Partial Purification of a Peroxisomal Polyamine Oxidase from *Candida boidinii* and its Role in Growth on Spermidine as Sole Nitrogen Source

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Candida boidinii grows well on spermidine as sole nitrogen source, but poorly on spermine. Cells grown on spermidine, cadaverine, putrescine and 1,3-diaminopropane contained a polyamine oxidase which attacks spermine and spermidine at the secondary amino groups, forming putrescine and a product thought to be 3-aminopropionaldehyde. The enzyme was synthesized before growth began when *C. boidinii* that had been grown in medium containing glucose + ammonium was transferred to medium in which spermidine replaced ammonium. Other enzymes increasing in specific activity during this adaptation were catalase, benzylamine oxidase and NAD-dependent glutamate dehydrogenase. The polyamine oxidase was purified to 50% homogeneity, but was too unstable to obtain completely pure. It had a pH optimum of 10.0, and could be stabilized by addition of inert protein. It oxidized spermine, spermidine, *N*¹-acetylspermidine, *N*-*n*-butylpropylamine, di-*n*-butylamine and di-*n*-hexylamine. It did not oxidize di-*n*-propylamine, diethylamine or *N*¹,*N*⁸-diacetylspermidine. Apparent *K*_m values were determined for the active substrates. The enzyme was potently inhibited by quinacrine and by divalent cations. The stoichiometry of the enzyme reaction was established using di-*n*-butylamine as substrate. The enzyme has a molecular weight in the range 80000 to 110000. Putrescine (the oxidation product of spermidine) was not oxidized by cell-free extracts, but evidence of aminotransferase activity was found. The oxidation/transamination product of putrescine, 4-aminobutyraldehyde (1-pyrroline), was oxidized by extracts and a scheme is presented by which spermidine could be catabolized. Polyamine oxidase was shown to co-sediment with NAD-dependent glycerol 3-phosphate dehydrogenase and catalase in sucrose gradients after mechanical breakage of spheroplasts, and is thus a peroxisomal enzyme. Polyamine oxidase was present in some other yeasts when grown on spermidine, *C. nagoyaensis*, *Hansenula polymorpha* and *Trichosporon melibiosaceum*, but absent from *C. steatolytica*, *Pichia pastoris* and *Sporopachydermia cereana*. These latter yeasts probably contained an enzyme resembling benzylamine/putrescine oxidase which attacks the primary amino groups of spermidine.

INTRODUCTION

Candida boidinii when grown on *n*-butylamine as sole nitrogen source produces two primary-amine oxidases, neither of which can oxidize the diamine putrescine (1,4-diaminobutane) nor the polyamines spermine (1,12-diamino-4,9-diazadodecane) and spermidine (1,8-diamino-4-azaooctane) (Haywood & Large, 1981). Other yeasts, e.g. *Pichia pastoris*, contain a primary-amine oxidase which can liberate ammonia from the primary amino groups of all three compounds (Green *et al.*, 1983). It was thus of interest to attempt to grow *C. boidinii* on polyamines as nitrogen source in order to determine their mode of degradation. *Candida boidinii* turned out to grow well on spermidine and putrescine, but only slowly on spermine, and in this paper we attempt to identify the enzymes responsible for liberating the primary and secondary amino groups of spermine and spermidine in a form utilizable by the yeast.

Abbreviation: ABTS, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonate).

METHODS

Materials. 2-Aminobenzaldehyde was synthesized as described by Albrecht *et al.* (1962). 1-Pyrroline (4-aminobutyraldehyde) was synthesized from DL-ornithine as described by Jakoby (1962). *N*¹-Acetylspermidine was prepared as described by Tabor *et al.* (1971) and purified on a Dowex 50-X8 (H⁺ form) column. It contained a trace (0.5%) of spermidine. It was standardized using benzylamine/putrescine oxidase of *Pichia pastoris*. *N*¹,*N*⁸-Diacetylspermidine was isolated from the same reaction mixture, as described by Bolkenius & Seiler (1981). *N*-*n*-Butylpropylamine was obtained from K & K Rare and Fine Chemicals (ICN Pharmaceuticals) via Kodak; di-*n*-butylamine, di-*n*-propylamine and 3-aminopropanol were from BDH; and di-*n*-octylamine, 3,3'-iminobispropylamine and diethylenetriamine were from Aldrich Chemical Co., Gillingham, Dorset, UK. All other chemicals were obtained from Sigma or Fisons Scientific Apparatus. Benzylamine/putrescine oxidase was purified from *Pichia pastoris* as described by Green *et al.* (1983), and yeast aldehyde dehydrogenase, catalase and horseradish peroxidase were obtained from Sigma. Zymolyase 5000 (Kitamura *et al.*, 1971) was obtained from Miles Laboratories, Stoke Poges, Bucks, UK.

