase enzyme activity is a result of the regulated, de novo synthesis of laccase protein. Based on the results of RNA inhibitor studies and characteristics of fungal nuclear pre-mRNA populations, we suggest that enhanced laccase protein synthesis is due to transcriptional activation of the *yA* gene.

(This research was conducted in partial fulfillment of the requirements of the Ph.D. degree awarded to D.J.L. from the Department of Biological Science, Wayne State University.)

MATERIALS AND METHODS

Growth of cultures. *A. nidulans* (Glasgow wild type, Fungal Genetics Stock Center no. 4) was grown by inoculating YG medium (0.5% yeast extract, 2.0% D-glucose) (1), in polypropylene flasks, with 5×10^4 conidia/ml and incubating the cultures at 37°C on a rotary shaker at 300 rpm. Under these conditions, the dry mass of the cultures increased exponentially for at least 20 h (doubling time = 1.5 h). To induce conidiation, 50-ml portions of cultures grown for 16 to 17 h were harvested onto unwashed, 9-cm Whatman no. 1 filter paper circles by filtration with suction. Care was taken to remove as much of the growth medium as possible. The filters and adherent cells were transferred to 10-cm petri dishes, each containing a single layer of sterile, 3-mm glass beads and 10 ml of fresh YG medium. Uncovered petri dishes were incubated at 37°C in enamel pans which contained moist paper towels and were covered with aluminum foil. The cultures were aerated by passing a stream of filtered, humidified laboratory air (250 ml/min per dish) through the pans. After 10 and 24 h of incubation they were supplemented with 5 ml of fresh YG medium. Dishes and media were examined microscopically at intervals to moniter conidiophore development and to ensure absence of microbial contamination. We have noted that many lots of yeast extract are unsatisfactory for preparing conidiating filter paper cultures. Yeast extract obtained from the Sigma Chemical Co. (control no. 59C 02581) was used for the experiments reported in this paper. However, we have also obtained satisfactory results with isolated lots of yeast extract obtained from Difco Laboratories or the GIBCO Laboratories.

Filter paper cultures were radioactively pulse-labeled by removing the filter paper and mycelium from the petri dish, blotting the filter on paper towels, and returning the cells to a dish containing glass beads, YG medium, and 2 mCi of $^{35}SO_4^{2-}$ (carrier-free). After an additional 2 h of incubation at 37°C, the cells were harvested and frozen in liquid N_2. An analogous protocol was used to treat the cultures with inhibitors of RNA or protein synthesis.

Preparation of protein extracts. Cultures were harvested by scraping the cells off of the filter paper and freezing them in liquid N_2. They were ground to a fine powder in a mortar and then suspended and thawed in 100 mM sodium phosphate buffer, pH 6.9 (PB), using 2 ml of buffer per g of cells. After stirring for 1 h at 4°C, the homogenates were clarified by centrifugation at 15,000 × *g* for 10 min and then dialyzed overnight against several changes of PB (4°C). The extracts were once again clarified by centrifugation at 15,000 × *g* and then assayed for laccase activity.

Assay for laccase activity. *p*-Diphenol oxidase activity (laccase) was assayed as described by Clutterbuck (5) by measuring the oxidation of *N,N*-dimethyl-*p*-phenylenediamine (DMP) spectrophotometrically. A unit of activity is expressed as that amount of enzyme oxidizing 1 nmol of DMP/min at 25°C in a 1-ml reaction mixture. We found that reaction velocities were constant for at least 10 min.

