INFECTION AND IMMUNITY, Oct. 1989, p. 3081–3090
0019-9567/89/103081-10$02.00/0

Vol. 57, No. 10

Regulation of *Trichophyton rubrum* Proteolytic Activity

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Received 3 March 1989/Accepted 23 June 1989

***Trichophyton rubrum* is the most common dermatophyte of humans and normally colonizes the superficial layers of the epidermis (stratum corneum). Several proteinases with a possible role in the metabolism of host proteins have been purified from this fungus. The regulation of these enzymes and their role in fungal metabolism were studied at the biochemical level. General proteolytic (azocollytic) activity was repressed when log-phase cultures of *T. rubrum* were grown in a minimal medium that contained readily metabolized sources of carbon, nitrogen, sulfur, and phosphorus. When either carbon, nitrogen, or sulfur was deleted from this minimal medium, azocollytic activity was derepressed. In all cases a high-molecular-weight activity (M_r, >200,000) was expressed. A 71,000-M_r proteinase was observed in nitrogen-depleted cultures, and proteolytic species of M_r 124,000 and 27,000 were secreted in sulfur-depleted cultures. The addition of either inorganic ($MgSO_4$, Na_2SO_3, NaS_2O_3) or organic (methionine, cysteine) sulfur to the sulfur-depleted medium repressed the expression of azocollytic activity. In contrast, keratinolytic activity was not repressed by carbon, nitrogen, or sulfur but instead was induced when a protein source was included in the minimal medium. Stationary-phase cultures of *T. rubrum* secreted all proteolytic activities constitutively. Unlike log-phase cultures, the stationary-phase cultures secreted azocollytic, elastinolytic, and keratinolytic activity in minimal medium. These activities fell in the carbon-, nitrogen-, and phosphorous-depleted media but remained high in sulfur-depleted medium. The following model is proposed for the regulation of *T. rubrum* proteolytic activity. In the initial stages of infection, *T. rubrum* grows logarithmically. In this state, proteolytic activity is derepressed whenever carbon, nitrogen, or sulfur is lacking in the fungal milieu. The general proteinases produced would act on the nonkeratinous proteins in the stratum corneum. There are probably peptidases, as yet unidentified, that would cleave the peptides generated by the initial proteolysis into amino acids. These amino acids would provide the cell with a source of carbon, nitrogen, and sulfur. Under these conditions, the expression of general proteinases would be repressed, whereas specific keratinases would be induced in this nutrient-rich environment. Disease may occur when the fungus reaches stationary phase, when proteinases are secreted constitutively. These enzymes may directly or indirectly incite a host response, resulting in the inflammatory manifestations of dermatophytosis.**

Trichophyton rubrum is a dermatophytic fungus that parasitizes keratinized tissues (stratum corneum and nails) of humans. It is hypothesized that this organism produces exocellular proteinases to utilize host proteins as a nutrient source. Several proteinases have been described and purified from the culture medium of *T. rubrum*. In this paper the culture medium into which proteins have been secreted will be referred to as conditioned medium (CM). Meevootisom and Niederpruem (20) have shown that the CM of keratin-grown *T. rubrum* contains proteolytic activity capable of degrading casein, bovine serum albumin (BSA), collagen, elastin, guinea pig hair, and keratin. A serine proteinase with an M_r of 35,000 has been purified from the CM of Sabouraud dextrose broth-grown *T. rubrum* (24). This enzyme has an alkaline pH optimum and degrades casein, azoalbumin, and α-*N*-benzoyl-L-arginine ethyl ester but has no activity against defatted human hair. Two proteinases with keratinolytic activity have been purified from the CM of *T. rubrum* (2). These serine proteinases have molecular weights of 93,000 and 71,000 under nonreducing conditions. Both enzymes exist as dimers of a smaller molecular-weight-subunit, M_r 44,000 and 36,000, respectively, and are serine proteinases with a pH optimum of 8.0 and a calcium dependence of 1 mM. They degrade azocoll, elastin, keratin, and synthetic peptide substrates with hydrophobic amino acids in their P-1 site, e.g., succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (*p*NA). Finally, a 27,000-M_r, serine proteinase has been purified from *T. rubrum* CM (1; G. Apodaca and J. H. McKerrow, J. Cell Biol. **107**:183, 1989). This enzyme degrades azocoll, succinyl-Ala-Ala-Pro-Phe-*p*NA, *t*-butyloxy carbonyl (Z)-Ala-Ala-Leu-*p*NA, and several components of the extracellular matrix, including type III collagen, type IV procollagen, laminin, and fibronectin.

At this time little is known about how *T. rubrum* regulates the expression of these proteinases. Meevootisom and Niederpruem (20) have reported that glucose or amino acids can repress the expression of extracellular proteinase activity. Dermatophytes can clear particulate elastin in an agar-plate assay; however, the addition of 2% glucose to their agar medium prevents the clearing of the elastin for several species of dermatophytes, including *T. rubrum*. Glucose also represses the production of guinea pig-hair-hydrolyzing enzymes for cultures of liquid-grown *T. rubrum*, as does erythrose, sorbitol, galactose, glucose, fructose, or mannitol. Amino acids are also inhibitory, suppressing proteinase expression, except in the case of isoleucine, serine, and a complex of Casamino Acids. The results of these studies indicate that as easily metabolized substrates are made available to *T. rubrum* proteinase secretion is not required.

Clues to how proteinase expression might be regulated in *T. rubrum* were sought from the more extensive examination of proteinase regulation in other filamentous fungi, especially *Aspergillus nidulans* and *Neurospora crassa*. Previous work has demonstrated that the expression of proteolytic enzymes is reduced when these organisms are grown in a

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rich medium, or one that contains easily metabolized substrates such as glucose, amino acids, nitrate, or ammonium (4–7, 18). In contrast, nutrient depletion or growth as stationary-phase cultures increases production of proteinases (18). Early studies with *Aspergillus niger* showed that the depletion of sulfur from its medium resulted in the secretion of an acid proteinase (24).

The purpose of this study was to confirm and extend the earlier work of Meevootisom and Niederpruem on proteinase regulation in *T. rubrum*. In particular, we were interested in determining whether *T. rubrum* proteinases are regulated in a similar fashion to other filamentous fungi and whether there were any differences in the way log-phase and stationary-phase fungi regulate expression of their proteolytic enzymes. We have found that when *T. rubrum* medium was depleted of a source of carbon, nitrogen, or sulfur, log-phase cultures of fungus responded by secreting proteinases. When the cultures reached stationary phase, the proteinases were expressed constitutively.

MATERIALS AND METHODS

Culture of *T. rubrum*. *T. rubrum* IFO 9185 is a stock culture obtained from M. Asahi, Kyushu University, Fukuoka, Japan. The strain was routinely maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) slants stored at 4°C and was subcultured by streaking the fungus onto the same medium every 2 months. *T. rubrum* spore suspensions were also maintained as stock cultures. To obtain the spore suspensions the fungus was grown on Sabouraud dextrose agar slants at 30°C, and when the fungus completely covered the surface of the slant the tube was flooded with approximately 5 ml of sterile distilled water. The water was pipetted rapidly up and down to dislodge the spores, but careful attention was paid to not disrupt the integrity of the actual agar surface. If necessary, this spore suspension was filtered through sterile gauze to remove bits of agar and mycelium. These spore suspensions were stored at ambient temperature in tightly stoppered, sterile containers.