Maintenance and growth of the organism. This has been described previously (Haywood & Large, 1981). The carbon source was 55 mM-glucose and the nitrogen sources were used at 30 mM (ammonia) or 5 mM (spermidine). Nitrogen sources in the experiment of Table 3 were 10 mM.

Enzyme assays. Polyamine oxidase (EC 1.5.3.—) was assayed by two different methods. Spermine was used as substrate except where indicated. (a) Polarographic assay. Enzyme and 200 µmol sodium borate buffer pH 10.0 were incubated in the chamber of a Rank oxygen electrode (Rank Instruments, Bottisham, Cambridge, UK) at 25 °C. The reaction was started by the addition of 10 µmol spermine tetrahydrochloride. The reaction volume was 3 ml. One unit of enzyme is the amount required to catalyse the uptake of 0.5 µmol oxygen min⁻¹. (b) Spectrophotometric assay. Enzyme, 200 µmol sodium borate buffer pH 10.0, 2.5 µmol of the sodium salt of ABTS and 16 units (200 µg) of horseradish peroxidase (total volume 3 ml) were incubated at 25 °C in polystyrene cuvettes (light path 1 cm). The reaction was started by addition of 10 µmol spermine tetrahydrochloride and the increase in absorbance at 405 nm was followed against a blank containing all components except substrate (Haywood & Large, 1981). One unit of enzyme is the amount required to catalyse the formation of ABTS radical cation at a rate of 1 µmol min⁻¹ under these conditions. An extinction coefficient of 18.4 l mmol⁻¹ cm⁻¹ was used (Werner *et al.*, 1970).

Other oxidases were assayed in an analogous fashion (Haywood & Large, 1981; Green *et al.*, 1982, 1983). Diamine aminotransferase (EC 2.6.1.29) was assayed as described by Kim (1964). Catalase (EC 1.11.1.6) was assayed as described by Haywood & Large (1981), cytochrome *c* oxidase (EC 1.9.3.1) as described by Wharton & Tzagoloff (1967), NADH dehydrogenase (EC 1.6.99.3) as described by Mackler (1967), NAD-linked glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) as described by Kawamoto *et al.* (1979), isocitrate dehydrogenase (EC 1.1.1.42) as described by Bergmeyer (1974), and NAD- and NADP-linked glutamate dehydrogenases (EC 1.4.1.2 and 1.4.1.4) by the method of Doherty (1970).

Purification of polyamine oxidase. (a) Preparation of crude extract. Frozen *C. boidinii* grown on spermidine or 1,3-diaminopropane was suspended in 2 vol. 50 mM-potassium phosphate pH 7.0 and passed through a French pressure cell (Haywood & Large, 1981). The extract was centrifuged at 40000 *g* at 4 °C for 20 min and the pellet resuspended in 1 vol. of the same buffer and passed through the French press. After centrifuging as before the supernatants from both treatments were combined.

(b) Ammonium sulphate concentration. Solid ammonium sulphate was added slowly at 4 °C with stirring to the crude extract to 90% saturation (3.5 M). The precipitated protein was dissolved in the smallest possible volume of 50 mM-potassium phosphate buffer pH 7.0. Purification was typically 1.3- to 2-fold.

(c) Sepharose 6B chromatography. The material from step (b) was applied to a column (100 cm × 2.5 cm diameter) of Sepharose 6B equilibrated in 20 mM-potassium phosphate pH 7.0 and washed at 4 °C with the same buffer. Fractions containing more than 50% of the maximum activity were combined. Purification was typically 2- to 3-fold.

(d) Chromatography on 4-aminobutyl-agarose. The material was then applied to a column (10 cm × 2.5 cm diameter) of 4-aminobutyl-agarose (Sigma) and washed with the same buffer (20 mM-potassium phosphate). The column was then eluted at 4 °C with a linear gradient of 0 to 1.5 M-NaCl in 20 mM-potassium phosphate pH 7.0. Fractions containing more than 50% of the maximum activity were combined and bovine serum albumin added to a final concentration of 5 mg ml⁻¹. Purification was typically 6- to 12-fold.

This preparation produced overall 20- to 100-fold purified polyamine oxidase in a yield of about 20%. The specific activity of the purified material varied from 15 to 30 units (mg protein)⁻¹.