Purification of laccase. All procedures were carried out at 4°C unless otherwise stated. Cells aerated for 30 to 36 h were scraped from 32 filter papers and extracted as described above. After the first centrifugation, the supernatant fraction was extracted three times with equal volumes of $CHCl_3$ (5). The protein solution (300 to 400 ml) was dialyzed overnight against several 2-liter changes of PB. Greater than 95% of the enzyme activity was recovered after dialysis. The sample was then loaded onto a DEAE-Sephadex column (2 by 15 cm) equilibrated with PB. The column was washed with PB until the absorbancy at 280 nm of the effluent was <0.05 and the enzyme was eluted with a linear, 100 to 600 mM NaCl gradient (in PB, total volume = 150 ml). Fractions (3 ml) were collected and those containing enzyme activity were pooled, dialyzed against PB, and then adjusted to 60% of saturation with $(NH_4)_2SO_4$ (at 4°C) by slow addition of the solid salt. The solution was stirred for 30 min and centrifuged at 15,000 × *g*. The supernatant was brought to 90% of saturation with $(NH_4)_2SO_4$, stirred for 30 min, and centrifuged. The pellet was dissolved in 2 to 3 ml of PB, and the proteins were fractionated by chromatography on a Sephadex G-200 column (2.5 by 40 cm) developed in PB. Fractions (5 ml) were collected and those containing laccase activity were pooled, concentrated by ultrafiltration, and electrophoresed on linear, 10 to 20% polyacrylamide gradient gels (100 by 140 by 1.5 mm) under nondenaturing conditions (7). After electrophoresis, gels were stained enzymatically by immersing them in a solution containing 5 mM DMP dissolved in 37 mM citric acid–124 mM Na_2HPO_4, pH 6.0 (5). Bands with enzyme activity were then excised, and the proteins were extracted by grinding the gel to a fine slurry in 1 mM PB. Gel fragments were removed by centrifugation, and the solution was dialyzed against several changes of 1 mM PB and concentrated to 1 to 2 ml. Animals were initially immunized with proteins prepared in this way (see below). One animal was later given booster injections with an enzyme fraction that was purified further. In this case, the partially purified enzyme, in 1 mM PB, was loaded onto a column containing 0.3 g of hydroxyapatite (Bio-Rad, DNA grade) equilibrated in 1 mM PB. The column was eluted with a 5-ml linear 1 to 100 mM PB gradient, fractions (0.3 ml) were collected, those containing laccase activity were pooled, and the enzyme solution was concentrated by ultrafiltration. With this procedure, we obtained an overall purification of about 400-fold.

Immunization of animals. New Zealand white rabbits weighing 3 to 4 kg were injected intradermally at 2-week intervals with 50 to 100 μg of antigen in Freund complete adjuvant (9). Immune animals were given booster injections consisting of 50 to 100 μg of antigen in Freund incomplete adjuvant. Antibody titers were monitored by rocket immunoelectrophoresis (15, 26).

Determination of molecular weights. The molecular weight of laccase was estimated by chromatography of partially purified enzyme preparations on a Sephadex G-200 column (2.5 by 40 cm) calibrated with the following molecular weight markers: rabbit lactic dehydrogenase (135,000), equine alcohol dehydrogenase (83,000), bovine serum albumin (67,000), ovalbumin (45,000), bovine chymotrypsinogen (25,000), and whale myoglobin (18,000).

Other procedures. Sodium dodecyl sulfate (SDS)-

polyacrylamide gel electrophoresis was according to Laemmli (14); fluorography of gels, Bonner and Laskey (3); preparation of immunoglobulin G, Palacios et al. and Palmiter et al. (17, 18); immunoprecipitation of ^{35}S-labeled proteins, Payvar and Schimke (19); mutagenesis with 1,2,7,8-diepoxyoctane, Hynes (11); protein determinations, Bio-Rad dye-binding assay.

RESULTS

Conidiation in filter paper cultures. Previously reported procedures for inducing conidiation in *A. nidulans* under controlled conditions (1, 2, 5) are not entirely suitable for many biochemical investigations because they either do not lend themselves to the preparation of large quantities of staged cells, do not allow the convenient addition or radioisotopes or metabolic inhibitors, or are expensive. We have developed a modification of the earlier methods which overcomes these problems. We found that suspension cultures consisting of undefined medium (yeast extract-glucose) inoculated with 5×10^4 conidia/ml grew exponentially as dispersed hyphae if they were incubated at 37°C in polypropylene flasks with vigorous agitation. We have never observed conidiophores or conidiophore-like structures in such cultures. When samples of these cultures were harvested onto filter paper disks, transferred to petri dishes each containing a supporting layer of glass beads and 10 ml of growth medium, and incubated at 37°C with controlled aeration, they rapidly began to differentiate conidiophores and to produce conidia. The kinetics of conidium formation by filter paper cultures are shown in Fig. 1. At about 4 h after filtration and plating, numerous aerial hyphae were produced uniformly over the entire surface of the culture. Between 4 and 10 h, the aerial hyphae elongated, formed apical vesicles, and began to differentiate metulae (primary sterigmata) and phialides (secondary sterigmata). Immature, unpigmented conidia began to be produced at about 10 h, and conidial pigmentation was evident at 16 h. Between 16 and 48 h, conidia were produced at a nearly constant rate (Fig. 1), forming a uniform, green lawn in the petri dish. Under these conditions, single 9-cm filter paper cultures produced about 10^{10} conidia over a 2-day interval. Cells could be easily harvested from these cultures by scraping them from the filter papers with a spatula.