To obtain stationary-phase cultures of this fungus a sample of the spore suspension, typically 500 μl, was added to 100 ml of Sabouraud dextrose broth in 75-cm^2 plastic tissue culture flasks (Costar, Cambridge, Mass.) and incubated at 30°C for approximately 3 to 4 weeks. At this time, a thick, white, floating mat of fungus had formed on the surface of the medium. The stationary-phase mats were washed three times with phosphate-buffered saline (Cell Culture Facility, University of California, San Francisco), and then circles (diameter, approximately 1 cm) were cut out of the stationary-phase mats with the open end of a sterile 120- by 17-mm conical centrifuge tube (Sarstedt, Hayward, Calif.). The circles of fungus were placed in 24-well tissue culture plates (Corning Glass Works, Corning, N.Y.) that contained 1 ml of minimal medium or a medium depleted of either sulfur, carbon, nitrogen, or phosphorus. Minimal medium contained 0.25% (wt/vol) glucose as a carbon source, 50 mM $(NH_4)_2PO_4$ as a nitrogen source, 3.4 mM KH_2PO_4 and 5.75 mM K_2HPO_4 as phosphorus sources, and 2 mM $MgSO_4 \cdot 7H_2O$ as a sulfur source (all obtained from Sigma Chemical Co., St. Louis, Mo.). Carbon-, nitrogen-, sulfur-, or phosphorus-depleted medium was made by deleting the appropriate compound from the minimal medium, with the following changes: in the phosphorus-depleted medium the potassium phosphate salts were left out of the medium and 50 mM NH_4HCO_3 (Sigma) was substituted for the $(NH_4)_2PO_4$, and in the sulfur-depleted medium, 2 mM $MgCl_2$ (Sigma) replaced the 2 mM $MgSO_4$. At times, 0.25% (wt/vol) elastin powder (Sigma) or 0.5% (wt/vol) keratin powder (ICN Pharmaceuticals Inc., Cleveland, Ohio) was added to the minimal medium. Cultures were incubated at 30°C for up to 1 week, and samples of CM were assayed for proteolytic activity.

To culture log-phase fungus, a 2-liter Erlenmeyer flask containing a spin bar was filled with 1 liter of sterile keratin salts or minimal medium and inoculated with 5 ml of spore suspension. Keratin salts medium (20) contained the following (per liter): 5 g of keratin powder, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.46 g of KH_2PO_4, 1 g of K_2HPO_4, and 100 μg of thiamine hydrochloride (Sigma). The fungus was incubated at ambient temperature with constant stirring on a magnetic stir plate (Corning) for 5 to 7 days in the case of minimal medium or 2 to 3 weeks in the case of keratin salts. A 30-ml sample of this culture was placed in a 50-ml sterile plastic centrifuge tube (Corning) and spun at a setting of 7 in an International Machine Corp. (Needham Heights, Mass.) tabletop centrifuge for 10 min at ambient temperature. The supernatant was discarded, and the pellet was suspended in 45 ml of sterile distilled water and centrifuged for an additional 10 min at the same setting. After two additional washes the pellet was finally suspended in 5 ml of sterile distilled H_2O, which gave an A_{600} of approximately 2.6. A 300-μl sample (equivalent to 1.5 mg [dry weight] of fungus grown in keratin salts medium) of these sterile washed hyphae (1.5 ml in the case of the carbon- or nitrogen-depleted cultures) was added to 10 ml of the above-mentioned media in 60- by 15-mm sterile plastic petri dishes (Fisher Scientific Co., Pittsburgh, Pa.) and incubated at 30°C for up to a week. In some experiments the sulfur-depleted medium was supplemented with 0.5 to 50 mM L-methionine or L-cysteine, 50 mM L-glycine, L-isoleucine, L-proline, L-alanine, L-phenylalanine, or L-serine, or 2.5% (wt/vol) Casamino Acids (all obtained from Sigma). At times, proteins such as 0.5% (wt/vol) elastin powder, 0.5% (wt/vol) keratin powder, 0.5% (wt/vol) type I swine skin gelatin (Sigma), or 0.5% (wt/vol) pentax fraction V bovine serum albumin (Sigma) were added to a medium that lacked a source of carbon, nitrogen, and sulfur, i.e., the protein plus the potassium phosphate buffer plus 2 mM $MgCl_2$. These cultures were incubated at 30°C for up to 1 week, and samples of CM were assayed for proteolytic activity. All culture media were sterilized by autoclaving; when media were appended with glucose, proteins, or amino acids, all ingredients were autoclaved at the same time.

The kinetics of *T. rubrum* growth in minimal medium and Sabouraud dextrose broth was also determined. The fungus was cultured as described above. After incubation for up to 30 days the log-phase fungal cultures were filtered onto glass fiber filters (Whatman Ltd., Maidstone, England), and Sabouraud dextrose broth-grown fungal cultures were filtered onto filter paper (Whatman). The 1-cm-diameter circles of fungus cut from stationary-phase mats were placed on glass fiber filters, and excess liquid was removed by vacuum filtration. Then the fungus, on pretared filters, was placed in a vacuum oven and dried for 3 to 5 h at 140°C. The dried fungus was then placed in a petri dish whose bottom was covered with drierite (W. A. Hammond Drierite Co., Xenia, Ohio) and allowed to cool to room temperature before being weighed on a Metler (Hightstown, N.J.) model AE 163 balance.

Azocoll degradation. CM, typically 50 to 100 μl, was incubated with 3 to 5 mg of azocoll (Sigma or Calbiochem, La Jolla, Calif.) in 100 mM Tris hydrochloride (Bio-Rad

Laboratories, Richmond, Calif.) (pH 8.0)–1 mM $CaCl_2$ (Sigma) buffer in 1.6-ml Eppendorf tubes for 4 to 24 h at 37°C. The final reaction volume was 1 ml. After incubation, the samples were centrifuged for 5 min in a Beckman (Palo Alto, Calif.) microfuge. Degradation of azocoll was measured by determining the A_{520} of the supernatant in a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). One unit of azocoll-degrading activity was defined as a change of 0.1 A_{520} unit per hour.

Elastin degradation. Purified ox ligamentum nuchae elastin (60 mesh; Elastin Products Corp., Pacific, Mo.) was labeled with $NaB[^3H]_4$ and assayed as described by McKerrow et al. (19). The radiolabeled elastin was suspended to a final concentration of 2 mg/ml in a 300 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$ buffer. A 100-μl sample was mixed with 50 to 100 μl of CM and sufficient H_2O to bring the reaction volume up to 300 μl and incubated at 37°C for 18 to 24 h. After this incubation period, the undigested elastin was sedimented by centrifugation for 5 min in a microfuge, 100 μl of the supernatant was mixed with 7 ml of Opti-Fluor scintillation fluid (Packard Instruments Co., Downers Grove, Ill.), and the radioactivity was counted in a liquid scintillation spectrophotometer (Beckman model LS 100C). Control incubations with water were included with each assay. The total amount of radioactive elastin available for degradation was determined by adding 10 μl (160 μg) of 2× crystallized procine pancreatic elastase (Sigma) to a control reaction. One unit of elastase activity will degrade 1 μg of elastin per h.