Enzymic changes during adaptation from growth on ammonia to growth on spermidine. *Candida boidinii* was grown in 3 l medium containing 15 mM-ammonium and 55 mM-glucose. The cells were centrifuged at 13000 *g* at 30 °C and resuspended in approximately 1000 ml sterile carbon- and nitrogen-free medium and 500 ml of this was added to each of two 20 l fermenters, one containing 15 mM-ammonium and 55 mM-glucose, the second containing 55 mM glucose and 5 mM-spermidine. The two cultures were thus equivalent in molarity of nitrogen. Growth was followed

by measuring A_{663} . Samples were taken at approximately 4 h intervals, centrifuged at 13000 g , the supernatant retained for analysis, and the cells washed in 50 mM-potassium phosphate pH 7.0, and stored at -15°C . The samples of media were used to determine the pH, glucose concentration and nitrogen-source concentration. Ammonia and other amines were known from preliminary experiments not to accumulate in the spermidine growth medium. Cell-free extracts were prepared as described by Haywood & Large (1981).

Stoichiometry of the polyamine oxidase reaction. The stoichiometry was determined using di-*n*-butylamine as substrate. The reaction mixture contained 135 μg purified enzyme, 200 μmol sodium borate buffer pH 10.0 and water, in a final volume of 3.4 ml. The reaction was started by addition of 0.5 μmol di-*n*-butylamine hydrochloride and oxygen consumption followed at 25°C in a Rank oxygen electrode calibrated by the method of Robinson & Cooper (1970). When no further oxygen was consumed, 60 μg beef liver catalase was added and oxygen evolution followed to a plateau. The reaction was then stopped by addition of 0.3 ml 2.5 M- HClO_4 and neutralized with KOH. A control incubation was followed over the same period of time in which enzyme was replaced by boiled enzyme. Oxygen and H_2O_2 were determined from the oxygen electrode readings, the other components of the reaction mixture were estimated as described below. Similar experiments were done in which di-*n*-butylamine was replaced by either spermidine or spermine.

Chemical estimations. Ammonia was determined by the indophenol method (Chaney & Marbach, 1962), and 1-pyrroline with 2-aminobenzaldehyde (Bachrach & Oser, 1963); di-*n*-butylamine was estimated by GLC at 200°C on a column of Carbowax B coated with Carbowax 20M and 0.3% KOH as described by Di Corcia & Samperi (1974). Glucose was measured with a Boehringer GOD-Perid blood sugar test combination. Protein was determined by the Bradford (1976) method. Butyraldehyde was determined with yeast aldehyde dehydrogenase as described by Lundquist (1958), *n*-butylamine, spermine, spermidine and putrescine were determined enzymically as follows. The neutralized sample (2–100 nmol primary amino groups) in a volume of up to 1.5 ml was added to a polystyrene cuvette containing 2.5 μmol disodium ABTS, 200 μmol potassium phosphate buffer pH 7.0 and 100 μg horseradish peroxidase in a total volume of 3 ml. The reaction was started by adding 25 μg purified benzylamine/putrescine oxidase from *Pichia pastoris* (Green *et al.*, 1983). The increase in absorbance at 405 nm was followed to completion (about 2 min) and the amount of ABTS radical cation formed was calculated using an absorption coefficient of $18.4 \text{ l mmol}^{-1} \text{ cm}^{-1}$. One mol of ABTS is equivalent to 0.5 mol of primary amino group. This method cannot be applied to mixtures of the above amines, only to individual compounds.

Thin-layer and ion-exchange chromatography. 2,4-Dinitrophenylhydrazones of aliphatic aldehydes were separated on cellulose thin-layer plates in heptane saturated with methanol as described by Eady *et al.* (1971). Aliphatic amines were separated on cellulose in butan-1-ol/ HCl /water (7:2:1, by vol.) (Blau, 1961) and putrescine and polyamines on cellulose in propan-2-ol/ HCl /water (80:30:2, by vol.) (Yamada *et al.*, 1980a). Putrescine, spermidine and spermine were also separated by ion-exchange on a Zeo Karb 225 column, eluting with a linear gradient of 0.5–5 M- HCl , as described by Gahl & Pitot (1982). After separation, combined fractions of each peak were identified by thin-layer chromatography in propan-2-ol/ HCl /water and estimated as described above.

Molecular weight determinations. The molecular weight of polyamine oxidase was determined by molecular exclusion chromatography on Sephadex G-150 as described by Andrews (1970). The molecular weight standard proteins were cytochrome *c* (12400), peroxidase (40000), malate dehydrogenase (70000), lactate dehydrogenase (130000), alcohol dehydrogenase (141000), aldolase (158000) and pyruvate kinase (235000). The molecular weight was also determined by polyacrylamide gel electrophoresis by the method of Hedrick & Smith (1968), as described in detail by Haywood & Large (1981). A third method used was the sucrose gradient centrifugation method of Martin & Ames (1961), comparing the sedimentation of polyamine oxidase with that of pyruvate kinase and lactate dehydrogenase.

