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Purification and Characterization of a Cysteine Dioxygenase from the Yeast Phase of *Histoplasma capsulatum*†

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ABSTRACT: A cysteine dioxygenase, cysteine oxidase (EC 1.13.11.20), has been purified from the cytosolic fraction of yeast phase cells of the dimorphic fungus *Histoplasma capsulatum*. The cysteine oxidase is an iron-containing dioxygenase with a molecular weight of 10 500 (±1500) and is present only in the yeast phase of the fungus. The enzyme is highly specific for L-cysteine, with a K_m of 2×10^{-5} M in vitro. The product of cysteine oxidation is cysteinesulfinic acid, as analyzed by thin-layer chromatography and mass spectroscopy. To our knowledge, this is the first cysteine oxidase isolated from a fungus, and it probably plays an important role in the mycelial to yeast phase transition of *H. capsulatum* during which redox potential and cysteine levels are crucial factors.

Histoplasma capsulatum is a dimorphic pathogenic fungus which exists in a multicellular form in nature and a unicellular yeast in infected tissue. In axenic culture, the organism grows as a mycelium at 25 °C and a yeast at 37 °C. Phase transitions can be induced by raising or lowering the temperature.

We have prviously identified unique features of each phase of *H. capsulatum* which may be importnat in the morphologic transition. One of these is a cysteine oxidase or cysteine dioxygenase (L-cysteine:oxygen oxireductase, EC 1.13.11.20) activity found in the cytosol of yeast phase cells (Maresca et al., 1981). As a first step in trying to understand the regulation of its activity and its role in the transition, we have purified the cysteine oxidase from yeast cells and characterized the enzyme and its product.

Experimental Procedures

Materials

DEAE-cellulose was purchased from Whatman Chemical Co. (Clifton, NJ). Cysteine, potassium cyanide, glutathione, SHAM,[1] cysteinesulfinic acid, cysteic acid, *S*-methylcysteine, glutamine, cysteine, cyanogen bromide activated Sepharose, β-mercaptoethanol, and PMSF were all obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bis(acrylamide), ammonium persulfate, crystalline $NaDodSO_4$, and TEMED were obtained from Bio-Rad Laboratories (Richmond, CA). Ampholines were supplied by LKB Instrument, Inc. (Rockville, MD). Glass beads were obtained from B. Braun Melsungen AG (Fernruf, West Germany). TPCK-trypsin was supplied by Millipore Corp. (Freehold, NJ). The molecular weight standards phosphorylase *b* (M_r 94 000), bovine serum albumin

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[1] Abbreviations: DEAE, diethylaminoethyl; SHAM, salicylhydroxamic acid; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine; KCN, potassium cyanide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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(M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), and lactalbumin (M_r 14 400) were obtained as a mixture from Pharmacia Chemicals (Uppsala, Sweden). Spectral grade 1-butanol and pyridine were supplied by Burdick & Jackson Laboratories Inc. (Muskegon, MI).

Methods

Growth of the Fungus. The dimorphic funugs *Histoplasma capsulatum* [Down's strain, mating type (−) from the permanent stock culture collection of this laboratory] was used throughout this study. A constant inoculum was grown to mid-log phase in liquid GYE medium (2% glucose and 1% yeast extract) as previously described (Maresca et al., 1981). Incubation with constant aeration was carried out at 37 °C for the yeast phase and 25 °C for the mycelial phase.

Phosphate Determination. Phosphate concentrations were measured according to Taussky & Shorr (1953).

Protein Measurements. Protein concentrations were measured by the spectrophotometric method of Groves et al. (1968).

Preparation of Ion-Exchange Resins. Sephadex G-50 and DEAE-cellulose were processed for chromatography according to the directions of the manufacturer.

Cysteine Oxidase Assay. Cysteine oxidase activity was defined previously by us (Maresca et al., 1981) as cysteine-dependent oxygen consumption after addition of KCN (1 mM) and SHAM (0.58 mM) which completely inhibited mitochondrial respiration. KCN was dissolved in aqueous solution adjusted to pH 7.0, and SHAM was dissolved in absolute ethanol. Both were prepared fresh for each experiment. Ethanol had no effect on oxygen uptake at the concentrations used in these experiments. Oxygen consumption was measured by a polaragraphic method on a KIC-oxygraph equipped with a Clark-type oxygen electrode with constant temperature water circulation (Gilson Instruments, Middleton, WI) (Maresca et al., 1981). The reaction was run at 37 °C and was initiated by the addition of 1.6 mM cysteine in 10 mM Tris-HCl, pH 7.0 (filtered through Millipore filters before use), to the cultures or enzyme fractions.