Keratin degradation. Keratin degradation was assayed by using keratin azure (Sigma), a dyed wool product. CM (50 to 100 μl) was incubated with 3 to 5 mg of keratin azure in a 100 mM glycine–NaOH (pH 9.0)–1 mM $CaCl_2$ buffer for 48 to 72 h at 37°C. The final reaction volume was 1 ml. After incubation, the reaction was spun in a microfuge for 5 min. The degradation of keratin azure was measured by determining the A_{595} of the supernatant in a spectrophotometer. A change of 0.01 A_{595} units per h equals 1 U of keratinase activity.

Substrate gel analysis. General proteinase activity was also assayed in sodium dodecyl sulfate-polyacrylamide gels copolymerized with substrate (2), in which proteinases can be detected and molecular weights can be determined simultaneously. The resolving gel (10 or 12% polyacrylamide) was polymerized in the presence of 0.12% pig skin type I gelatin (Sigma). The samples were not boiled or reduced, and the stacking gel (4% polyacrylamide) did not contain substrate. The gels (0.75 mm thick) were prepared in a small-slab-gel apparatus (Hoefer, San Francisco, Calif.). CM was diluted with an equal volume of 2× sample buffer (0.25 M Tris hydrochloride [pH 6.8], 20% [vol/vol] glycerol, 0.2% [wt/vol] sodium dodecyl sulfate, and 0.005% [wt/vol] bromophenol blue), and 15 to 25 μl was loaded on the gel. After electrophoresis, the gels were washed in 100 ml of 2.5% (vol/vol) Triton X-100 (Sigma) for 30 min to remove the sodium dodecyl sulfate. The gels were then incubated for 12 to 24 h at 37°C in the same buffered solution used in the azocoll assay. We later found that this incubation period could be cut down to 2 to 4 h with no significant loss of visualization of proteinase activity. The gels were stained with 0.1% Coomassie brilliant blue R-250 (Bio-Rad) in water-methanol-acetic acid (5:5:1, vol/vol) for 30 min and then destained in 45% (vol/vol) methanol–3% (vol/vol) acetic acid until the proteolytic bands could be visualized. Molecular weight standards (Bio-Rad) were myosin (200,000), β-galactosidase (116,300), phosphorylase *b* (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,500).

Inhibition assays. Inhibitors were preincubated with 50 to 100 μl of sulfur-depleted CM for 20 min at ambient temperature before azocoll substrate was added to the reaction mixture. 1,10-Phenanthroline and *N*-ethylmaleimide (both from Sigma) were made up as 150 mM stocks in ethanol, whereas the chloromethylketone inhibitor Phe-Gly-Ala-Leu-CH_2Cl (Enzyme System Products, Livermore, Calif.) was made up as a 1-mg/ml stock in dimethyl sulfoxide (Sigma). For inhibitors requiring ethanol or dimethyl sulfoxide in stock solutions, an equivalent amount of solvent was run as a control.

Hydrolysis of peptide substrates. The rate of hydrolysis of peptide-*p*NA substrates was measured at 410 nm on a Gilford spectrophotometer fitted with a model 6050 chart recorder. The reactions were initiated by adding 20 to 50 μl of sulfur-depleted CM to 0.5 ml of a solution containing 1 mM substrate, succinyl-Ala-Ala-Pro-Phe-*p*NA (Vega, Tucson, Ariz.), in 100 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$. Because of solubility problems associated with Z-Ala-Ala-Leu-*p*NA (Peninsula Laboratories, Belmont, Calif.), the conditions were different for this substrate. For these reactions the substrate was made up as a 0.5-mg/ml stock in dimethylformamide (Sigma); then 200 μl of this substrate solution was added slowly to 925 μl of 100 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$, and 75 μl of the sulfur-depleted CM was added to this buffered solution of substrate. An ε value of 8800 M/cm at 410 nm was used for the liberated 4-nitroaniline.

RESULTS

Kinetics of *T. rubrum* growth. The kinetics of log-phase *T. rubrum* growth in minimal medium are shown in Fig. 1A. There was a period of continuous growth for several days until the fungus reached a maximum dry weight of 15 mg at day 9. At this time, the organism completely covered the liquid surface; however, unlike cultures grown to stationary phase in Sabouraud dextrose broth, these cultures did not form mats. Growth at the slower rate continued until day 12 or 13, at which time the fungus appeared to be dying and its mass decreased (data not shown). *T. rubrum* growth in Sabouraud dextrose broth inoculated with a spore suspension was also assessed (Fig. 1B). The fungus reached a maximal dry weight of approximately 550 mg after 3 to 4 weeks of incubation. At this time, a thick, white mat of fungus formed at the surface of these liquid cultures. Once the mat formed, its mass appeared to remain constant even when fresh medium was added to these cultures. To quantify this observation, 1-cm-diameter plugs of the stationary-phase mats were washed and placed in fresh minimal medium. The dry weight of stationary-phase fungus remained relatively constant throughout the 7-day incubation (Fig. 1C); the fungus did not appear to be growing.

Repression of log-phase proteinase expression. Fungi respond to a lack of carbon, nitrogen, sulfur, or phosphorus in their environment by producing a number of proteins responsible for the assimilation of important nutrients. Proteinase production is one aspect of that response. Log-phase *T. rubrum* was washed and then cultured in minimal medium or a medium lacking a carbon, nitrogen, sulfur, or phosphorus source. The results of nutrient depletion on proteinase production are shown in Table 1.

Very little azocollytic or elastinolytic activity was produced in the minimal medium, although there was some