Preparation of spheroplasts. Freshly harvested cells of *C. boidinii* were centrifuged, washed and resuspended in solution A (3 M-sorbitol, 1 mM-EDTA, 1 mM- MgCl_2 in 70 mM-potassium phosphate pH 7.0) for osmotic stabilization. After 10 min at 0°C the cells were centrifuged at 10000 g for 10 min at 4°C , and the pellet resuspended in solution A (200 mg wet wt ml^{-1}). Zymolyase 5000 was added to a final concentration of 1 mg ml^{-1} (Osumi *et al.*, 1975) and the mixture incubated at 30°C for 2 h. Mercaptoethanol was omitted because it inhibits amine oxidase activity (Zwart *et al.*, 1980). Spheroplast formation was monitored in the microscope. An equal volume of solution B (1 mM-EDTA, 1 mM- MgCl_2 in 70 mM-potassium phosphate pH 7.0) was added and the mixture left for 15 min at 0°C . The spheroplasts were centrifuged at 5000 g for 5 min and the pellet resuspended in solution C (1.5 M-sorbitol, 1 mM-EDTA, 1 mM- MgCl_2 in 70 mM-potassium phosphate pH 7.0).

Cell fractionation. Spheroplasts were broken in a Potter hand homogenizer (with a Teflon plunger), and the product was examined in the microscope to confirm breakage. Unbroken spheroplasts were removed by centrifuging at 5000 g for 5 min. Organelles were then sedimented at 30000 g for 15 min and resuspended in solution C. Any unsuspended material was removed by centrifuging at 5000 g for a further 5 min.

The material so obtained was layered (2 ml) on to a discontinuous sucrose gradient prepared in a 15 ml Sorvall centrifuge tube. The gradient was made by pipetting successive samples of 75% (1 ml), 60% (1 ml), 45% (1 ml), 40% (2 ml), 35% (2 ml), 30% (2 ml) and 25% (1 ml) sucrose (w/v) in 0.2 M-potassium phosphate pH 7.0. The

gradient was then centrifuged at 50000 *g* for 60 min in a Sorvall SS34 angle head at 4 °C and 1 ml fractions were removed successively from the top of the tube and assayed for the activity of polyamine oxidase, cytochrome *c* oxidase, NADH dehydrogenase, catalase and NAD-linked glycerol-3-phosphate dehydrogenase. The sucrose concentration was determined by measuring the refractive index of the individual fractions.

RESULTS

Candida boidinii grew poorly and unpredictably with a long lag period on a defined medium with 5 mM-spermine tetrahydrochloride as sole nitrogen source and glucose as carbon source, but grew well when spermine was replaced by 5 mM-spermidine trihydrochloride. This is similar to the observations of Yamada *et al.* (1980*a*) with *Aspergillus terreus*. The precipitate from a 95% saturation ammonium sulphate treatment of crude extracts of cells grown on spermidine when tested by spectrophotometric assay at pH 7.0 showed low activity with the following amines [rates are nmol ABTS radical cation formed min⁻¹ (mg protein)⁻¹]: spermidine 0.43 and spermine 4.7 when tested at 3.3 mM; 0.33 mM-benzylamine and *n*-butylamine were also active [23 and 76 munits (mg protein)⁻¹, respectively]. Lower concentrations of polyamines showed no activity at pH 7.0. When the pH optimum for polyamine oxidation was examined, spermidine and spermine both showed a pH optimum in the region of 10.0. At this pH value activity was not very stable, and so the more rapid spectrophotometric assay was usually preferred. No activity could be detected in the region of pH 5.0 with *C. boidinii* extracts, in contrast to the results with spermine and spermidine oxidation in *Penicillium* species (Isobe *et al.*, 1980*a*; Kobayashi & Horikoshi, 1982).

The *Penicillium* polyamine oxidases were also stable at acid pH values (Isobe *et al.*, 1980*a*; Kobayashi & Horikoshi, 1982), but when crude extracts of spermidine-grown *C. boidinii* were held at various pH values in the range 3.8 to 11.2 at 0 °C for 64 h, 8.6% of the initial activity survived at pH 7.0 and 3.1% of the activity at pH 8.0, but no activity survived at other pH values. Essentially similar observations were made in experiments involving shorter incubation periods. Thus the enzyme was unstable even at neutral pH values.