Cysteine Oxidase Purification. Yeast cells of *H. capsulatum* were harvested by filtration through Whatman No. 1 paper. The packed cells were washed thoroughly with cold distilled water and resuspended in buffer A which is 20 mM sodium phosphate, pH 7.0 (obtained by mixing equal volumes of equimolar solutions of Na_2HPO_4 and NaH_2PO_4 and then adding 0.2 mg/mL of PMSF). The cells were then mixed with twice their weight of glass beads (acid washed) and homogenized for 2 min in a Braun homogenizer (Model MSK) cooled with CO_2. The homogenate was centrifuged at 8000*g* for 20 min. in a Sorvall SS-34 rotor, and the resultant supernatant was then centrifuged at 100000*g* for 1 h in a Beckman Type 50 Ti rotor.

The protein concentration of the supernatant was adjusted to 14 mg/mL with buffer A and applied to a DEAE-cellulose column (2.5 × 10 cm) previously equilibrated with buffer A. The protein bound to the column was eluted by using a linear gradient of 20–200 mM sodium phosphate in buffer A containing 10% glycerol. Fifty fractions of 5 mL were collected and assayed for cysteine oxidase activity.

The DEAE-cellulose fractions containing the enzyme activity were pooled and concentrated to about 1 mL by dialysis against carbowax 20 000 and then applied to a Sephadex G-50 column (1 × 25 cm), equilibrated with buffer A containing 10% glycerol, and 80 fractions of 0.5 mL were collected and assayed for cysteine oxidase activity.

The active fractions from the Sephadex G-50 column were pooled, again concentrated to 1 mL by dialysis against carbowax 20 000, and applied to a freshly prepared cysteine–Sepharose column (0.5 × 6 cm). Cysteine was attached to Sepharose as described by Iverius (1971). Oxidase was eluted from the column with a linear gradient of 0–1.2 M NaCl in buffer A containing 10% glycerol and 0.05 mM dithiothreitol. Twenty-four fractions of 0.8 mL were collected, and cysteine oxidase activity was assayed in every fraction.

Iodination. Active fraction(s) from the cysteine Sepharose column was (were) iodinated with ^{125}I (Kumar et al., 1982); 100 μL of protein solution was mixed at 25 °C with 1 μL of 100 mCi/mL ^{125}I supplied as NaI and 5 μL of 15 mg/mL Chloramine T. After 2 min, 5 μL of 100 mg/mL KI was added. The reaction was allowed to continue for 1 more min, and then 2.5 μL of $Na_2S_2O_5$ was added to stop the reaction. Excess ^{125}I was removed by extensive dialysis against 62.5 mM Tris-HCl, pH 6.8.

Polyacrylamide Gel Electrophoresis. Purified iodine-labeled cysteine oxidase from the cysteine–Sepharose column was electrophoresed under nondenaturing and denaturing conditions. The method of Laemmli (1970) was used, with a 15% polyacrylamide gel at a constant voltage of 100 V. The gel was dried and autoradiographed. The molecular weight of cysteine oxidase was estimated by using the method of Shapiro et al. (1967).

Electrofocusing. Purified ^{125}I-labeled cysteine oxidase was analyzed by isoelectric focusing on an LKB multiphor flat-bed electrofocusing unit. The iodinated and noniodinated enzymes were focused in a 4.5% polyacrylamide gel supplied by LKB Instruments, containing ampholytes ranging from 3.5 to 9.5 pH units. The focusing at 400 V was performed until human serum proteins were resolved into visible bands on the gel. It usually took 1.5–2 h at 4 °C.

Two-Dimensional Electrophoresis. Cytoplasmic fractions from both yeast and mycelial cells were obtained by breaking the cells in Tris–glycine buffer, pH 7.5, in 10% glycerol and 0.2% PMSF by four 30-s bursts in a Braun homogenizer with 30-s intervals of cooling in liquid CO_2. The resulting homogenate was centrifuged at 2000*g*, followed by centrifugation at 105000*g* for 1 h; 100 μg of protein from the clear supernatants of both mycelial and yeast phase cells were mixed with ^{125}I-labeled purified oxidase and analyzed by two-dimensional electrophoresis as described by O'Farrel (1975). For the first dimension, each fraction was electrofocused in a 4.5% polyacrylamide gel containing ampholytes ranging from 5 to 7 and 3 to 10 pH units at a 4:1 ratio. Electrofocusing was done for 2.5 h at a constant wattage of 0.5 W/gel. The gels were then equilibrated and electrophoresed simultaneously in the second dimension on 15% $NaDodSO_4$–polyacrylamide gels. One set of gels was soaked in 50% methanol, 10% acetic acid, 2% trichloroacetic acid, and 1% sulfosalicylic acid and stained with silver (Merril et al., 1981). The other gel was dried and subjected to autoradiography to identify the spot corresponding to cysteine oxidase.