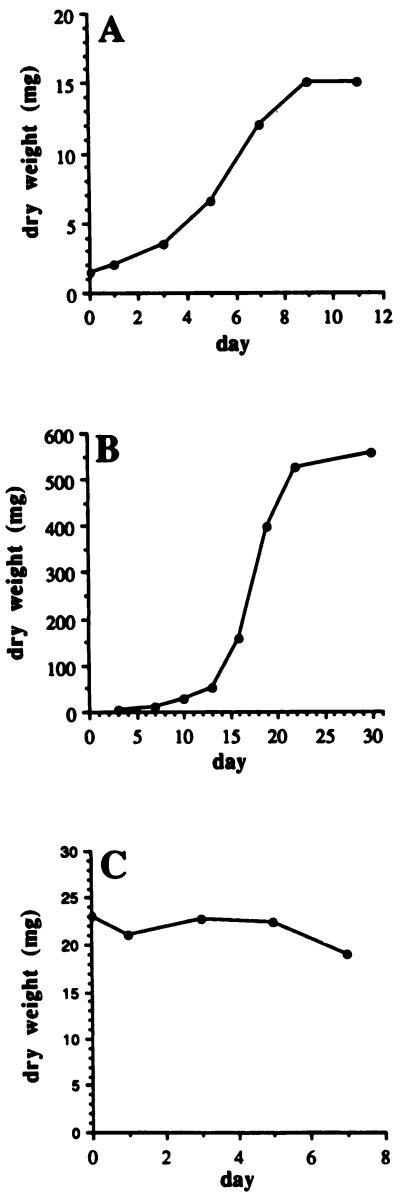

FIG. 1. Kinetics of *T. rubrum* growth. (A) Fungus grown in keratin salts medium was washed and inoculated onto 60- by 15-mm petri dishes filled with 10 ml of minimal medium. This medium contains 0.25% (wt/vol) glucose as a carbon source, 50 mM $(NH_4)_2PO_4$ as a nitrogen source, 3.4 mM KH_2PO_4 and 5.75 mM K_2HPO_4 as phosphorus sources, and 2 mM $MgSO_4 \cdot 7H_2O$ as a sulfur source. After incubation at 30°C for up to 11 days, the fungus was dried in a vacuum oven and weighed (mean; $n = 3$). (B) *T. rubrum* spores were inoculated into 100 ml of Sabouraud dextrose broth and incubated for up to 30 days at 30°C before the fungus was dried and weighed (mean; $n = 2$). (C) Circles (1-cm diameter) of stationary-phase mats were placed in 24-well culture plates filled with 1 ml of minimal medium. The mats were dried and weighed after incubation for up to 7 days at 30°C (mean; $n = 3$).

keratinase activity produced. Clearly, *T. rubrum* responds to nutrient deprivation by elaborating proteinases. When either carbon, nitrogen, or sulfur was deleted from the minimal medium, this fungus responded by secreting proteinases capable of degrading azocoll. Little elastase or keratinase was produced under these conditions. The addition of keratin or elastin to the minimal medium did not induce azocollytic or elastinolytic activity; however, it did induce keratinolytic activity.

TABLE 1. Effect of carbon, nitrogen, sulfur, or phosphorus deprivation, and protein on the expression of azocoll-, elastin-, and keratin-degrading activities in log-phase cultures of *T. rubrum*

Growth medium	Substrate degraded (U/ml± SD)[a]			Mean wt (mg)[b]
	Azocoll	Elastin	Keratin	
Minimal	0.03 ± 0.01	0.14 ± 0.12	0.57 ± 0.31	12
Minimal-elastin	0.11 ± 0.02	0.25 ± 0.19	1.20 ± 1.02	ND
Minimal-keratin	0.04 ± 0.05	0.60 ± 0.22	2.15 ± 1.45	ND
Carbon depleted	1.00 ± 0.06	0.10 ± 0.14	0.08 ± 0.14	5
Nitrogen depleted	1.35 ± 0.14	0.57 ± 0.58	0.30 ± 0.30	5
Sulfur depleted	1.62 ± 0.16	0.32 ± 0.10	0.08 ± 0.14	4
Phosphorus depleted	0.16 ± 0.07	0.19 ± 0.09	0.20 ± 0.20	ND

[a] Log-phase hyphae of *T. rubrum* were inoculated into 10 ml of medium, and after 1 week of incubation at 30°C a sample of the CM was analyzed for proteolytic activity ($n = 3$).

[b] After 1 week of incubation, the mycelial mat was dried and weighed ($n = 3$). ND, Not determined.

It should be noted that in early experiments the nitrogen- and carbon-depleted cultures died before their CM could be assayed. These experiments were repeated by using five times the inoculum of fungus, which yielded positive results. No azocollytic activity was detected when the same inoculum of fungus was cultivated in minimal medium. Although the starting inoculum was increased for these cultures, leaching of nitrogen and carbon from dead or living cells did not appear to be a major problem. If this were a problem, then leaching of carbon or nitrogen from the cells into nitrogen- or carbon-depleted media would have, in effect, created minimal medium. Fungus in minimal medium does not express azocollytic activity. In addition, as described below, the proteinase species expressed by the carbon- and nitrogen-depleted cultures were different from those expressed by cultures of fungus in minimal medium, again suggesting that leaching was not a significant problem. The larger starting inoculum could have also contributed to proteolytic activity by the release of intracellular stores of proteinases from dead or dying cells. However, the equivalent of 24 mg (dry weight) of ground, log-phase fungus suspended in phosphate-buffered saline (lysate) had about 0.15 U of azocollytic activity per ml, and when assayed by gelatin substrate gel analysis proteinases of M_r 53,000 and >200,000 were detected (data not shown). As described below, the carbon- and nitrogen-depleted cultures expressed either different proteolytic species than the lysate or only one of the proteinases present in the lysate.

The concentration of glucose in the minimal medium did not appear to matter, since similar results were obtained when the glucose concentration was increased to either 1 or 2%. Growth of *T. rubrum* was greatest in the minimal medium; the other conditions were inhibitory to mycelial production, although the sulfur-depleted cultures of fungus appeared to be growing as their mass increased from 1.5 to 4 mg in the 1-week incubation. Finally, the same derepression of proteolytic activity was detected when the fungus was incubated for only 3 days before its CM was assayed.

When the CM of the nutrient-starved cultures was analyzed on gelatin substrate gels, proteolytic activities were detected (Fig. 2). In the minimal medium a high-molecular-

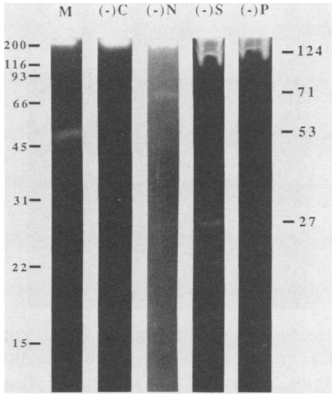

FIG. 2. Expression of gelatin-degrading enzymes in log-phase cultures of *T. rubrum* depleted of carbon, nitrogen, sulfur, or phosphorus. Log-phase *T. rubrum* was cultivated in a minimal medium (M). The carbon-, nitrogen-, sulfur-, and phosphorus-depleted media [(−)C, (−)N, (−)S, and (−)P]) were made by deleting the appropriate compound from the minimal medium, with the following changes. In the phosphorus-depleted medium the potassium phosphate salts were left out of the medium, and 50 mM NH_4HCO_3 was substituted for the $(NH_4)_2PO_4$. The 2 mM $MgSO_4$ was replaced by 2 mM $MgCl_2$ in the sulfur-depleted medium. After 1 week of incubation at 30°C a sample of CM from each of the cultures was analyzed by electrophoresis on gelatin-containing substrate gels. Molecular weight markers (in thousands) are indicated at the left, and relative molecular weights (in thousands) of the proteolytic species are indicated at the right.

