Role of cysteine in regulating morphogenesis and mitochondrial activity in the dimorphic fungus *Histoplasma capsulatum*

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ABSTRACT Three stages can be distinguished in the temperature-induced mycelial-to-yeast phase transition of *Histoplasma capsulatum*. Stage one is characterized by a progressive decrease in the respiration rate and in the intracellular concentrations of cysteine and other amino acids. By stage two, respiration has ceased completely and free cysteine has fallen to low levels. Exogenous cysteine is required during the second stage for activation of mitochondrial respiration (stage three) and completion of the morphological transition. Mitochondria isolated from cells in the second stage show no respiration with NADH, succinate, or other substrates unless they are first incubated with cysteine. In addition, a novel, cytosolic cysteine oxidase appears during the latter part of the second stage. In stage three, the respiration rate rises, intracellular concentrations of free cysteine and other amino acids increase to levels characteristic of yeast, and the morphological transition is completed. The results support the idea that alterations in cysteine metabolism play a key role in this differentiation process.

Histoplasma capsulatum is a dimorphic pathogenic fungus that grows as a multicellular mycelium in soil and as a yeast in the tissues of infected humans and animals (1). Factors that affect morphogenesis and maintenance of one phase of the organism are of considerable interest because growth of the yeast phase appears to be required for pathogenicity (2). In culture, the transition from mycelial to yeast phase is triggered by a shift in temperature from 25°C to 37°C. Growth and maintenance of the yeast phase in culture are also dependent upon a low oxidation-reduction potential (3) and on the presence of cysteine in the culture medium (2, 4). Thus far, however, there has been little insight into the biochemical basis of any of these observations.

We have shown that cysteine stimulates oxygen consumption in the yeast phase but not in the mycelial phase (5). We reasoned that this observation might provide a good starting point for the investigation of biochemical mechanisms in phase determination and morphogenesis of *H. capsulatum*. In the present work, we show that the cysteine stimulation of oxygen consumption is due to a cytosolic cysteine oxidase activity, which is present only in yeast-phase cells. In addition, experiments monitoring cellular respiration, cysteine levels, and cysteine oxidase activity during the temperature-induced mycelial-to-yeast phase transition support the idea that alterations in cysteine metabolism play a key role in the differentiation process. They also demonstrate the occurrence of novel regulatory mechanisms involving the effect of cysteine on the mitochondrial respiratory chain. On the basis of our results and work by others, we pro-

pose a model that outlines biochemical events at specific stages of the morphological transition.

MATERIALS AND METHODS

Growth of *H. capsulatum*. The wild-type Down's strain of *H. capsulatum* mating type (-) was used in this study. Cells were grown in the defined medium R₃B₃ (6) or in 2% (wt/vol) glucose/1% yeast extract as described (7). Cultures were started with a constant inoculum of cells and were grown to midlogarithmic phase, which occurred after 48 hr of incubation.

Measurement of Cell Respiration. Cells were harvested by centrifugation and resuspended in a modified respiration buffer containing 1 mM dimethylglutaric acid (pH 7.2), 1 mM CaCl₂, and 1% mannose instead of glucose (7, 8). In some experiments, respiration was measured in fresh growth medium. Oxygen concentration was measured polarographically with a KIC-oxygenograph equipped with a Clark-type oxygen electrode (Gilson) (9). Respiration rates in whole cells are expressed as μ l of O₂ per hr/mg of cells (dry weight) at 37°C for yeast and at 25°C for mycelium (9).

KCN was dissolved in aqueous solution and adjusted to pH 7.0; salicylhydroxamic acid (Aldrich) was dissolved in absolute ethanol. Both were prepared fresh for each experiment (10). Ethanol had no effect on oxygen uptake at the concentrations used in these experiments. Cysteine was not oxidized spontaneously in the respiration buffer as determined by polarographic measurements (data not shown).

In the study on the appearance of cysteine oxidase activity in transforming mycelia, midlogarithmic-phase cells were centrifuged and resuspended in fresh synthetic medium R₃B₃ or in glucose/yeast extract and incubated at 37°C. Cysteine oxidase activity was defined as cysteine-dependent oxygen consumption after respiration in whole cells had been inhibited by KCN (1 mM) and salicylhydroxamic acid (0.58 mM). Some cultures were given daily additions of 2 mM cysteine or 2 mM 2-mercaptoethanol. Duplicate samples of the cultures used for respiration measurements were filtered and dried at 40°C for dry-weight measurements.