Thin-Layer Chromatography. For identification of the product of the cysteine oxidase, thin-layer chromatography was performed by using PEI–cellulose plates. Commercially available ^{14}C-labeled cysteine was purified by thin-layer chromatography on PEI–cellulose paper and incubated at 37 °C with freshly purified cysteine oxidase in 100 μL. At specified times, 5 μL of reaction mixture was spotted on a PEI–cellulose paper, and ascending chromatography was performed by using butanol–acetic acid–water (12:3:5) as the solvent (Bonker & Tonge, 1963). The paper was dried and

then analyzed by autoradiography.

Mass Spectroscopy of the Product. Cysteine was incubated with freshly prepared cysteine oxidase for 1 h at 37 °C. The reaction mixture was subjected to ascending chromatography as described above. The product was scraped from the TLC plate, eluted in sterile distilled water, and lyophilized. The lyophilized material was then analyzed by mass spectroscopy along with standard compounds in the cysteine metabolic pathway using an LKA Model 9000 single-focusing computer-interfaced mass spectrometer.

Tryptic Mapping. Tryptic peptide mapping was done by a modification of the procedure described by Gracy (1977). One to two micrograms of ^{125}I-labeled cysteine oxidase was subjected to purification by Sephadex G-200 column chromatography. This removed aggregation products and any unassociated ^{125}I. The purified cysteine oxidase fraction was carboxymethylated by using 5 μg of iodocetic acid at pH 8.0 for 15 min in 500 μL of 20 mM phosphate buffer, pH 8.0. Five microliters of β-mercaptoethanol was added, followed by 50 μg of trypsin in 50 μL, and the mixture was incubated for 2 h at 25 °C and then for 24 h at 4 °C. The incubation mixture was lyophilized and dissolved in 125 μL of 2% ammonium hydroxide. The undissolved material was removed by centrifugation at 2000*g* in a Sorval. One microliter (100 000 cpm) of clear solution was spotted at a corner of a cellulose TLC plate and electrophoresed for 3 h at 300 V with acetic acid–pyridine–deionized water (1:10:300) as the electrophoretic buffer. After electrophoresis, ascending chromatography was done with 1-butanol–acetic acid–pyridine–deionized water (50:1:33:40) as the solvent for the second dimension. Tryptic peptide mapping of the protein corresponding to the cysteine oxidase from the yeast two-dimensional gel was done as above after the protein was eluted from the spot electrophoretically (12 h at 25 V, 8 mA) into a dialysis bag in a destaining chamber containing a 10-fold dilution of phosphate-buffered saline. A spot on the yeast gel next to the cysteine oxidase and its corresponding protein on the mycelial gel was processed in the same way.

Determination of FAD Content. The pure enzyme was divided into two equal samples; in one half, the flavin content of the pure enzyme was estimated by a method described by Burch (1958). Known concentrations of FAD and cysteine oxidase in 0.5 mL of 20 mM phosphate buffer, pH 6.8, were treated with 1.5 mL of 10% trichloroacetic acid for 20 min at 4 °C. The samples were filtered through a Millipore filter (0.45-mm pore size). The filtrate was neutralized with 2 N sodium hydroxide and the fluorescence measured by using a Farrand Model A-2 fluorometer with a number 760 primary filter and numbers 372 and 373 as secondary filters. The other half was incubated at 38 °C for 24 h and neutralized and the fluorescence measured to obtain the total FMN content. FAD content and FMN content of the enzyme were then estimated by the method described by Burch (1958).

Results

Isolation of Cysteine Oxidase Activity from the Cytoplasm of Yeast Cells. Figure 1A shows the cysteine oxidase activity of the high-speed supernatant fraction from yeast cells eluted from the DEAE-cellulose column. The oxidase activity was eluted as a sharp peak at 0.05 M phosphate, a slightly lower phosphate concentration than an equivalent enzyme isolated from rat liver (Skakibara et al., 1976).

The Sephadex G-50 column resolved the protein into two major peaks, one eluting at the void volume and the other near the bed volume (Figure 1B). When alternate fractions were assayed for cysteine oxidase activity, it eluted close to the bed