TABLE 2. Effect of inorganic and organic sulfur sources on the expression of proteolytic activity in log-phase cultures of *T. rubrum*

Growth medium[a]	Substrate degraded (U/ml± SD)[b]		
	Azocoll	Elastin	Keratin
Minimal	0.19 ± 0.03	ND	ND
−S	2.41 ± 0.18	ND	ND
−S + 2 mM NaS_2O_3	0.21 ± 0.15	ND	ND
−S + 2 mM Na_2SO_3	0.16 ± 0.03	ND	ND
−S + 50 mM Met	0.62 ± 0.06	ND	ND
−S + 5 mM Met	0.34 ± 0.05	ND	ND
−S + 0.5 mM Met	0.27 ± 0.18	ND	ND
−S + 50 mM Cys	0.31 ± 0.05	ND	ND
−S + 5 mM Cys	0.40 ± 0.02	ND	ND
−S + 0.5 mM Cys	0.50 ± 0.10	ND	ND
−S + 50 mM Gly	3.04 ± 0.60	ND	ND
−S + 50 mM Ile	4.13 ± 0.43	ND	ND
−S + 50 mM Pro	2.99 ± 0.13	ND	ND
−S + 50 mM Ala	3.29 ± 0.36	ND	ND
−S + 50 mM Phe	3.04 ± 0.14	ND	ND
−S + 50 mM Ser	2.54 ± 0.06	ND	ND
−S + Casamino Acids	0.77 ± 0.24	ND	ND
Elastin −C, −N, −S	6.44 ± 0.76	30.50 ± 5.25	1.11 ± 0.59
Keratin −C, −N, −S	1.03 ± 0.40	0.96 ± 0.12	0.95 ± 0.30
Gelatin −C, −N, −S	0.79 ± 0.00	0.68 ± 0.09	0.00 ± 0.00
Albumin −C, −N, −S	7.34 ± 0.49	31.99 ± 2.52	3.29 ± 0.93

[a] Log-phase hyphae of *T. rubrum* were inoculated into 10 ml of sulfur-depleted medium supplemented with inorganic sources of sulfur or amino acids as described in Materials and Methods. The log-phase hyphae were also cultured in a medium were a protein served as the sole source of carbon, nitrogen, and sulfur. A minus sign before an element indicates that the medium did not contain that element.

[b] After 1 week of incubation at 30°C, a sample of the CM was analyzed for proteolytic activity (n = 3). ND, Not determined.

weight activity (M_r, >200,000) and a 53,000-M_r activity were detected. The carbon-depleted cultures produced the high-molecular-weight activity, as did the nitrogen-depleted cultures. The nitrogen-depleted cultures also secreted a 71,000-M_r proteinase. The high-molecular-weight activity, a 124,000-M_r species, and a 27,000-M_r proteinase were all secreted when the medium was depleted of sulfur. Finally, the phosphorus-depleted cultures secreted the high-molecular-weight activity and a 124,000-M_r activity.

Effects of organic and inorganic sulfur sources on proteinase production by sulfur-depleted cultures. The repression of the proteolytic activities of *T. rubrum* by sulfur was particularly interesting because one of the repressible activities, the 27,000-M_r proteinase, has recently been purified from *T. rubrum* CM (1; Apodaca and McKerrow, J. Cell Biol., 1989). Sulfur-depleted medium degraded azocoll and had little activity against elastin. The log-phase, sulfur-depleted CM was inhibited by Phe-Gly-Ala-Leu-CH_2Cl (77% inhibition) and not by 1,10-phenanthroline or *N*-ethylmaleimide. Finally, the sulfur-depleted CM degraded succinyl-Ala-Ala-Pro-Phe-*p*NA (5.7 μM/min per ml of sulfur-depleted CM) and Z-Ala-Ala-Leu-*p*NA (4.5 μM/min per ml of sulfur-depleted CM) substrates.

We were interested in determining whether both organic and inorganic sources of sulfur would repress this proteolytic activity, and what proteinases would be expressed if the fungus were grown in a medium where a single protein served as the sole source of carbon, nitrogen, and sulfur. Cultures of log-phase *T. rubrum* were washed and then cultured in sulfur-depleted medium. Various amino acids and inorganic sources of sulfur were added to these cultures. The addition of methionine or cysteine to the sulfur-depleted medium repressed proteinase production by fungus grown under these conditions (Table 2). Concentrations of sulfur-containing amino acids as low as 0.5 mM were effective. Although all concentrations of methionine and 0.5 mM cysteine had no visible effect on fungal growth, the 5 and 50 mM cysteine cultures were growth inhibited. The addition of any of the other amino acids tested actually appeared to stimulate the production of proteolytic activity. Grossly, these amino-acid-appended cultures did not grow better than the minimal or methionine-appended cultures. When the fungus was cultured in sulfur-depleted medium that contained Casamino Acids, a blend of amino acids that contains methionine and cysteine, expression of proteolytic activity was repressed, although some proteolytic activity was detectable by substrate gel analysis (Fig. 3). Inorganic sources of sulfur, e.g., $MgSO_4$, NaS_2O_3, and Na_2SO_3, were equally as effective as organic sources of sulfur in repressing proteinase activity. On gelatin substrate gels, the 27,000-M_r proteinase and a zone of hydrolysis from M_r 124,000 to the top of the gel were detected under conditions of derepression but not under conditions of sulfur repression (Fig. 3).

When *T. rubrum* was grown under conditions where a single protein served as the sole source of carbon, nitrogen, and sulfur, all proteolytic activities were derepressed when elastin, which contains no methionines or cysteines, or BSA was used. On gelatin substrate gels the 27,000-M_r activity and the high-molecular-weight activity were expressed (data not shown). Although expression of azocollytic and elastinolytic activities was lowered when the fungus was cultured on keratin as the sole source of carbon, nitrogen, and sulfur, the keratinase activity was not.

Effects of nutrient depletion on stationary-phase cultures. Stationary-phase cultures of *T. rubrum* were cultured in

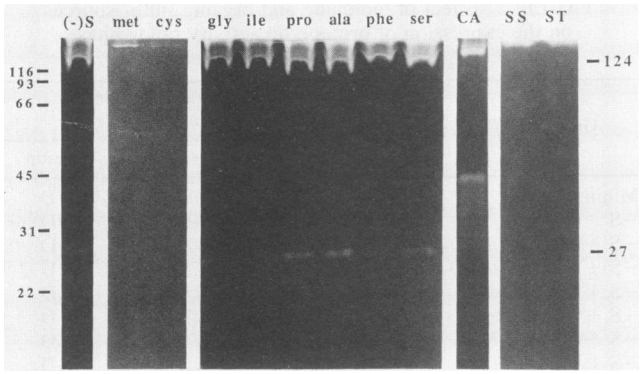

FIG. 3. Expression of gelatin-degrading enzymes in sulfur-depleted medium supplemented with amino acids and inorganic sources of sulfur. Log-phase *T. rubrum*, was cultured in sulfur-depleted medium [(–)S] or sulfur-depleted medium supplemented with 2.5% (wt/vol) Casamino Acids (CA), 2 mM Na_2SO_3 (SS), 2 mM NaS_2O_3 (ST), or any of the following amino acids (50 mM): L-methionine, L-cysteine, L-glycine, L-isoleucine, L-proline, L-alanine, L-phenylalanine, or L-serine. After 1 week of incubation at 30°C a sample of CM from each of the cultures was analyzed by electrophoresis on gelatin-containing substrate gels. Molecular weight markers (in thousands) are indicated at the left, and the relative molecular weights (in thousands) of the proteolytic species are indicated at the right.