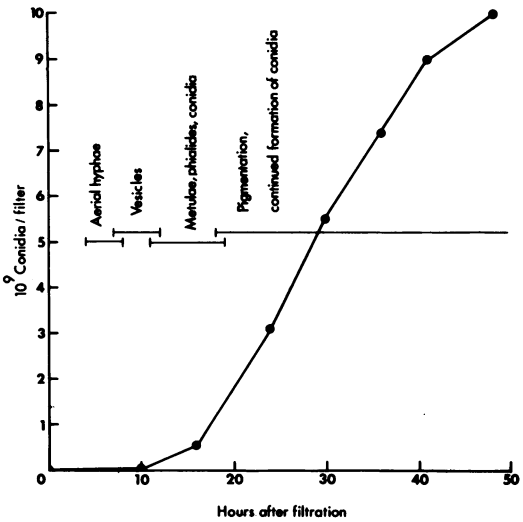

FIG. 1. *Kinetics of conidium production by filter paper cultures of Aspergillus. Exponentially growing, vegetative cells were harvested onto 9-cm filter paper disks; then the papers and adherent mycelium were transferred to petri dishes containing glass beads and YG medium and aerated at 37°C. At various times, cultures were removed and examined microscopically to determine the developmental stage. The cells were then suspended in 0.1% (vol/vol) Tween-80 and filtered through cotton, and the conidia in the filtrate were diluted and counted in a hemocytometer.*

Changes in laccase specific activity during conidiation. To determine how laccase levels varied when somatic cells were induced to conidiate under the conditions described above, we harvested filter paper cultures at various times, extracted soluble proteins, dialyzed the extracts, and assayed them for laccase activity. No activity could be detected in protein preparations from freshly plated cells, even when large amounts of protein (350 μg) were assayed (Fig. 2). Laccase activity could first be detected at 10 h after filtration, coinciding with the appearance of immature conidia on the conidiophores. Thereafter, laccase specific enzyme activity increased, reached a peak at 36 h, and then declined slowly. Enzyme activity could be reliably detected in extracts from 36-h cultures even when <1 μg of total protein was assayed. Since no activity was detected in extracts derived from freshly plated cells when samples containing 350 μg of protein were assayed, laccase specific activities increased at least 350-fold between 0 and 36 h after plating.

The procedure we used to homogenize the cells (grinding in liquid N_2) did not result in complete breakage of hyphae or in significant disruption of conidia. It was thus possible that some laccase was present in the cells but was not extracted and, therefore, not detected. To test this possibility, cells which had first been extracted as described above were further disrupted by blending them with 100-μm glass beads until >90% of the somatic cells and conidia (if present) were broken as determined by microscopic examination. Protein extracts from the rehomogenized cells were then assayed for laccase enzyme activity. In no case did the amount of laccase detected upon reextraction exceed 5%

of that obtained initially. In fact, we found that >90% of the laccase activity present in cells aerated for 36 h could be extracted simply by stirring them gently in buffer. These results support the suggestion that laccase is secreted into the periplasmic space (5).

Changes in laccase protein levels during conidiation. The increases in laccase enzyme specific activity occurring during conidiation (Fig. 2) could have been due to activation of previously synthesized, but nonfunctional, laccase protein, synthesis of new protein, or a combination of the two processes. To distinguish between these alternatives, we estimated laccase protein levels in extracts from conidiating cultures by an immunological procedure. We obtained from a rabbit which had been immunized with a partially purified enzyme preparation an antiserum that efficiently precipitated laccase. Preliminary characterization of the antiserum showed that it precipitated several other proteins present in conidiating cells as well. We were, nevertheless, able to use it to estimate unambiguously laccase protein levels because we found that the enzyme retained its activity even when immunoprecipitated. This property allowed us to identify which of several precipitates formed during rocket immunoelectrophoresis contained laccase by comparison of parallel gels stained either generally for proteins or enzymatically for *p*-diphenol oxidase.