Role of polyamine oxidase in growth on spermidine

The time course of the synthesis of various enzymes was examined during the adaptation of *C. boidinii* grown on ammonium as nitrogen source to growth on spermidine as nitrogen source (Fig. 1). The cells were compared with a control culture growing on ammonium (Fig. 2). No polyamine oxidase was detectable at any stage during growth on ammonium (Fig. 2*a*) but transfer to spermidine produced an immediate, rapid synthesis of the enzyme together with an increase in the specific activity of catalase (Fig. 1*b*) before growth on spermidine began. Activities of polyamine oxidase and catalase remained high well into the stationary phase of growth. (Both the spermidine and ammonium cultures become carbon-limited quite rapidly.) Benzylamine oxidase also increased in specific activity during exponential growth, but fell thereafter (Fig. 1*b*), while methylamine oxidase only became detectable towards the end of growth and remained at significant levels in the stationary phase. Growth on spermidine did not significantly affect the activities of isocitrate dehydrogenase or NADP-linked glutamate dehydrogenase, but produced significant elevation of NAD-linked glutamate dehydrogenase before growth began, and which remained at a high level (Fig. 1*a*). The control culture growing on ammonium as nitrogen source produced increased activity of NADP-linked glutamate dehydrogenase, and a small increase in catalase activity (Fig. 2*a*), but no benzylamine or methylamine oxidase activity was detected.

Attempted stabilization of polyamine oxidase

Possible stabilizing agents for the enzyme were tested on a partially purified preparation, namely spermine, ethanol and bovine serum albumin. Of these, only the last seemed to have a significant stabilizing effect, and this was of little use during purification. Thus it was necessary to perform the purification as quickly as possible.

Fig. 1. Effect on growth and enzyme activity of transfer of *Candida boidinii* grown on ammonium as nitrogen source to medium containing spermidine as sole nitrogen source. (a) ●, Growth; ▽, isocitrate dehydrogenase; ▲, NAD-linked glutamate dehydrogenase; ■, NADP-linked glutamate dehydrogenase (broken line). (b) ●, Growth; ○, polyamine oxidase; △, benzylamine oxidase; □, methylamine oxidase; ■, catalase (broken line). (c) ●, Growth; ▲, pH of culture medium (broken line); △, glucose concentration in the medium; ○, spermidine concentration.

Partial purification of the enzyme

Consistent yields of enzyme were obtained using the procedure described in Methods. Further purification was attempted but because of instability no improvement in specific activity was achieved. Activity of the partially purified material could only be retained by addition of 5 mg bovine serum albumin per ml of enzyme preparation as a stabilizing agent, but under these conditions it was stable at 4 °C for 1 to 2 months. A sample of enzyme of specific activity 15.1 units (mg protein)⁻¹ was examined by polyacrylamide gel electrophoresis as described by Davis (1964). Activity was detected using diaminobenzidine as described by Haywood & Large (1981). The enzyme was about 50% pure; some seven protein impurities were detected, of which two were present in significant amounts.

Properties of the partially purified enzyme

Substrate specificity. The substrate specificity of the enzyme was examined at pH 10.0 (borate buffer) by the polarographic assay. The structural requirements for activity seem to be that the substrate must contain a secondary amino group and one alkyl chain longer than C₄. The following were oxidized: spermine, spermidine, N¹-acetylspermidine, N-n-butylpropylamine,

Fig. 2. Effect on growth and enzyme activity of transfer of *Candida boidinii* grown on ammonium as nitrogen source to fresh medium of identical composition. (a) ●, Growth; ○, polyamine oxidase; ▽, isocitrate dehydrogenase; ▲, NAD-linked glutamate dehydrogenase; ■, NADP-linked glutamate dehydrogenase; ▼, catalase (broken line). (b) ●, Growth, ▲, pH of culture medium (broken line); △, glucose concentration; ▼, ammonium concentration.

Table 1. Apparent K_m and V_{max} values for substrates of *Candida boidinii* polyamine oxidase

Values were obtained from double-reciprocal plots of rate and substrate concentration. The best line was drawn using a linear regression program as described by Brook & Large (1976). Values are based on a single series of experiments using one enzyme preparation for all the data in the polarographic assay and a later preparation for the data in the spectrophotometric assay. The values are thus representative and are chiefly useful for comparison between different substrates.