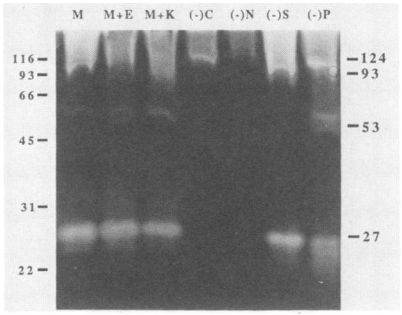

FIG. 4. Expression of gelatin-degrading enzymes in stationary-phase cultures of *T. rubrum* depleted of carbon, nitrogen, sulfur, or phosphorus. Fungus cultivated to stationary phase was placed in minimal medium (M) or in medium depleted of a source of carbon (−C), nitrogen (−N), sulfur (−S), or phosphorus (−P). After 1 week of incubation at 30°C a sample of CM from each of the cultures was analyzed by electrophoresis on gelatin-containing substrate gels. Molecular weight markers (in thousands) are indicated at the left, and relative molecular weights (in thousands) of the proteolytic species are indicated at the right.

minimal medium or a medium lacking a source of carbon, nitrogen, sulfur, or phosphorus, and proteolytic activity was assayed (Table 3). Unlike the case in log-phase cultures, all proteolytic activities were expressed in minimal medium, and the addition of elastin or keratin to this medium did not induce any activity above the controls. These stationary-phase cultures responded poorly to depletion of phosphorus, carbon, and especially nitrogen from their medium. Proteolytic activity was decreased under these conditions. The only exception was when sulfur was left out of the minimal medium. In this situation large amounts of azocoll-, elastin-, and keratin-degrading activity were detected.

The CM from these various culture conditions was analyzed on gelatin substrate gels (Fig. 4). The minimal, minimal plus elastin, minimal plus keratin, and phosphorus-depleted cultures all had the same proteinase pattern. There was a broad area of activity from M_r 71,000 to the top of the gel, a doublet of activity around M_r 53,000, and additional activities of M_r 31,000, 27,000, and 25,000. The carbon- and nitrogen-depleted cultures of fungus secreted a series of high-molecular-weight activities (M_r 93,000 to the top of the gel) and two activities of M_r 53,000 and 45,000. A broad smear of activity from M_r 71,000 to the top of the gel and the M_r 27,000 proteinase were detected in the sulfur-depleted cultures.

TABLE 3. Effect of carbon, nitrogen, sulfur, or phosphorus deprivation and protein on the expression of azocoll-, elastin-, and keratin-degrading activities in stationary-phase cultures of *T. rubrum*

Growth medium[a]	Substrate degraded (U/ml± SD)[b]		
	Azocoll	Elastin	Keratin
Minimal	7.65 ± 0.22	57.54 ± 12.37	1.59 ± 0.67
Minimal-elastin	6.84 ± 0.82	33.10 ± 19.89	0.77 ± 0.57
Minimal-keratin	6.20 ± 1.19	7.50 ± 3.69	1.07 ± 1.10
Carbon depleted	2.40 ± 0.66	2.99 ± 0.61	1.44 ± 1.36
Nitrogen depleted	0.58 ± 0.29	0.81 ± 0.42	0.28 ± 0.36
Sulfur depleted	7.94 ± 0.20	77.49 ± 21.08	1.82 ± 1.39
Phosphorus depleted	4.16 ± 0.99	12.89 ± 6.09	0.41 ± 0.33

[a] Stationary-phase mats of *T. rubrum* were inoculated into 1.0 ml of medium in 24-well plates as described in Materials and Methods.

[b] After 1 week of incubation at 30°C a sample of the CM was analyzed for proteolytic activity (n = 4).

DISCUSSION

Like *A. nidulans*, *A. niger*, and *N. crassa*, log-phase cultures of *T. rubrum* respond to a lack of carbon, nitrogen, and sulfur in their environment by secreting proteinases. Under carbon, nitrogen, and sulfur deprivation, a high-molecular-weight activity (M_r, >200,000) was secreted. An additional 71,000-M_r proteinase was produced by the nitrogen-depleted cultures. A 27,000-M_r proteinase was secreted when this fungus was deprived of sulfur, as was a 124,000-M_r species. The secretion of different proteolytic activities in response to carbon, nitrogen, and sulfur starvation is different from that in other fungi.

A number of extracellular proteolytic enzymes, named α, γ, ε, are produced by *A. nidulans* (5). There is a precursor of γ, referred to as δ, that is present in both extracts and CM from this fungus. Additionally, there is a β proteinase along with two precursors, β^1 and β^2, that are found only in extracts of mycelium. When *A. nidulans* cultures are starved for carbon, nitrogen, or sulfur the extracellular proteinases α, δ-γ, and ε are found in the CM of the fungus. Repression of the conversion of β^1 and β^2 to the intracellular β enzyme is also mediated by low-molecular-weight carbon, nitrogen, and sulfur sources. In addition, this intracellular proteinase is also repressed when phosphorus is present in the fungal milieu.

The proteinases of *N. crassa* are regulated in a similar manner, with one difference. Under conditions of carbon, nitrogen, or sulfur limitation, extracellular proteinases from this organism are expressed, but only in the presence of a high-molecular-weight protein in the medium (7–9, 11, 18). The induction is not seen when peptides or amino acids are added to the medium. The major extracellular, alkaline proteinase (M_r, 31,000) of *N. crassa* has been purified and is expressed whenever carbon, nitrogen, or sulfur is limited

(11, 12, 14). Other *N. crassa* proteinases are expressed in response to nutrient starvation, including two chelator-sensitive proteinases and an acid proteinase (15). The structural gene of the 31,000-M_r proteinase is thought to be regulated by signals arising from one of three circuits that control carbon, nitrogen, and sulfur assimilation. The carbon circuit effect is hypothetical; however, the control of this proteinase by the product of the regulatory gene *nit-2* has been described (12). Whenever glutamine or ammonia is not available to the fungus, the protein encoded by the nitrogen metabolism locus *nit-2* acts as a positive regulator of several genes involved in the utilization of secondary nitrogen sources including nitrate and nitrite reductase, L-amino acid oxidase, phenylalanine ammonia lyase, the purine catabolic enzymes, and of course extracellular proteinase (10, 12).

The sulfur-assimilating enzymes and proteins of *N. crassa* are also under the control of a master regulatory circuit. The expression of sulfate permeases, aryl sulfatase, choline sulfatase, choline sulfate permease, a methionine-specific permease, and an extracellular proteinase is repressed in the presence of methionine or inorganic sulfate and derepressed whenever sulfur is limited (11, 16). The *cys-3* locus encodes a positive regulatory protein that is required for the expression of all of these activities (12, 13, 16, 17, 22). A second unlinked regulatory gene, *scon*, has been described in the sulfur regulatory circuit. Its effect is opposite that of *cys-3*; the product of this gene acts as a negative regulator of sulfur assimilatory protein expression (3). The mutant allele, $scon^c$, results in a phenotype where the various enzymes are constitutively expressed.

In the studies on *T. rubrum* reported here, we found that although the CM from fungus grown in the minimal medium had little proteolytic activity, a 53,000-M_r proteinase and a high-molecular-weight activity were detected by substrate gel analysis. There was also a small amount of keratinolytic activity. By definition these proteinases are constitutively expressed, albeit at low levels. The 53,000-M_r activity may represent a glycosylated proteinase recently described in stationary-phase *T. rubrum* cultures (1; G. Apodaca, Ph.D. thesis, University of California, San Francisco, 1989). Keratinolytic activity was induced when either elastin or keratin was included in the minimal medium. In contrast, expression of azocollytic and elastinolytic activities was not significantly induced by the addition of these proteins to the minimal medium.