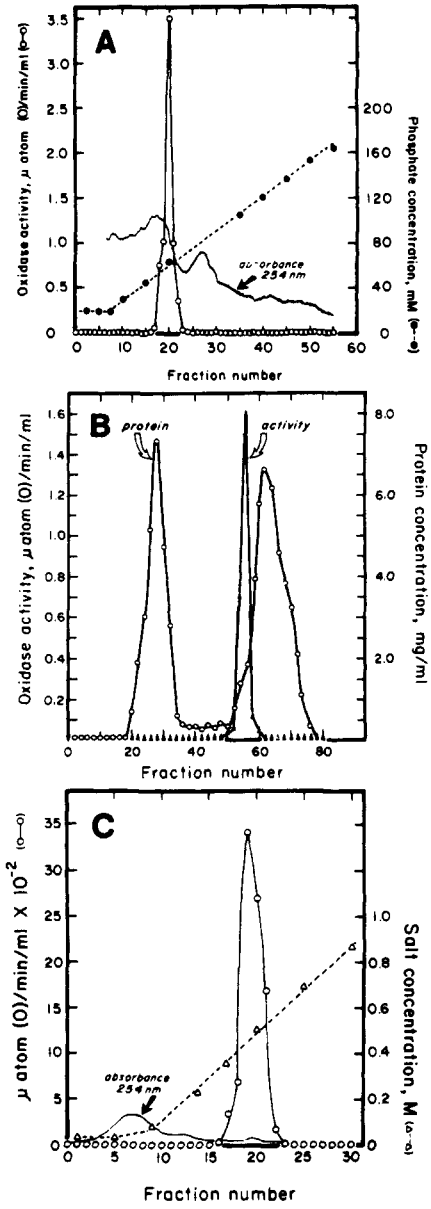

FIGURE 1: (A) DEAE-cellulose chromatography of the high-speed supernatant from the yeast phase cells. The protein was eluted with a linear gradient of 0.02–0.20 M phosphate, pH 6.8 (●). Sixty fractions were collected. Fractions were assayed for cysteine oxidase activity (○). The protein eluted was monitored by LKB UV scan (—). (B) Sephadex G-50 chromatography of the active fractions from the DEAE-cellulose column. The fractions were pooled, concentrated to 1/20th the volume, and loaded on a 1 × 25 cm Sephadex G-50 column equilibrated with 20 mM phosphate, pH 6.8, in 10% glycerol; 0.5-mL fractions were collected, and protein (○) and cysteine oxidase activity (△) were estimated in alternate fractions. Protein was measured by using the method of Groves et al. (1968). (C) Cysteine–Sepharose chromatograhy of the active fractions from the Sephadex G-50 column. The pooled fractions were loaded on a 0.5 × 6 cm column of cysteine–Sepharose. The activity was eluted with a linear gradient of 0–1.2 M sodium chloride in 20 mM phosphate buffer, pH 6.8, and 10% glycerol (△). Fractions of 0.8 mL were collected and assayed for cytsteine oxidase activity (○). Protein (—) was monitored by an LKB UV monitor.

volume. The fractions containing the highest activities were pooled and concentrated as described under Methods. The concentrated protein was applied to a cysteine–Sepharose column equilibrated in 20 mM phosphate buffer, pH 6.8, 0.1 mM DTT, and 10% glycerol. The cysteine oxidase activity eluted at 0.5 M NaCl (Figure 1C). In most of the preparations, the active fraction from cysteine–Sepharose was homogeneous and gave a single band on a $NaDodSO_4$–poly-

Table I: Purification of KCN- and SHAM-Resistant Cysteine Oxidase

fraction	volume (mL)	mg/mL	total protein	sp act.[a]	total units	purification (-fold)	yield[b] (%)
HSS	52	14.05	730.60	0.003	2.19	1	100
DEAE-cellulose (pool)	6	1.87	11.22	0.04	0.45	13.3	20.5
Sephadex G-50	4	0.91	3.64	0.11	0.40	36.6	18.3
cysteine-agarose	2	0.02	0.04	6.31	0.25	2103	11.4

[a] Atoms of oxygen ($\times 10^{-6}$) per minute per milligram of protein. [b] Starting material was 10 g wet weight of yeast cells.

acrylamide gel. In some cases, when more than one band was obtained, the active fractions from cysteine–Sepharose were further purified by gel filtration on a Sephadex G-200 column, 0.5 × 30 cm. The active fractions obtained from this column were invariably pure, as evidenced by the presence of only one band after polyacrylamide gel electrophoresis (see below).

The purity, recovery, and steps of purification of the cysteine oxidase activity are shown in Table I. The overall yield of the pure enzyme in terms of recoverable units of enzyme activity was 10%, and the specific activity of the enzyme increased by about 2100-fold. A unit of enzyme activity was defined as the amount of enzyme which consumed 0.398×10^{-6} atoms of oxygen/min at 37 °C.

Characterization and Properties of the Cysteine Oxidase. The purity of the enzyme preparation obtained from cysteine–agarose chromatography was tested by polyacrilamide gel electrophoresis on 15% gels. Because the amount of protein obtained was very low, it was necessary to iodinate the purified enzyme sample with ^{125}I. This allowed us to identify the enzyme on the gel and also increased the sensitivity of detection of any contaminants. An autoradiogram of an iodinated, purfied cysteine oxidase (0.1 μg equiv to 84 000 cpm) run on a 15% $NaDodSO_4$–polyacrylamide gel revealed one band with an estimated molecular weight of 10 500 ± 1500. A native 7.5–15% gradient gel also showed one band with approximately the same molecular weight. The molecular weight was also confirmed by chromatography with standards on a Sephadex G-200 column.

Isoelectric focusing of an ^{125}I-labeled sample revealed a band at p*I* = 5.8. This corresponded to the activity obtained from a sample of cysteine oxidase not labeled with ^{125}I which was focused simultaneously. These results are shown in Figure 2.