Protein extracts were prepared from cells harvested at various times, and samples containing equal amounts of protein were electrophoresed into duplicate gels containing antiserum (15, 26). The result of this experiment is presented in Fig. 3; Fig. 3A shows a gel stained with brilliant blue-

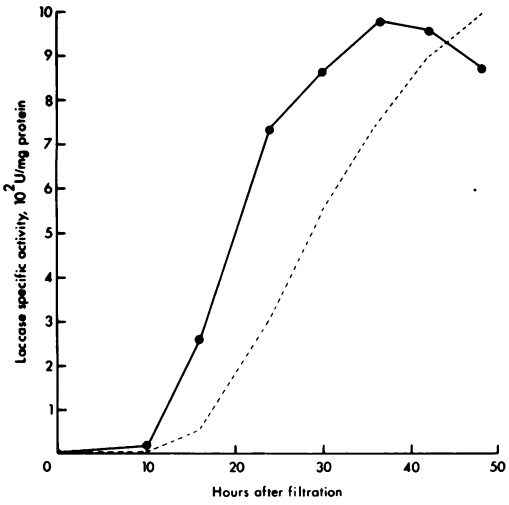

FIG. 2. *Changes in laccase specific activity during conidiation. Filter paper cultures were harvested at various times, frozen in liquid N_2, and suspended in 100 mM PB. Debris and intact cells were removed by centrifugation, and the supernatant fraction was dialyzed overnight against 100 mM PB. The extracts were again clarified by centrifugation and then assayed for laccase activity by measuring the oxidation of DMP spectrophotometrically. Protein concentrations were estimated by a dye-binding assay. Symbols: ●, laccase specific activity; -----, conidia per plate (from Fig. 1).*

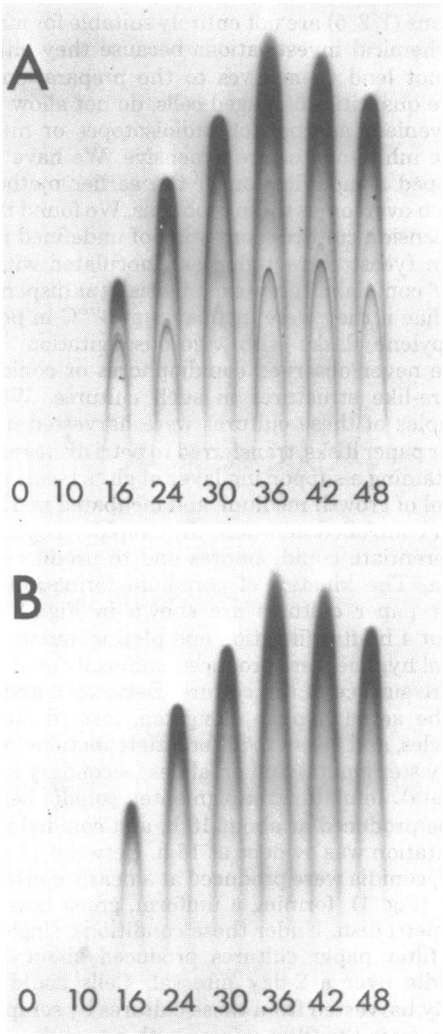

FIG. 3. *Quantitation of laccase protein levels by rocket immunoelectrophoresis. Protein extracts were prepared as described in the legend to Fig. 2 and concentrated by ultrafiltration. Equal amounts of protein (25 μg/well) were then electrophoresed into agarose gels containing laccase antiserum. The times after filtration at which the cultures were harvested are indicated. (A) Gel stained with brilliant blue R; (B) gel stained enzymatically with DMP.*

R. No immunoprecipitates were formed in the lane containing proteins from cells which had just been induced to conidiate. On the other hand, several were visible in each of the lanes containing proteins from cells harvested at 10 to 48 h. Figure 3B shows a parallel gel which was stained enzymatically by immersing it in a substrate (DMP) solution. Laccase immunoprecipitates were clearly congruent with one set of the generally stained precipitates shown in Fig. 3A.

The fact that each of the antigens lacking *p*-diphenol oxidase activity was present only in conidiating cells suggested the possibility that they were related to the enzyme itself in some way. If this were the case, the additional immunoprecipitates would influence the analysis of the data shown in Fig. 3. To investigate this possibility, we compared proteins extracted from mutant and wild-type cells. Conidia were treated with the mutagen 1,2,7,8-diepoxyoctane, which produces a high proportion of deletions (11), and several strains were isolated which formed yellow conidia. Protein extracts from these mutants were found to lack detectable laccase activity. A number of yellow mutants were tested for immunologically cross-reacting material by fused rocket immunoelectrophoresis with wild-type proteins (23). Reactions of identity were observed for all of the antigens except laccase. Since mutation at the *yA* locus only affected the laccase protein, the additional antigens precipitated by the antiserum are not directly related to the enzyme itself.

The relative amounts of laccase protein present in extracts from cultures which had been induced to conidiate for various times were estimated by calculating the areas enclosed by the immunoprecipitates (height × width at one-half height) shown in Fig. 3B. As shown in Fig. 4, relative laccase protein levels closely paralleled laccase enzyme specific activities.