The 71,000-M_r proteinase expressed in the nitrogen-depleted medium probably represents a proteinase purified from *T. rubrum* CM with an identical mobility on substrate gel electrophoresis (2). The high-molecular-weight activity and the 124,000-M_r activity have not been purified and have unknown properties. A 27,000-M_r proteolytic species was repressed by sulfur. This is probably the same 27,000-M_r activity that has been purified from stationary-phase cultures of *T. rubrum* (1; Apodaca and McKerrow, J. Cell Biol., 1989). There are several pieces of data that suggest this is true. Both activities migrated in a similar fashion when analyzed on gelatin substrate gels, and both the purified enzyme and the CM from the sulfur-depleted cultures degraded azocoll and had low activity against elastin. The log-phase, sulfur-depleted CM, like its 27,000-M_r proteinase counterpart, was inhibited by Phe-Gly-Ala-Leu-CH_2Cl and not by 1,10-phenanthroline or *N*-ethylmaleimide. Finally, both the purified enzyme and the sulfur-depleted CM degraded succinyl-Ala-Ala-Pro-Phe-*p*NA and Z-Ala-Ala-Leu-*p*NA substrates. Production of antibodies to the purified M_r 27,000 proteinase would allow one to determine whether this activity is actually present in the sulfur-depleted medium.

The following model for control of proteolytic activity in log-phase cultures of *T. rubrum* is proposed. Under conditions of carbon, nitrogen, or sulfur deprivation the fungus derepresses the expression of general proteolytic (azocollytic) activity. These proteinases probably act on the nonkeratinous proteins of the stratum corneum, generating peptides. There are peptidases secreted by *T. rubrum* that would cleave the peptides into amino acids. The amino acids would then be either recognized directly or metabolized into intermediates that could act as mediators of repression of not only proteolytic activity but also other enzymes responsible for the assimilation of carbon and nitrogen. If this model is correct, the addition of individual amino acids to the carbon- or nitrogen-depleted medium should result in the repression of proteolytic activity. Meevootisom and Niederpruem (20) have shown that the addition of individual amino acids to fungal medium (e.g., keratin salts) represses guinea pig-hair-degrading enzymes. If the proposed model is correct it should be possible to detect exopeptidase activity in *T.*

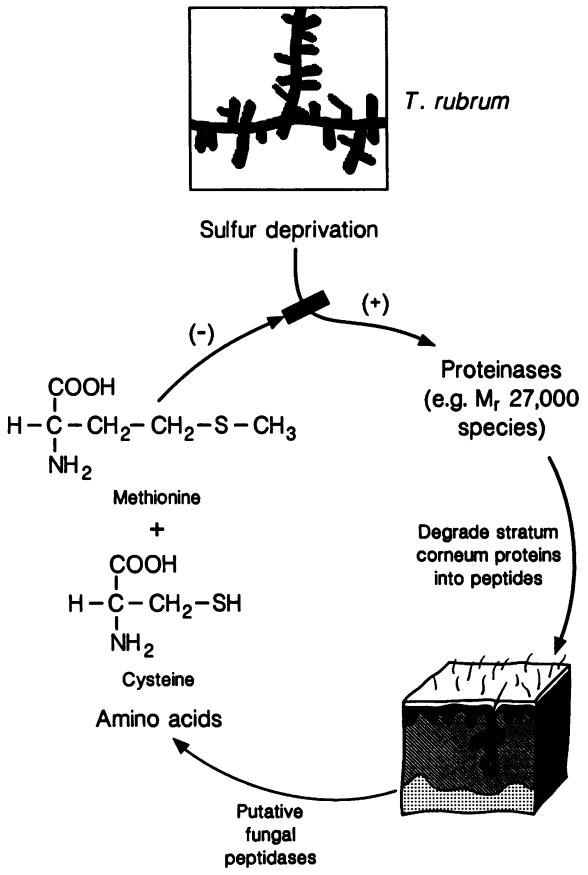

FIG. 5. Proposed model for the regulation of the sulfur-repressed proteinases of *T. rubrum*. Under conditions of sulfur deprivation, *T. rubrum* responds by secreting azocoll-degrading proteinases, including the 27,000-M_r species. These proteinases would act on proteins contained in the stratum corneum of the skin, releasing peptides into the fungal environment. There are putative peptidases that would cleave the peptides into amino acids, including methionine and cysteine. These sulfur-containing amino acids would then act as mediators of proteinase repression. The black box represents a regulatory element(s) that senses the concentration of sulfur in the cell and regulates the expression of proteolytic activity.

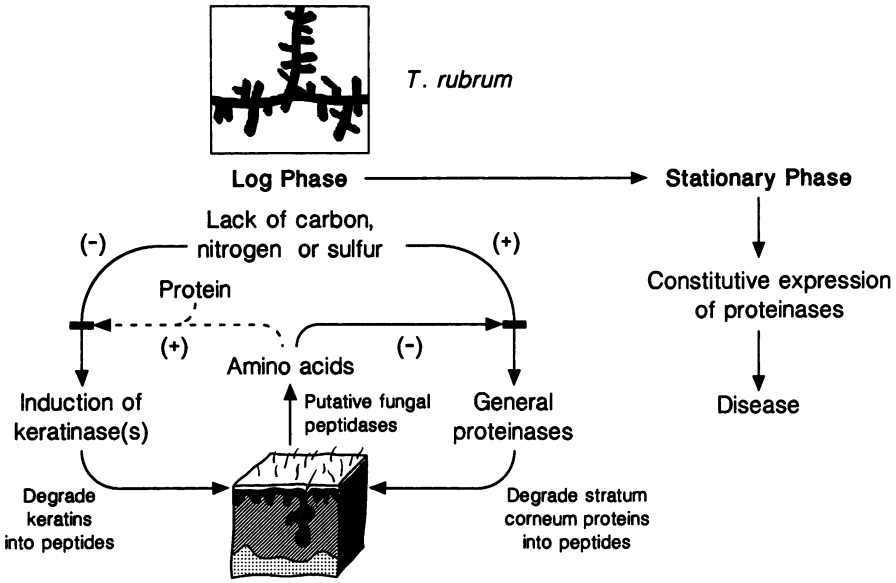

FIG. 6. Proposed model for the regulation of *T. rubrum* proteolytic activity. In the initial stages of infection, *T. rubrum* grows logarithmically. In this state, general proteolytic activity is derepressed whenever carbon, nitrogen, or sulfur is lacking in the fungal milieu. These proteinases would act on the nonkeratinous proteins contained in the stratum corneum of the skin, releasing peptides into the fungal environment. There are putative peptidases that would cleave the peptides into amino acids. These amino acids would provide the cell with a source of carbon, nitrogen, and sulfur. Under these conditions, the general proteolytic activity would be repressed, whereas specific keratinases would be induced in the presence of protein. Disease may occur when the fungus reaches stationary phase, when proteinases are secreted constitutively. These enzymes may directly or indirectly incite a host response, resulting in the inflammatory manifestations of dermatophtosis. The black boxes represent regulatory elements that sense the concentration of carbon, nitrogen, and sulfur in the cell and control the expression of proteolytic activity.

rubrum CM as well. In addition, when *T. rubrum* is grown in a medium where a single protein serves as the sole source of carbon and nitrogen (e.g., keratin salts medium), it responds by secreting azocoll- and elastin-degrading enzymes (Apodaca, Ph.D. thesis). It appears that the expression of elastinolytic activity is derepressed when more than one source of nutrition is depleted from the fungal medium (e.g., both nitrogen and carbon).