The activity of the cysteine oxidase was almost 2-fold higher at 37 °C than at 25 °C and was linear vs. time for 5–6 min.

Stability of the Enzyme Activity. The crude enzyme was stable for up to 4 weeks at −70 °C. The activity from DEAE-cellulose was stable for about 2 weeks. However, pure protein very quickly lost its activity in 1 or 2 days. Unlike the cysteine oxidase isolated from rat liver (Yamaguchi et al., 1978) the loss of activity could not be prevented by the addition of DEAE unabsorbed material.

The level of oxidation of cysteine could be enhanced by increasing the amount of enzyme added to the reaction mixture. The oxidase activity was saturable and obeyed Michaelis–Menten kinetics. If the reciprocal of the rate of reaction was plotted against the reciprocal of the concentration of cysteine, the K_m of the enzyme for cysteine was calculated to be 2×10^{-5} M. When activity was measured at different pHs, the optimum was obtained at about 8.0.

Several other cysteine-related substrates, some of which are found in the molecular pathways of cysteine, were tested with the cysteine oxidase fraction from DEAE-cellulose column, and these results are shown in Table II. Only high concentrations of β-mercaptoethanol caused some oxygen consumption in the presence of cysteine oxidase. The K_m for β-mercaptoethanol was 16.6×10^{-3} M, about 800-fold higher than

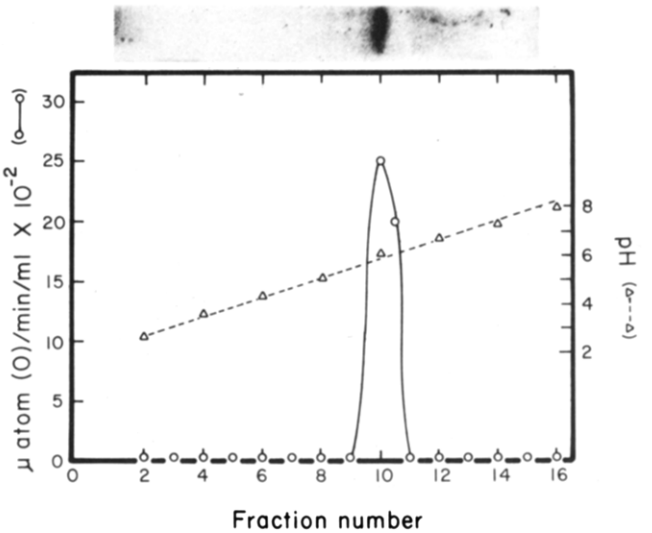

FIGURE 2: Isoelectric focusing of the pure cysteine oxidase. ^{125}I-Labeled cysteine oxidase was focused on a 4.5% acrylamide gel containing ampholytes of 3.5–9.5 pH values. The gel was dried, and autoradiography was performed. Another gel was simultaneously focused with active cysteine oxidase. The gel was sliced into 16 pieces of 0.5 mm each, and the enzyme was eluted from the gel slices by shaking them in phosphate buffer, pH 6.8, for 24 h at 20 °C, and each fracton was assayed for cysteine oxidase (○). The pH gradient (△) was measured by a LKB surface electrode.

Table II: Activity of Compounds Related to Cysteine as Substrates for Purified Cysteine Oxidase

agent	concn (mM)	atoms (O) consumed (mg of protein)$^{-1}$
L-cysteine	2.0	5
D-cysteine	2.0	0
cystine	2.0	0
taurine	2.0	0
cystamine	2.6	0
cysteinesulfinic acid	2.6	0
glutathione	2.0	0
cysteic acid	2.0	0
S-methylcysteine	2.0	0
pyruvic acid	2.0	0
β-mercaptoethanol	3.3	0.7

that for cysteine. Other amino acids were also tested as substrates for the cysteine oxidase, and none were oxidized by the enzyme (data not shown).

Effect of Various Agents on the Enzyme Activity. Incubation of the enzyme with Pronase (20 μg/mL) destroyed the enzymatic activity Fe^{2+}, and NADH stimulated the activity of the enzyme. Agents like *o*-phenanthroline, EDTA, and EGTA totally inhibited the activity of the cysteine oxidase at very low concentrations. The inhibition by 30 μM EDTA could be overcome by subsequent addition of Fe^{2+}. Twenty-six percent of the activity could be restored by the addition of 12 μM Fe^{2+}, and a maximum of 60% of the original activity was achieved with 22 μM Fe^{2+}. KCN and SHAM added individually or together had no effect on the activity.