Substrate	Polarographic assay		Spectrophotometric assay	
	V_{max} [units (mg protein) ⁻¹]	K_m^{app} (mM)	V_{max} [units (mg protein) ⁻¹]	K_m^{app} (mM)
Spermine	8.30	0.20	8.57	0.13
Spermidine	0.92	0.28	1.17	0.17
N ¹ -Acetylspermidine	23.40	0.054	34.0	0.03
N-n-Butylpropylamine	0.50	6.89	—	—
Di-n-butylamine	0.72	0.31	1.18	0.57
Di-n-hexylamine	0.10	0.05	—	—

—, Not determined.

di-n-butylamine and di-n-hexylamine. The following amines were not substrates: diethylamine, di-n-propylamine, 3,3'-iminobispropylamine (1,7-diamino-4-azaheptane, *sym*-norspermidine), diethylenetriamine (1,5-diamino-3-aza-pentane), putrescine (8-amino-4-aza-octanoate), N¹,N⁸-diacetylspermidine, methylamine, n-butylamine, benzylamine, agmatine, 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), 1,6-diaminohexane, 1,8-diaminooctane and 2-phenylethylamine. Unlike the rat liver enzyme (Hölttä, 1977), there was no stimulation of activity by 5 mM-benzaldehyde.

Kinetic properties. Apparent K_m values and V_{max} values for active secondary amine substrates (oxygen concentration 0.25 mM) were determined by both assay methods (Table 1), which agreed reasonably well.

Table 2. *Inhibitors of polyamine oxidase*

Inhibitor	Concn tested	Percentage inhibition*	Inhibitor	Concn tested	Percentage inhibition*
Carbonyl reagents			Substrate analogues		
Semicarbazide-HCl	167 μ M	0	Diethylamine	167 μ M	3
	3.3 mM	40		3.3 mM	96
Hydroxylamine-HCl	3.3 mM	36	Triethylamine	3.3 mM	6
Iproniazid	3.3 mM	47	Tri- <i>n</i> -propylamine	3.3 mM	15
Hydrazine-HCl	3.3 mM	14	3-Dimethylaminopropylamine	3.3 mM	8
Chelating agents			Lilly 53325†	0.33 mM	26
2,2'-Bipyridyl	33 μ M	67	Lilly 18947‡	0.33 mM	21
	3.3 mM	100	Miscellaneous		
Amino oxidase inhibitors			Quinacrine-HCl	1.33 μ M	2
<i>trans</i> -2-Phenyl-	33 μ M	54		66 μ M	97
cyclopropylamine	3.3 mM	100	Sodium mersalyl	8.3 μ M	100
Harmaline-HCl	0.66 mM	80			
Pargyline-HCl	3.3 mM	78			

* The uninhibited rate was 0.13 μ mol O₂ consumed min⁻¹ in a reaction volume of 3 ml.

† Lilly 53325 is 2,4-dichloro-6-phenylphenoxyethylamine-HCl.

‡ Lilly 18947 is 2,4-dichloro-6-phenylphenoxyethyl-diethylamine.

Effect of various possible inhibitors. Inhibitors were tested in the polarographic assay after 5 min preincubation with enzyme in the absence of substrate (3.3 mM-spermine). Those compounds that did inhibit the enzyme are listed in Table 2. The following compounds showed no inhibition: isoniazid (3.3 mM), putrescine (3.3 mM) and phenylhydrazine (1.3 mM). The following compounds stimulated oxygen uptake rather than inhibited it (although they showed no oxygen uptake alone): cuprizone (84 μ M), FAD (17 μ M), bromoethylamine (0.13 and 1.33 mM), aminoacetonitrile (0.33 and 3.33 mM), aminoguanidine (3.3 mM), KCN (3.3 mM), *n*-butylamine (3.3 mM), EDTA (3.3 mM), di-*n*-propylamine, 3,3'-iminobispropylamine and diethylenetriamine. The stimulation by chelating agents suggested that the enzyme might be inhibited by metal ions, and 0.1 mM-MgCl₂, NiCl₂, ZnSO₄ and CuCl₂ were all shown to be potent inhibitors. Addition of EDTA at 3.3 mM partially relieved this inhibition.

Attempted resolution of the enzyme. By analogy with polyamine oxidase of moulds and mammals, it seemed likely that the enzyme should contain FAD. However, attempts to resolve the enzyme by treatment with 1.5 M-KBr followed by acidified saturated ammonium sulphate as described by Hölttä (1977) produced an inactive precipitate which showed no reactivation even after incubation for 2 h at 0 °C in the presence of 33 μ M-riboflavin, FMN or FAD. This loss of activity is probably due to the much greater instability of the yeast enzyme. No direct spectral measurements on the purified enzyme were possible because it was too dilute and lost activity on pressure concentration.