T. rubrum also expressed azocollytic activity when its medium was depleted of sulfur (Fig. 5). Under this condition the fungus responded by turning on the expression of 27,000- and 124,000-M_r proteinases. Again, these proteinases would cleave extracellular proteins, presumably the nonkeratinous proteins found in the stratum corneum and nails, into peptides. The proposed peptidases would cleave the peptides into individual amino acids. The fungus would take up sulfur-containing amino acids, i.e., methionine and cysteine. It is hypothesized that, as in *N. crassa* and *A. nidulans*, there are mediators of catabolite repression that would sense the increased concentration of sulfur in the cell, down-regulating several enzymes responsible for sulfur assimilation, including proteinases.

In confirmation of this model of sulfur regulation, the addition of methionine or cysteine in concentrations as low as 0.5 mM to the sulfur-depleted medium repressed proteinase production by fungus grown under these conditions. The addition of several other amino acids, including glycine, isoleucine, proline, alanine, phenylalanine, and serine, actually stimulated the production of proteolytic activity. When the fungus was cultured in sulfur-depleted medium that contained Casamino Acids, a blend of amino acids that contains methionines and cysteines, expression of proteinases was also repressed. It appears that the effect of sulfur-containing amino acids, even in a blend of amino acids, is still repressive to proteinase expression. When the fungus was grown under conditions where a single protein, such as gelatin, elastin, or keratin, was the sole source of carbon, nitrogen, and sulfur, proteinase expression was derepressed only in the case of elastin, which contains no methionines or cysteines. To confirm that only non-sulfur-containing proteins would allow for the expression of the proteinases, BSA was also tried under the same conditions. Surprisingly, large amounts of azocoll-, elastin-, and keratin-degrading activities were expressed under these conditions. It may be that the proteolysis of BSA results in specific peptides that stimulate the expression of proteinases. Another possibility is that BSA is not a good substrate for the fungal proteinases, such that no amino acids, sulfur containing or not, would be released, and proteinase activity would remain derepressed. Clearly, other proteins need to be studied. Inorganic sources of sulfur were equally effective at repressing proteinase expression. For example, 2 mM $MgSO_4$, NaS_2O_3, or Na_2SO_3 quelled azocollytic activity.

Although azocollytic and elastinolytic activities were repressed by carbon, nitrogen, and sulfur, keratinolytic activity was not, indicating that it is regulated by a different mechanism. This activity was induced when insoluble proteins, such as elastin or keratin, were added to the minimal medium. The data suggest that keratinase is induced whenever there is a source of available carbon, nitrogen, sulfur, and protein. How, then, is the keratinase activity induced if it requires carbon, nitrogen, and sulfur to be present in the fungal medium? We propose that at the onset of a *T. rubrum* infection the carbon-, nitrogen-, and sulfur-repressed activities would become derepressed and act on the proteins other than keratins present in the stratum corneum. Once the

proteins were cleaved, peptides derived from them would be further degraded by peptidases into individual amino acids. These would provide the cell with a source of carbon, nitrogen, and sulfur. Under these conditions, the azocollytic and elastinolytic activities would be repressed, whereas keratinases could be secreted.

Proteinase activity in stationary-phase cultures appeared to be regulated differently than proteolytic activity in log-phase cultures. Unlike the log-phase cultures, the stationary-phase fungus produced azocollytic, elastinolytic, and keratinolytic activity in the minimal medium. The addition of protein to this medium did not induce any proteolytic activity. Proteinases were not derepressed when sources of carbon, nitrogen, or phosphorus were deleted from the minimal medium. In fact, expression of all proteolytic activities decreased under these conditions. The exception was in the sulfur-depleted cultures. In this case, there was an increase in all activities. In stationary-phase cultures, all activities were derepressed and secreted constitutively.

We suggest that proteinases are important to *T. rubrum* as a means of releasing carbon, nitrogen, and sulfur from the keratinized tissues of humans. Two lines of evidence suggest that this may be true. First, easily metabolized substrates such as glucose and amino acids can repress proteinase secretion (20), arguing that as other nutrient sources are made available to this organism, proteinase secretion is not required as long as other nutritive molecules are present in its environment. Second, when either carbon, nitrogen, or sulfur is absent from the fungal milieu the fungus responds by expressing proteinases, strongly suggesting that proteinases do play an important role in nutrition gathering by this organism.

The determination of which proteinases are actually being expressed in vivo during a typical *T. rubrum* infection will have to wait for production of probes, such as cloned proteinase genes or antibodies to purified *T. rubrum* proteinases. Also, at this time there is no way to tell whether during infection the fungus is in a log-phase or stationary-phase growth cycle. Presumably, in the initial stages of infection the fungus is growing logarithmically. This log-phase growth may actually continue for long periods of time, since the fungus can be continually shed as old keratinocytes are replaced by new cornified tissues. There would be neither a lack of substrate upon which proteinases could act nor a buildup of toxic substances under these conditions. In this log phase of growth, general proteolytic (azocollytic) activity would be derepressed whenever carbon, nitrogen, or sulfur is lacking in the fungal environment (Fig. 6). These proteinases would act on the nonkeratinous proteins in the stratum corneum. There are probably peptidases, as yet unidentified, that would cleave the peptides generated by the initial proteolysis into amino acids. These amino acids would provide the cell with a source of carbon, nitrogen, and sulfur. Under these conditions, the expression of general proteinases would be repressed, whereas keratinases would be induced in this nutrient rich environment. Disease may occur when the fungus reaches a stationary phase of growth. In this state proteolytic activity appears to be constitutively expressed and could act to incite an inflammatory response. There is a correlation of high proteolytic activity and the more acute and inflammatory infections of *Trichophyton mentagrophytes* (21, 23). Based on this model, it should be possible to prevent the colonization of the stratum corneum and the ensuing disease by inhibiting the proteinases produced by *T. rubrum* with specific inhibitors. Regardless of the stage of growth that *T. rubrum* is in during human infections, this organism clearly possesses the proteolytic armamentarium necessary to parasitize the cornified tissues of the body.

ACKNOWLEDGMENTS

This work was supported by a National Science Foundation Minority Fellowship and a Ford Foundation Dissertation Year Fellowship (G. Apodaca) and by Public Health Service grant AI 20452-06 from the National Institute of Allergy and Infectious Diseases (J. H. McKerrow).

We thank M. Banda, C. Craik, C. Halde, J. Railey, and Z. Werb for their helpful comments and discussion and M. Banda and J. Railey for their critical review of this manuscript.

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