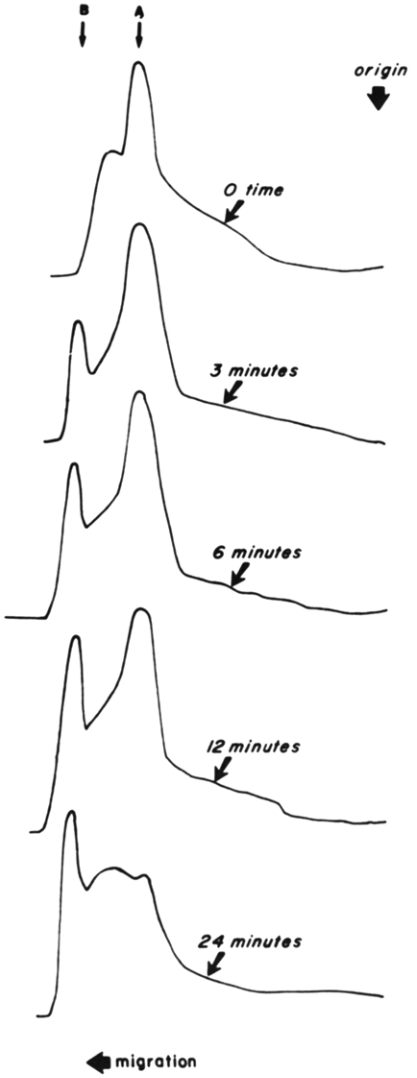

FIGURE 3: Thin-layer chromatography of the product of the cysteine oxidase reaction. Twenty-five microliters of the purified enzyme preparation was added to a solution of 25 μL of radiolabeled [^{14}C]cysteine (24 μCi/μmol) resulting in a final concentration of 2 mM cysteine. The reaction was initiated by placing the sample in a 37 °C water bath, and 2-μL samples were taken at 0-, 3-, 6-, 12-, and 24-min intervals and were spotted individually onto 13 cm separate TLC plates of PEI–cellulose 400, with the appropriate markers. They were subjected to ascending chromatography with 0.2 M sodium citrate, pH 2.2, as the solvent. The strips were dried and subjected to autoradiography at −70 °C using a Cronex screen. The autoradiograms were scanned on a Joyce-Loebel scanner. The arrows indicate the location of cysteine (A) and cysteinesulfinic acid (B).

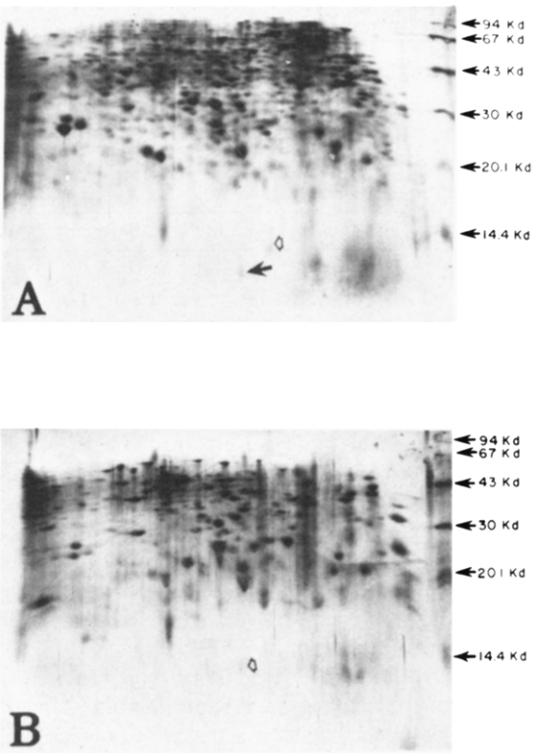

FIGURE 4: Two-dimensional gel electrophoresis of the cytoplasmic fractions of yeast and mycelia. Cytoplasmic fractions of yeast and mycelia phase cells of *H. capsulatum* were mixed with ^{125}I-labeled cysteine oxidase purified from the yeast phase and subjected to two-dimensional electrophoresis as described in the text. After the electrophoresis, the gels were stained with silver nitrate and subjected to autoradiography as described in the text. The large dark arrow in (A) indicates the position of the radioactive spot obtained with ^{125}I-labeled oxidase. The arrows in (A) and (B) indicate the other spots subjected to tryptic mapping. (A) Yeast phase; (B) mycelial phase.

FAD added at concentrations varying from 1.6 to 16.6 μM had an inconsistent effect on enzyme activity. In some experiments, there was a low level of stimulation; in others there was none. When the flavin content of the pure enzyme was estimated, 1 mol of enzyme was found to contain about 0.1 mol of flavin. At the highest purity, the amount of flavin detected as FAD was 70% of the total flavin content (Burch, 1958). When the enzyme was purified in the presence of 6 μM FAD (Krohne-Ehrich et al., 1977), the final flavin content of the enzyme was still only 0.1 mol/mol of enzyme protein, and the activity was not stimulated by FAD.