Identity of reaction products and stoichiometry. The fact that longer chain secondary amines are substrates suggests that the enzyme oxidizes only secondary amino groups. When di-*n*-butylamine was incubated with enzyme for 24 h, the reaction products contained no ammonia. Half of the reaction mixture was made alkaline and extracted with butan-1-ol. The butanol layer contained a ninhydrin-positive material which on TLC on cellulose in butan-1-ol/HCl/water had an *R_F* of 0.81 (*R_F* values of primary amine markers were 0.54, 0.69, 0.81 and 0.86 for ethylamine, *n*-propylamine, *n*-butylamine and *n*-amylamine, respectively). The other half of the reaction mixture was treated with 1 mM-2,4-dinitrophenylhydrazine in 2 M-HCl. After 10 min it was extracted with chloroform and on TLC on cellulose in heptane saturated with methanol, a 2,4-dinitrophenylhydrazone was identified with an *R_F* of 0.83 (*R_F* values of dinitrophenylhydrazones of standards were: acetaldehyde 0.58, propionaldehyde 0.74, butyraldehyde 0.82). In order to establish the stoichiometry for the cleavage of di-*n*-butylamine into monobutylamine and butyraldehyde, the enzyme was incubated with substrate in the oxygen electrode as described in Methods. The oxidation of 305 nmol of di-*n*-butylamine was accompanied by an

oxygen consumption of 315 nmol and 315 nmol H_2O_2 , 283 nmol *n*-butylamine and 279 nmol butyraldehyde were formed in accordance with the following equation:

In the case of spermidine it seemed likely that here also the reaction would involve oxidation of the secondary amino group. Determination of ammonia formed with spermidine as substrate showed the formation of 0.26 mol NH_3 per mol oxygen consumed, and for spermine the ratio was 0.043, compared with a ratio of 1.19 mol NH_3 per mol O_2 for spermidine oxidation by the benzylamine/putrescine oxidase of *Pichia pastoris* (Green *et al.*, 1983). The question also arose as to whether the cleavage was between the C_3 carbon chain of spermidine and the secondary nitrogen atom (giving 1,4-diaminobutane and 3-aminopropionaldehyde) or between the C_4 chain and the secondary nitrogen atom (giving 1,3-diaminopropane and 4-aminobutyraldehyde, which spontaneously cyclizes to 1-pyrroline). Incubation of the reaction products with 2-aminobenzaldehyde failed to give a yellow colour (due to 1-pyrroline; Bachrach & Oser, 1963), so that it seemed likely that the cleavage was between the C_3 chain and the secondary nitrogen atom. This was confirmed by an experiment in which the reaction products of spermine and spermidine oxidation were separated on a Zeo-Karb 225 ion-exchange column. The oxidation of 433 nmol spermidine was accompanied by an oxygen consumption of 320 nmol and of the products formed there were 320 nmol H_2O_2 and 330 nmol putrescine. In the case of spermine the oxidation of 325 nmol by 450 nmol of oxygen gave 75 nmol spermidine and 234 nmol putrescine.

The unknown C_3 derivative which was also formed gave a thiosemicarbazone with λ_{max} 262 nm and a 2,4-dinitrophenylhydrazone with λ_{max} 372 nm. The latter was insoluble in chloroform. On TLC of the aqueous layer on silica gel G in butan-1-ol/ammonia (70:30, by vol.) or ethanol/ammonia (70:30, by vol.) a spot was identified, R_F 0.24 in the first solvent and 0.33 in the second, which had the same R_F values as the 2,4-dinitrophenylhydrazone of the oxidation product of 3-aminopropanol by liver alcohol dehydrogenase, which, according to Hölttä (1977), is 3-aminopropionaldehyde. The product certainly cannot be acrolein (Kimes & Morris, 1971; Gahl & Pitot, 1982), whose 2,4-dinitrophenylhydrazone is soluble in chloroform.

Molecular weight of the polyamine oxidase. Considerable variation in the molecular weight value was found, depending on the method by which it was determined. By using Sephadex G-150 with molecular weight markers in the range 12 400 to 235 000 (see Methods), a value of 79 500 was obtained for the partially purified enzyme and 90 000 for crude enzyme. With the method of Hedrick & Smith (1968), in which the retardation coefficient (Ferguson, 1964) determined by non-denaturing polyacrylamide gel electrophoresis is compared with those of standard proteins, crude cell extracts gave a molecular weight value of 200 000. In this experiment the enzyme was detected by activity staining (see Green *et al.*, 1982). Using the sucrose density gradient method of Martin & Ames (1961) with appropriate standard proteins, a value of 111 000 for the molecular weight was obtained.