As mentioned above, the homogenization procedure we used might not result in the release of proteins sequestered within the cells. Thus, it was possible that an inactive laccase precursor might not have been detected in the experiments just described. We therefore disrupted previously extracted cells by blending with glass beads, prepared protein extracts, and tested them for the presence of immunoprecipitable proteins. No such proteins were observed in the extracts from cells which had just been induced to conidiate, and in no case did the amount of immunoprecipitable protein exceed 5% of that extracted by grinding the cells in liquid N_2 and stirring them in buffer.

Changes in the relative rates of laccase protein synthesis. The data presented above suggested that increased laccase enzyme activity

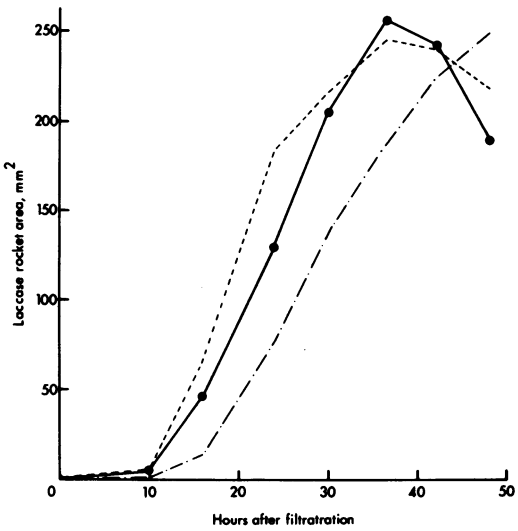

FIG. 4. *Alterations in laccase protein levels during conidiation. The gel shown in Fig. 3B was analyzed by determining the area enclosed by the laccase immunoprecipitate (rocket area). Rocket areas are plotted versus time after filtration and plating* (●). *Conidium production* (—·—) *and laccase enzyme specific activities* (·····) *are indicated for comparison.*

was the result of de novo synthesis and concomitant accumulation of laccase protein. However, other explanations for these results are also possible. We therefore estimated the relative rates of laccase protein synthesis in cultures which had been induced to conidiate for various times. The strategy we used for this analysis involved radioactively pulse-labeling cells, extracting proteins, preparing immunoprecipitates, and fractionating them on SDS-polyacrylamide gels. Preliminary experiments showed that, as expected, several radioactively labeled peptides were immunoprecipitated. To determine which of the several denatured peptides was laccase, we used one of the yellow mutants which did not produce laccase cross-reacting material.

A mutant and the wild-type strain were induced to conidiate on medium containing $^{35}SO_4^{2-}$. After 24 h of incubation, we prepared protein extracts and then fractionated immunoprecipitates prepared from them by SDS-polyacrylamide gel electrophoresis (14). A fluorogram (3) of one such gel is shown in Fig. 5. Several labeled peptides were precipitated from the wild-type and mutant protein extracts which comigrated on the gels. One peptide, having an apparent molecular weight of 85,000, was present in the extract from wild-type cells and absent from that from the mutant. We have determined that the molecular weight of laccase under nondenaturing conditions is 90,000 ± 3,000 (see Ma-

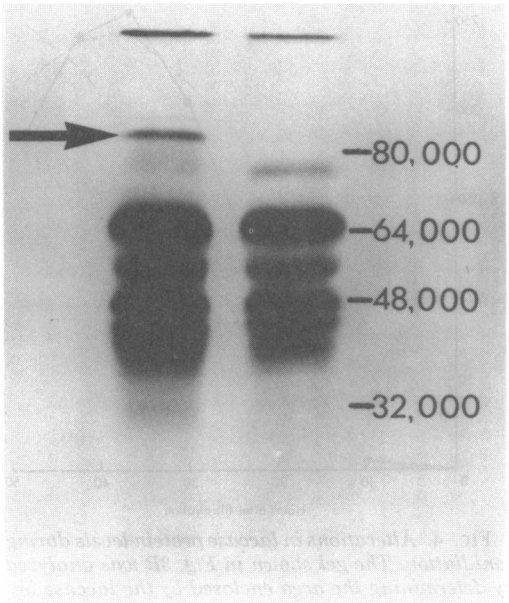

FIG. 5. *SDS-polyacrylamide gel electrophoresis of immunoprecipitated protein from the wild-type strain and a yellow-spored mutant. A yellow-spored mutant (produced by treatment of conidia with diepoxyoctane) which lacked laccase enzyme activity and immunologically cross-reacting material was selected. Mutant and wild-type cells were induced to conidiate on medium containing $^{35}SO_4^{2-}$ and harvested after 24 h of incubation, and protein extracts were prepared as described in the legend to Fig. 2. Immunoprecipitates formed by treatment of samples (containing equal radioactivity) with laccase antiserum were prepared, and fractionated on 12% SDS-polyacrylamide gels, and the gels were analyzed by fluorography. The positions and molecular weights of cross-linked hemoglobin standards are shown. The arrow indicates the position of an 85,000-dalton peptide which is absent from the mutant. Wild-type proteins are in the left lane; mutant proteins are in the right.*