Preparation of Mitochondria, High-Speed Centrifugation, and Measurement of Respiration. Yeast or mycelia were harvested by filtration through Whatman no. 1 filter paper, washed with cold water, and resuspended in 0.33 M sucrose/1 mM ethyleneglycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, pH 7.0/0.3% bovine serum albumin. Glass beads, 1/3 of the suspension volume, were added, and the cells were broken by shaking for 2 min in a Braun cell homogenizer that was con-

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stantly cooled with liquid CO₂. Subsequent steps were carried out at 4°C. The homogenate was centrifuged twice at 800 × *g* (10 min) and the mitochondria formed a pellet at 17,000 × *g* (30 min). The mitochondrial pellets were resuspended in the mitochondrial respiration buffer (0.3 M sucrose/8 mM NaH₂PO₄/8 mM Tris-HCl, pH 7.0/5 mM MgCl₂/0.7 mM EDTA). The supernatant formed at 17,000 × *g* was centrifuged at 100,000 × *g* in a Beckman Ti 50 rotor to produce a high-speed supernatant.

Measurements of oxygen uptake by mitochondria were carried out at 25°C in a chamber containing the mitochondrial respiration buffer saturated with air. Respiration rates are expressed in μmol of O per min/mg of mitochondrial protein. Mitochondrial protein was measured by the Lowry method (11).

Measurements of Intracellular Amino Acid Concentrations. Approximately 5 g of cells (wet weight) were suspended in 10 ml of 5% (wt/vol) trichloroacetic acid, homogenized in a Braun cell homogenizer for 3 min, and then centrifuged at 18,000 × *g*. A₂₂₄₋₂₃₃ of the supernatants was measured against an appropriate trichloroacetic acid blank on samples diluted 1:250 to 1:500. The original supernatants were then diluted so that A₂₂₄₋₂₃₃ was equivalent to 15 mg of bovine serum albumin per ml (see ref. 12). Fractions (0.5 ml) were lyophilized and resuspended in H₂O. The fractions were adjusted to pH 8.0 with NaOH. Free cysteine in the samples was carboxymethylated at room temperature with 15 μmol of iodoacetic acid, pH 8.0 (13). The reaction was stopped by the addition of 65 μmol of 2-mercaptoethanol. The samples were lyophilized, resuspended in 0.1 M citrate buffer, pH 2.2, and analyzed in a Beckman 119 C amino acid analyzer by using a single-sulfonated-polystyrene-column technique. This method measures cysteine and cystine independently.

The recovery of cysteine in our extraction procedure was determined by adding [³⁵S]cysteine to the harvested yeast and mycelia before homogenization in the Braun cell homogenizer and then determining the percentage of radioactivity recovered in the form of cysteine from the amino acid analyzer. Recoveries were 85–92%.

RESULTS

Previous cell respiration studies showed that *H. capsulatum* respire through a branched electron-transport system consisting of two terminal oxidase pathways: (i) a standard cytochrome system, which can be inhibited by cyanide or antimycin, and (ii) an unidentified alternate oxidase, which is insensitive to these inhibitors but can be specifically inhibited by salicylhydroxamic acid (7).

Under standard culture conditions, both oxidases are present constitutively in yeast- and mycelial-phase cells and are active enough so that either oxidase can accommodate almost the entire respiratory flux when the other is inhibited (7).

In yeast- and mycelial-phase cells, cyanide and salicylhydroxamic acid were added together to inhibit mitochondrial respiration. In yeast (Fig. 1A), subsequent addition of cysteine stimulated oxygen consumption to ca. 60% of the original rate, whereas cysteine had no effect on respiration of mycelial-phase cells under the same conditions (Fig. 1B). The cysteine-stimulated oxygen consumption of yeast-phase cells was not inhibited by 1 mM azide (data not shown), which has been reported to block a third mitochondrial respiratory pathway in some fungi (e.g., see ref. 14).