Identification of the Product of Cysteine Oxidase. In Figure 3, a densitometry tracing of an autoradiograph shows the product of cysteine oxidase activity when [^{14}C]cysteine (A) is used as substrate. The accumulated product (B) corresponds to a cysteinesulfinic acid standard run on the same thin-layer chromatogram. This was further confirmed by mass spectrometric analysis of the product (data not shown). The spectrum obtained corresponded to cysteinesulfinic acid, the product of other known eucaryotic cysteine oxidases (Skakibara et al., 1976; Yamaguchi et al., 1978; Sorbo & Ewetz, 1965; Lombardini et al., 1969; Wainer, 1965).

Cysteine Oxidase Activity in Mycelium. The mycelial cytoplasm showed no cysteine oxidizing activity. No activity could be found in mycelial extracts even after blind purification. Mixing mycelial extracts with the yeast enzyme had no effect on yeast enzyme activity so it was unlikely that the mycelia contained an inhibitor of the enzyme. We prepared cytoplasmic fractions from the yeast and mycelial phases as described under Methods and added ^{125}I-labeled purified cysteine oxidase to 100 μg of cytoplasmic protein of mycelial or yeast phases, and these were then subjected simultaneously to two-dimensional electrophresis. The gels were stained with silver nitrate and subjected to autoradiography as described under Methods.

The silver nitrate stained spot corresponding to the spot of cysteine oxidase seen in the autoradiograph of the yeast phase cytoplasmic fraction is marked by the large dark arrow in Figure 4A. No corresponding spot is seen in the mycelial gel (Figure 4B). The cysteine oxidase spot was cut out of the gel and radiolabeled with ^{125}I, and a trypic fingerprint was done as described under Methods. The pattern of spots was the same as that obtained when the pure cysteine oxidase was treated with trypsin and chromatographed in the same manner. Five major and several minor spots with almost identical patterns were seen on both chromatograms (Figure 5C,D).

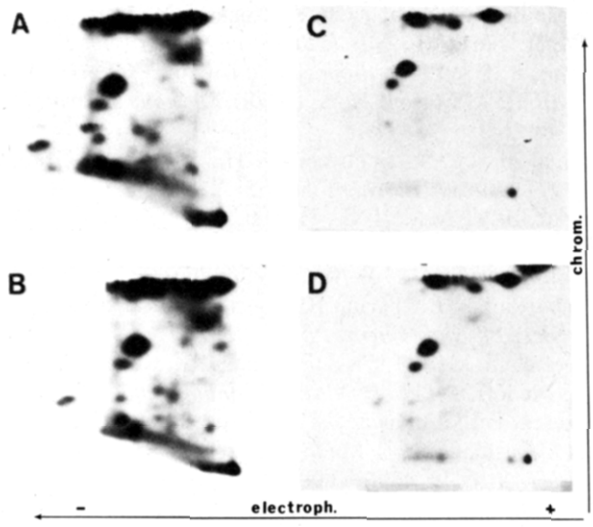

FIGURE 5: Tryptic mapping of the protein spots extracted from two-dimensional gels of cytoplasmic fractions of *H. capsulatum*. The non-oxidase protein spot from the yeast phase (A), the corresponding spot from the mycelial phase (B) and purified cysteine oxidase (C), and the spot corresponding to the cysteine oxidase from the yeast phase gel (D) were labeled with ^{125}I and subjected to tryptic fingerprinting as described in the text.

The neighboring spot on the yeast gel and its corresponding protein in the mycelial gel (labeled with a small arrow) were also subjected to a trypsin digest by using the same method. The patterns of these two spots were identical with each other and completely different from the cysteine oxidase pattern (Figure 5A,B). Therefore, the yeast form of the cysteine oxidase appears to be absent from mycelial proteins, as is any detectable activity of the enzyme. However, the presence of an altered inactive form of the enzyme in mycelia cannot presently be ruled out. Our attempts to raise antibody to purified cysteine oxidase have proven unsuccessful. The enzyme appears to have a very low antigenicity, but if we can obtain larger quantities for immunization, antibodies to yeast cysteine oxidase might be used to detect cross-reacting protein among the mycelial proteins.

In addition to the cysteine oxidase, there are a considerable number of other differences in the patterns of spots in the gels from the two phases. These differences were not analyzed further at this time.

Discussion

Cysteine oxidases have been described in bacteria (Yu & Devoe, 1981; Duerre & Chakrabarty, 1975; Chen et al., 1971) and animal cells (Skakibara et al., 1976; Hosokawa et al., 1980; Sorbo & Ewetz, 1965; Yamaguchi et al., 1978); here we describe such an enzyme from a fungus. Although the assay system we used differs from those used in the bacterial and animal systems by other workers, it is certain we are dealing with a similar enzyme. Like the oxidases isolated from other sources, the cysteine oxidase from *H. capsulatum* has a high level of specificity for L-cysteine. (D-Cysteine is not used as a substrate; see Table II.) Moreover, the product of oxidation, cysteinesulfinic acid, is also the same as that from the other sources.