terials and Methods), which is in reasonable agreement with that reported by Clutterbuck (5) (107,000) and very similar to that of the 85,000-dalton peptide shown in Fig. 5. Since only one major peptide band present in the wild-type strain was absent from the mutant, and because the molecular weight of this peptide was very similar to that of nondenatured laccase, this experiment argues that the 85,000-dalton peptide was laccase. This conclusion is supported by experiments in which fractions obtained during Sephadex G-200 chromatography were analyzed by SDS-gel electrophoresis. We found that the 85,000-dalton peptide precisely coeluted with laccase enzyme activity, whereas other peptides did not. Since mutation at the *yA* locus affected only the laccase protein, the results shown in Fig. 5 support our earlier conclusion (see above) that the additional peptides precipitated by the antiserum are not related to the enzyme itself.

Cultures were pulse-labeled for 2 h with $^{35}SO_4^{2-}$ as described in Materials and Methods, and protein extracts were prepared. Immunoprecipitates from samples containing equal amounts of acid-insoluble radioactivity (5×10^5 cpm) were then solubilized and fractionated by SDS-polyacrylamide gel electrophoresis. Fluorograms of these gels were analyzed by densitometry to determine the relative amount of ^{35}S which had been incorporated into the 85,000-dalton laccase protein (see Fig. 5). The results of this experiment are summarized in Fig. 6. Cells which were exposed to $^{35}SO_4^{2-}$ between 0 and 2 h after filtration and plating contained no detectable, labeled laccase even when films were heavily overexposed for samples from later times. Significant incorporation of ^{35}S was apparent at 8 to 10 h. Thereafter, incorporation rates increased, reached a peak at 22 to 24 h, and then

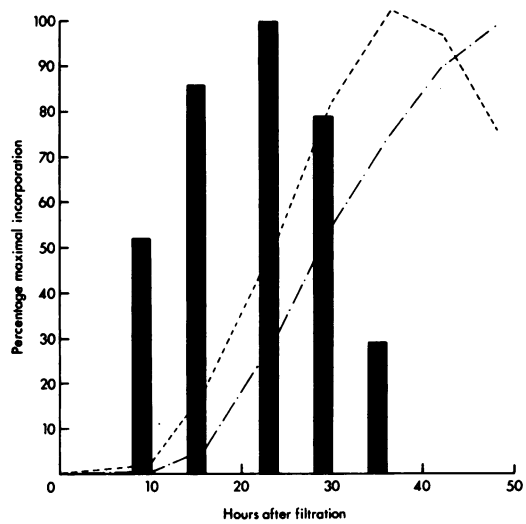

FIG. 6. *Changes in relative rates of laccase synthesis. Filter paper cultures were labeled for 2 h with $^{35}SO_4^{2-}$ at various times after inducing conidiation as described in Materials and Methods, and protein extracts were prepared. Immunoprecipitates from samples containing equal amounts of acid-insoluble radioactivity were fractionated on 12% acrylamide gels, and fluorograms were prepared (see legend to Fig. 5). The films were scanned in a Gilford spectrophotometer at 600 nm, and the relative intensities of the 85,000-dalton laccase bands (relative incorporation rates) were estimated by cutting out the peaks from the recorder chart and weighing them. No incorporation into laccase was detected in the 0-to-2-, 40-to-42-, or 46-to-48-h samples. Conidium production* (○) *and accumulation of immunoprecipitable laccase protein* (-----) *are shown for comparison.*

declined rapidly. No laccase synthesis was detected in cells labeled at 40 to 42 or 46 to 48 h after filtration. Assuming that proteins in general are synthesized from a common pool of sulfur-containing amino acids, these incorporation rates should be directly proportional to relative rates of synthesis.