FIG. 1. O₂-electrode recordings of respiration of yeast (A) and mycelium (B) of *H. capsulatum*; 0.1 ml of yeast [5 mg (dry weight)/ml] and 0.2 ml of mycelium [2.5 mg (dry weight)/ml] were used. Additions were: KCN, 1 mM in yeast and 0.5 mM in mycelium; salicylhydroxamic acid (SHAM), 0.58 mM; cysteine, 1 mM. The values given in parentheses are rates of oxygen consumption expressed as μl of O₂ per hr/mg (dry weight). Assays were at 37°C for yeast and 25°C for mycelium.

FIG. 2. O₂-electrode recordings of isolated mitochondria (A) and supernatant obtained after high-speed centrifugation (B). Additions to yeast mitochondria were 40 μM KCN and 0.39 mM salicylhydroxamic acid; additions to mycelial mitochondria were 0.39 mM salicylhydroxamic acid and 100 μM KCN. Additions to the supernatant were 0.5 mM KCN and 0.58 mM salicylhydroxamic acid for both phases. Cysteine was 1 mM. Yeast extracts had 0.5 mg of protein per ml, and mycelia had 0.26 mg of protein per ml. Assays were at 25°C in mitochondrial respiratory buffer with 0.5 mM ADP. NADH at a concentration of 1 mM was the mitochondrial substrate. Respiration rates are expressed as μmol of O per min/mg of mitochondrial protein. Both the yeast and mycelial respiration were more sensitive to KCN than salicylhydroxamic acid.

The above results show that yeast-phase but not mycelial-phase cells contain a novel cysteine oxidase activity that is insensitive to cyanide, salicylhydroxamic acid, or azide. To determine the intracellular location of this oxidase, cells were homogenized and centrifuged at high speed to yield a mitochondrial fraction and supernatant. Both fractions were treated with KCN and salicylhydroxamic acid. The cysteine oxidase activity, assayed as cysteine-dependent oxygen consumption in the presence of cyanide and salicylhydroxamic acid, was found only in the supernatant fraction of yeast obtained after high speed centrifugation (Fig. 2). Mitochondria from the yeast and mycelia and the supernatant from mycelia showed no cysteine oxidase activity (Fig. 2). The oxygen-electrode recordings also showed that the salicylhydroxamic acid-sensitive alternate oxidase is located in the mitochondrial fraction because both salicylhydroxamic acid and cyanide are required to inhibit mitochondrial respiration (Fig. 2).

To gain insight into the role that alterations in cysteine metabolism play in the differentiation process, we carried out experiments in which we monitored cellular respiration, cysteine oxidase activity, and intracellular concentrations of free cysteine during the temperature-induced transition from the mycelial to the yeast phase (Fig. 3; Table 1).

Three stages in the transition could be distinguished. Stage one, immediately after the temperature increase, was characterized by a progressive decrease in the respiration rate (Fig. 3A), which was paralleled by a decrease in intracellular cysteine concentration (Fig. 3B). Other amino acid pools also were depleted during stage one. At 24 hr, phenylalanine and histidine decreased to 60% of mycelial levels; valine, tyrosine, and cystine decreased to 20%; and aspartic acid, threonine, serine, glutamic acid, proline, methionine, glycine, alanine, isoleucine, leucine, lysine, and arginine decreased to <10% of mycelial-phase levels. The values for cystine and cysteine at different times after the temperature shift are shown in Table 1. The depletion of free amino acids may reflect a decreased rate of amino acid biosynthesis or a decreased rate of amino acid

FIG. 3. Respiration (Δ), cysteine levels ($\bullet$), and cysteine oxidase ($\circ$) activities during the mycelial-to-yeast phase transition. (A) Oxygen uptake in mycelia (dry weight) shifted to 37°C. Respiration was measured both in respiration buffer and fresh medium, with essentially identical results. (B) Level of cysteine in mycelia incubated at 37°C. Recoveries of cysteine were 85–92% as determined by addition of radioactive cysteine just before cell breakage.

Table 1. Levels of intracellular cysteine and cystine during the mycelial-to-yeast phase transition*

Time,† hr	Cystine‡	Cysteine‡
0	5.70	7.20
6	11.50	5.57
16	3.40	0.91
24	0.73	0.70
48	0.76	Not measurable
72	1.06	0.52
144	1.50	0.48
Control yeast at 37°C§	4.1	0.45

* Recoveries of cysteine were 85–92% as determined by addition of radioactive cysteine just before cell breakage. The experiment was repeated with essentially the same results. The increase in cystine levels at 6 hr was reproducible both times the experiment was done.