Our purification procedure was almost the same as that used for the cysteine oxidase from rat liver (Yamaguchi et al., 1978). An important difference between the methods is that we used cysteine–Sepharose as an affinity resin which provided a quick efficient additional purification step after DEAE-cellulose and Sephadex G-50 column chromatography. We were able to achieve greater than 2000-fold purification to a level of homogeneity as evidenced by the one band seen on $NaDodSO_4$–polyacrylamide gels.

The cysteine oxidase isolated from *H. capsulatum* is an iron-containing dioxygenase with a molecular weight of 10 500 (±1500). This estimate of molecular weight may be inaccurate becuase it is based on use of the iodinated protein and the assumption that iodination does not affect molecular weight. However, the estimate of molecular weight is probably accurate because the position of the iodinated oxidase was identical with the position of the nonlabeled oxidase on the two-dimensional gels (Figure 4A). Further, the ^{125}I band obtained upon isoelectric focusing corresponded to the position of activity of the enzyme on simultaneously run gels (Figure 2). In contrast to the enzyme in *H. capsulatum*, the rat liver enzyme has a molecular weight of 22 500 (Yamaguchi et al., 1978). The latter oxidase activity appears to be composed of two distinct proteins which are separable by DEAE-cellulose column chromatography. One, protein B, is thought to be the catalytic protein, whereas the other, protein A, has little or no catalytic activity but activates protein B. The separation of the two proteins which occurs during purification is thought to be a factor in the extreme lability of the oxidase activity.

The cysteine oxidase from *H. capsulatum* is also extremely labile. However, unlike the rat liver enzyme, we have no evidence for a specific activating protein or subunit. Addition of the protein which failed to bind to the DEAE-cellulose column did not increase the activity of the purified enzyme nor did addition of various other protein extracts from the yeast phase of *H. capsulatum*.

Most of the dioxygenases described thus far have been metal-containing enzymes (Keevil & Mason, 1978). The increase in activity that we observed after incubation with Fe^{2+} suggests that the cysteine dioxygenase from *H. capsulatum* contains iron. The evidence for the metal requirement for activity is supported by the observed inhibition of enzyme activity in the presence of chelating agents like *o*-phenanthroline, EDTA, or EGTA and the subsequent restoration of most of the activity by Fe^{2+}.

FAD had little or no effect on enzyme activity, and the amount of FAD contained in the enzyme was not stoichiometric. We have no explanation for the small amount of FAD that was present; it could represent contamination by cytoplasmic flavin that copurified with our enzyme. It is also possible that we are dealing with a flavoprotein and FAD along with its enzyme receptor lost during purification of the cysteine oxidase. We think this possibility is unlikely, and we believe the cysteine dioxygenase is an iron-containing enzyme.

Like the rat liver enzyme, the oxidase from *H. capsulatum* is located in the cytoplasm. It is not a mitochondrial enzyme and does not utilize the respiratory chain or the alternate oxidase (Maresca et al., 1981). This is the basis for the resistance of its activity to inhibition by KCN and SHAM. The pH for optimal activity of the enzyme from both sources is similar, and the enzyme from *H. capsulatum* has a p*I* of 5.8 compared to a p*I* of 5.5 in the rat liver enzyme. The K_m of the rat liver enzyme for cysteine is 4.5×10^{-4} M compared to 2×10^{-5} M of the enzyme from *H capsulatum*.

The function of the cysteine oxidase in rat liver and *H. capsulatum* is unknown. The activity in the former appears to be regulated by hormones and also by the level of development of the rat embryo (Hosokawa et al., 1980). The activity of the enzyme in *H. capsulatum* also appears during a developmental process which may be regulated by cyclic AMP (Maresca et al., 1977). The enzyme activity and probably the protein are absent in the mycelial phase, appear

within 3–4 days after the temperature shift from 25 to 37 °C, and precede the dramatic morphologic changes of the mycelial to yeast transition (Maresca et al., 1981). Cysteine oxidase catalyzes the oxygenation of cysteine to cysteinesulfinic acid, considered to be a key intermediate of cysteine metabolism to cysteic acid, hypotaurine, taurine, and pyruvate (Giorgio et al., 1975). Our previous studies and the work of others have indicated that the level of intracellular cysteine is a critical factor in the mycelial to yeast transition of *H. capsulatum* (Maresca et al., 1981, 1977; Scherr, 1957; McVeigh & Houston, 1972). The cysteine oxidase may therefore provide a mechanism to regulate the level of intracellular free cysteine in the cell or provide a metabolic product of the oxidation of cysteine important to the transition to yeast. The time of appearance of the cysteine oxidase and the striking dependence of the mycelial to yeast transition and yeast growth on the presence of cysteine in the cultures indicate that it plays an important role in the differentiation process.

Acknowledgments

We are grateful to David Schlessinger, Alan Lambowitz, Helen Burch, George Drysdale, and Claire Birge for helpful discussion. Audrey Painter provided excellent technical help.

Registry No. Cysteine, 52-90-4; cysteine dioxygenase, 37256-59-0.

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