Effect of cycloheximide on laccase accumulation. If enhanced laccase expression were due to de novo synthesis of enzyme protein, as indicated by the data presented above, then inhibition of protein synthesis at any time after inducing conidiation should also inhibit further laccase accumulation. We therefore examined the effect on laccase specific activities of addition of cycloheximide to conidiating cultures. Filter paper cultures which had been incubated for various times were transferred to petri dishes containing 25 μg of cycloheximide per ml. At later times, the cultures were harvested and protein extracts were prepared and assayed for enzyme activity (Fig. 7). Addition of the antibiotic at any time after inducing conidiation completely inhibited further accumulation of laccase. Addition of cycloheximide after 10 h of incubation resulted in a rapid decline in laccase specific activity. The reason for this rapid decline is currently unclear. As shown in Fig. 6, laccase protein synthesis rates were nil after about 40 h of incubation. Therefore, the reduction in laccase enzyme specific activity occurring between 42 and 48 h in untreated cultures (Fig. 2 and 7) should reflect normal turnover of the protein. The decline induced by cycloheximide was much more rapid than this. A similar result was obtained when 5-fluorouracil was added to cultures (see below). Neither of the antibiotics inhibited laccase enzyme activity in vitro.

Effect of inhibitors of RNA synthesis on laccase accumulation. If de novo synthesis of laccase protein were the result of increased availability of laccase mRNA for translation, and if increases in translatable laccase mRNA required continuous RNA synthesis, then cessation of RNA synthesis should prevent further enzyme accumulation. We therefore investigated the effects of addition of inhibitors of RNA function or synthesis on laccase specific activities. Filter paper cultures were treated with 50 μg of 5-fluorouracil per ml (21) as described above for cycloheximide (Fig. 8). The concentration of the compound used was the lowest which inhibited conidiophore development when added at the time the cells were plated. As we observed with cycloheximide, 5-fluorouracil completely inhibited accumulation of laccase when added at any time after inducing conidiation. When added at times after 10 h, this compound caused a rapid decline in laccase specific activities.

We also investigated other inhibitors. We found that actinomycin D did not effectively inhibit conidiophore differentiation or spore production even when added to a final concentra-

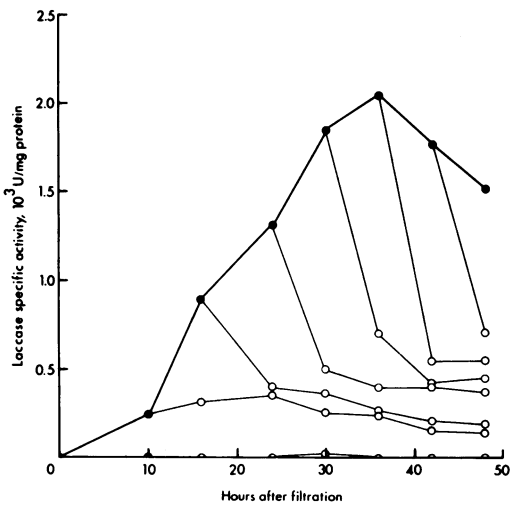

FIG. 7. *Effect of cycloheximide on laccase accumulation. Cultures were induced to conidiate by filtration and plating. At various times, sets of filters were transferred to medium containing 25 μg of cycloheximide per ml. At later times, cultures were harvested and protein extracts were prepared and assayed as described in the legend to Fig. 2. Symbols: ●, enzyme activities before addition of cycloheximide; ○, specific activities at times after the addition of the antibiotic.*

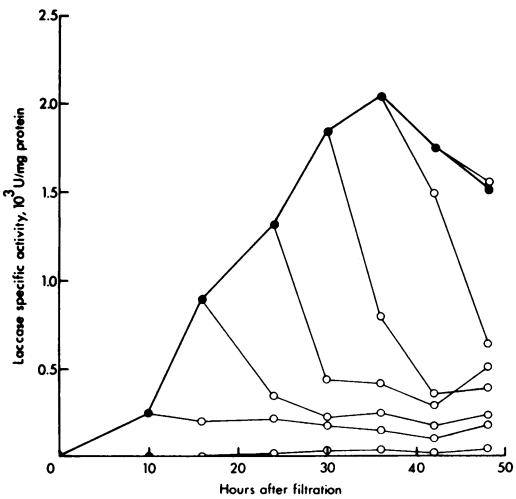

FIG. 8. *Effect of 5-fluorouracil on laccase accumulation. Conidiating cultures were treated with 5-fluorouracil (50 μg/ml), and laccase was assayed as described in the legend to Fig. 7. Symbols: ●, enzyme activities at the time of addition; ○, specific activities at times after the addition of the compound.*

tion of 200 μg/ml. However, a mixture of actinomycin D and daunomycin (200 μg/ml each) (8) was partially effective. These antibiotics inhibited laccase accumulation when added at any time after inducing conidiation, although not completely (data not shown). We observed that restricted areas of cultures treated with the actinomycin D/daunomycin mixture, above regions where the supporting filter was not in direct contact with the medium, were not affected by the antibiotics (in terms of conidiophore and spore development), whereas the majority of the mycelium was. The unaffected areas were probably responsible for the production of the limited amounts of laccase activity observed in these experiments.