† Time after the temperature shift from 25°C to 37°C.

‡ nmol/0.1 mg of protein.

§ Passed through fresh medium three times.

transport resulting from decreased respiratory activity and depletion of ATP. Stage one continued for 40 hr, at which time the cells ceased to respire (stage two). Free amino acid concentrations decreased to low levels during this period. The dormant stage lasted 4–6 days, at which time the cells entered the third stage, characterized first by an increase in concentrations of intracellular cysteine (Fig. 3) and other amino acids and then by return of respiration to levels characteristic of the yeast phase. Mechanisms that could have contributed to the buildup of cysteine levels include any or all of (i) protein degradation, (ii) a low level of biosynthesis, and (iii) transport supported by ATP from glycolysis. Cysteine oxidase activity appeared during the latter part of the second stage. Colony counts, dry weight measurements, and levels of respiration during the third stage

FIG. 4. Oxygen uptake during phase transition. Mycelia-phase cells were centrifuged and resuspended in growth medium, and the temperature was shifted from 25°C to 37°C. Daily additions of 2 mM cysteine (Δ) and 2 mM 2-mercaptoethanol ($\square$) were begun 24 hr after the temperature shift (day 1) and continued until the transition was complete. $\circ$, Control.

indicated that no cell death had occurred (i.e., that the number of viable cells was equal to that of the mycelial culture prior to the phase transition). The earliest change observed microscopically was an increase in branching of the mycelia which occurred during stages one and two. True yeast cells were seen after the onset of the third stage.

Further experiments showed that cysteine plays a key role in the transition from the dormant or second stage to the third stage. When cysteine and cystine were absent from the medium, the transition could not proceed beyond the second stage (ref. 15, unpublished data). The normal culture medium contained sufficient cysteine so that the transition could proceed to completion. Addition of more cysteine (2 mM) or other reducing agents (2 mM 2-mercaptoethanol or 2 mM dithiothreitol) to the complete medium shortened the dormant stage and accelerated the mycelial-to-yeast phase transition (Fig. 4). Boguslawski *et al.* (16) have reported that yeast-phase cells do not

FIG. 5. O_2 -electrode recordings of isolated mitochondria from dormant cells (i.e., stage two or 24 hr after the temperature shift). Assays were carried out in mitochondrial respiration buffer. Additions were 1 mM NADH, 1.6 mM cysteine, 2 mM 2-mercaptoethanol, 0.72 mM salicylhydroxamic acid, and 0.5 mM KCN. In control experiments, 1.6 mM cysteine incubated with 1 mM NADH in the absence of mitochondria shows no substantial oxygen consumption (<5% of the rate of oxygen consumption when mitochondria were included). Respiration rates are expressed in parentheses in μmol of O per min/mg of mitochondrial protein.

Table 2. Respiration rates of isolated mitochondria from dormant (i.e., stage two) cells*

Substrate	Concentration mM	Respiration rate with 1.6 M cysteine†
Succinate	20	0.064
Pyruvate plus malate	5	0.032
Malate	1	0.060
α -Ketoglutarate plus malate	5	0.079
Acetate	1	0.048
None	20	0.002

* Assays were carried out as described in the legend to Fig. 5. Respiration rates were zero in the absence of cysteine.

† Respiration rates are in μmol of O per min per mg of mitochondrial protein.

synthesize cysteine and hypothesized that cysteine biosynthetic enzymes are repressed after the temperature increase. Our findings (Fig. 4) suggest that cysteine accelerates the transition by acting as a reducing agent in addition to fulfilling any nutritional requirement of the yeast phase.