DISCUSSION

The asexual spores, or conidia, of *A. nidulans* contain in their walls a green pigment which is not found in other cell types. Synthesis of the spore pigment is mediated, in part, by a *p*-diphenol oxidase, or laccase, enzyme encoded at the *yA* genetic locus (5, 20). This enzyme acts to convert a yellow pigment intermediate to the mature green form. Laccase activity, which cannot be detected in vegetative hyphae, begins to accumulate soon after the onset of conidiophore differentiation. Thus, laccase is a developmentally regulated enzyme which is well defined genetically, biochemically, and physiologically. For these reasons, we have begun to investigate the molecular processes controlling expression of the laccase gene of *A. nidulans*. In this paper we have presented results which (i) show that increases in laccase enzyme activity occurring during conidiation are the result of regulated, de novo synthesis of the protein and (ii) suggest that increased laccase synthesis rates may be the result of transcriptional activation of the *yA* gene.

We found that cells growing exponentially in submerged culture lacked detectable laccase enzyme activity and neither contained nor synthesized immunoprecipitable laccase protein. When such cultures were induced to conidiate under defined conditions, they began to accumulate laccase enzyme activity coincident with the first appearance of immature conidia on the conidiophores. Accumulation of enzyme activity was closely paralleled by increases in the amount of immunoprecipitable laccase protein and was completely dependent on continued protein synthesis. In addition, we observed that laccase protein synthesis rates underwent a transient increase which was directly correlated with the kinetics of laccase accumulation. These results are most consistent with regulated translation of laccase mRNA.

Increased rates of laccase synthesis could be controlled at the level of translation, by post-transcriptional modifications of laccase mRNA, by changes in processing or transport patterns of nuclear, laccase pre-mRNA, or by increased availability of laccase mRNA due to transcriptional activation of the *yA* gene. We observed that both 5-fluorouracil, an inhibitor of RNA function, and a mixture of actinomycin D and daunomycin, inhibitors of RNA synthesis, prevented further accumulation of laccase enzyme activity. Ryder and Peberdy (21) used 5-fluorouracil and cycloheximide to investigate mechanisms controlling production of chitin synthetase in germinating spores of *A. nidulans*. They found that cycloheximide prevented spore germination and synthesis of the enzyme, whereas 5-fluorouracil inhibited germination but allowed increases in chitin synthetase activity. Their results suggest (i) that chitin synthetase is synthesized de novo from preformed mRNA and (ii) that cycloheximide and 5-fluorouracil can be used to selectively distinguish translational and transcriptional control mechanisms in *Aspergillus*. If the action of the inhibitors we used in this study is reasonably specific, as seems to be the case with 5-fluorouracil, our results indicate that laccase accumulation is dependent on continued RNA synthesis. This implies that increased laccase protein synthesis rates were not due solely to translational control mechanisms or post-transcriptional modifications involving preformed laccase mRNA.

Accumulation of a translationally active mRNA could be regulated by the transcriptional activity of its structural gene or by the selection of nuclear pre-mRNA transcripts for transport to the cytoplasm. Regulation at the level of RNA processing and transport may be common in animals and higher plants (6, 12, 13, 27). Inhibitor studies of the type reported here cannot distinguish between transcriptional control mechanisms and selection for transport of constitutively synthesized transcripts. Therefore, in the absence of further evidence, we cannot exclude one or the other alternative in the case of laccase expression. However, it appears that the fungi in general lack heterogeneous nuclear RNA populations of the sort observed in higher eucaryotic forms in that the complexity of nuclear RNA is the same as that of mRNA (10, 25, 28). This observation implies that the majority of nuclear transcripts are transported to the cytoplasm. We therefore suggest that it is likely that control of laccase expression during development occurs at the level of gene transcription.

We are currently investigating this possibility by using recombinant DNA clones.

ACKNOWLEDGMENTS

This research was supported by a grant from the National Institutes of Health. D.J.L. was a Wayne State University Fellow.

We thank Robert Goldberg, Albert Siegel, John Wireman, and T. T. Tchen for helpful discussions; Donald Schwartz for his contributions; Sue Lyman for her assistance in preparing the manuscript; and Roberta Simone for her valuable assistance.

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