Remarkably, cysteine and the other reducing agents appeared to act by activating the mitochondrial electron-transport chain. Mitochondria isolated from dormant cells showed no respiration with any substrates tested: 1 mM NADH [in fungal mitochondria, exogenous NADH interacts with the cytochrome system through a separate NADH dehydrogenase located on the outside of the inner mitochondrial membrane (17)]; 20 mM succinate; 5 mM pyruvate/1 mM malate; 5 mM malate; 5 mM α -ketoglutarate/1 mM malate; or 20 mM acetate. However, these substrates were oxidized if the mitochondria were first treated with 1.6 mM cysteine or 2 mM 2-mercaptoethanol (Fig. 5 shows the results for NADH and Table 2 lists results for other substrates). Respiration with NADH showed maximal stimulation with 1.6 mM cysteine ($.095 \mu\text{mol}$ of O per min/mg of proteins) whereas addition of 1.6 mM cysteine or 2 mM 2-mercaptoethanol in the absence of substrate supported only a slow rate of oxygen consumption by the mitochondria (less than $0.002 \mu\text{mol}$ of O per min/mg of protein) (Fig. 5).

Whole cell respiration during the dormant stage was also activated and restored to values characteristic of the yeast phase (7) by addition of 1.6 mM cysteine, 2.0 mM 2-mercaptoethanol, or 2 mM dithiothreitol (data not shown).

DISCUSSION

In this report, we describe a series of biochemical events in the temperature-induced transition of *H. capsulatum* from the mycelial phase to the yeast phase. Following the temperature jump, there is a decrease in cell respiration and in the intracellular concentrations of free cysteine and other amino acids. The immediate cause for inhibition of mitochondrial respiration is not clear. In view of the finding that cysteine is required to activate mitochondrial respiration in the dormant phase, we can speculate that inactivation of mitochondrial respiration results from a temperature-induced increase in oxidation-reduction potential. This view is supported by the data in Table 1, which show that the ratio of cysteine to cystine decreases after the temperature increases.

The depletion of free amino acids, which follows the temperature increase, could result from the decrease in respiration rate and Krebs-cycle activity, although repression of biosynthetic enzymes could also play a role. Boguslawski *et al.* (16) hypothesized that enzymes involved in cysteine biosynthesis

are repressed after mycelial-phase cells are shifted from 25°C to 37°C.

Exogenous cysteine is required during the dormant phase to permit cells to complete the transition to yeast morphology. Previous studies showed that cysteine is required for maintenance of the yeast phase (2, 4, 15, 16). Our results show that cysteine also may be required to activate mitochondrial respiration and that this requirement can be met by other reducing agents (2-mercaptoethanol and dithiothreitol). As yet, it is not clear whether the sustained recovery of respiration during the final stage of the transition is due to the buildup of intracellular cysteine or to further modification of the mitochondria so they can respire in the absence of cysteine. On one hand, addition of cysteine to the culture medium can be shown to decrease the time required for reactivation of oxygen consumption *in vivo* (Fig. 4). On the other hand, the increase in cysteine concentration in stage three actually appears to precede the activation of respiration (Fig. 3). However, the latter discrepancy may only be apparent and could reflect compartmentalization of free cysteine.

Our results show that the previously demonstrated cysteine-stimulation of respiration in the yeast phase (5, 18) is due to a novel cytosolic cysteine oxidase activity and that this activity appears during the latter part of the dormant stage. The time of appearance of the cysteine oxidase is consistent with the idea that it plays a role in the differentiation process, perhaps by contributing to the synthesis of some compound required for the maintenance of yeast morphological appearance.

The role of cysteine suggested by our studies is in agreement with older observations by McVeigh and Houston (15). They showed that the initiation of the mycelial-to-yeast phase conversion can occur in the absence of a reduced sulfhydryl-containing compound and molecular oxygen (stages one and two), but that cysteine and oxygen are required later in the conversion to complete the transition and to support growth in the yeast phase (stage three).

Assuming that mycelial morphological characteristics are advantageous for saprophytic growth and that yeast morphological characteristics are favorable for parasitic growth, we can speculate that the requirement for cysteine to complete the transition from mycelium to yeast is an important biological control mechanism for *H. capsulatum*. The shift in temperature from 25°C to 37°C converts the organism from a heterotroph to a

cysteine "auxotroph." The requirements for cysteine to activate mitochondrial respiration and for maintenance of the yeast phase assure that random temperature increases will not result in morphological conversion and that yeast-phase growth will occur only in an appropriate host environment, where exogenous cysteine is available.

The present work sets the stage for more detailed analysis of the morphological transition by raising specific questions about biological processes in different stages.

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