Distribution of the enzyme in C. boidinii grown on different nitrogen sources

The enzyme was found to be present in lower but significant levels in cells grown on a number of other nitrogen sources (Table 3) besides spermidine, namely 1,3-diaminopropane, putrescine and cadaverine.

Metabolism of putrescine by C. boidinii

Cells of *C. boidinii* grew well on putrescine, but although they contained polyamine oxidase activity, which presumably is gratuitously induced (Table 3), no putrescine oxidase activity was detected. However crude extracts did give a positive result when tested for the activity of diamine aminotransferase (EC 2.6.1.29) by the method of Kim (1964) (Table 3).

The product of either oxidation or transamination of putrescine is 4-aminobutyraldehyde (1-pyrroline) and it has been established that 1-pyrroline can be oxidized by crude extracts of a number of yeasts (Table 4), as well as by purified benzylamine oxidase from *C. boidinii*.

Table 3. *Distribution of enzymes of polyamine and nitrogen metabolism in Candida boidinii, C. utilis and Pichia pastoris*

Growth substrate	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹ ; except catalase, μmol min ⁻¹ (mg protein) ⁻¹]							
	Methylamine oxidase	Benzylamine oxidase	Polyamine oxidase	Putrescine oxidase	Putrescine amino- transferase	Glutamate dehydrogenase		
						NAD	NADP	Catalase
<i>Candida boidinii</i>								
Ammonium	0	0	0	0	0.7	0	350	19
Methylamine	81	3	0	0	4.9	16	670	133
Dimethylamine	196	0	0	0	6.9	10	490	216
<i>n</i> -Butylamine	39	20	0	0	4.8	0	630	180
Choline	54	0	0	0	4.8	23	290	151
Lysine	0	0	0	0	0.3	133	290	25
1,3-Diaminopropane	1	24	110	0	3.9	83	590	146
Putrescine	0	16	47	0	2.1	121	146	62
Cadaverine	5	30	7	0	1.4	204	530	177
1,6-Diaminohexane	0	1	0	0	0	20	1490	155
Spermidine	0	5	514	0	2.2	178	138	67
<i>Candida utilis</i>								
Ammonium	0	0	0	0	0	4	27	45
<i>n</i> -Butylamine	36	118	0	11	8.8	64	291	171
1,3-Diaminopropane	4	182	0	25	5.9	4	18	132
Putrescine	0	141	0	10	1.8	2	22	143
<i>Pichia pastoris</i>								
Ammonium	0	0	0	0	2.5	10	21	21
Putrescine	6	5	0	16	2.3	206	156	101
Spermidine	37	7	0	47	0.7	87	143	104

Peroxisomal location of polyamine oxidase

A cell-fractionation technique based on that of Zwart (1983) was used to determine the intracellular location of polyamine oxidase activity. The mitochondria/peroxisome fraction, isolated from mechanically broken spheroplasts (see Methods) by centrifugation at 30000 *g* for 15 min was centrifuged on a discontinuous sucrose gradient as described in Methods, to separate mitochondria and peroxisomes. Due to the size of the organelles in *C. boidinii*, good separations between the two fractions were not easily obtained, but it is clear from Fig. 3(c) that polyamine oxidase co-sediments with catalase and glycerol-3-phosphate dehydrogenase, both of which are known to be peroxisomal enzymes in yeast (Yamada *et al.*, 1980*b*; Zwart *et al.*, 1980; Kawamoto *et al.*, 1979). The mitochondrial marker enzymes cytochrome oxidase (Delaissé *et al.*, 1981; Zwart, 1983) and NADH dehydrogenase (Von Jagow & Klingenberg, 1970) end up at a slightly lower sucrose concentration (Fig. 3*b*). Some catalase activity was also found in this fraction (Fig. 3*c*). Poor separation between peroxisomes and mitochondria was also observed in *C. stellatoidea* by Jenkins *et al.* (1983).

Presence of polyamine oxidase in other yeast species

Polyamine oxidase activity at pH 10.0 was found in the following yeast species when grown on spermidine as sole nitrogen source: *C. boidinii*, *C. nagoyaensis*, *Hansenula polymorpha* and *Trichosporon melibiosaceum* (Table 4). Other organisms which contained polyamine oxidase activity when tested at pH 7.0, but not at pH 10.0 were: *C. steatolytica*, *Pichia pastoris* and *Sporopachydermia cereana*. These organisms also contained putrescine oxidase activity (Table 4), and it seems likely that both activities are due to a benzylamine/putrescine oxidase similar to that already characterized in *P. pastoris* (Green *et al.*, 1983).

Fig. 3. Separation of mitochondrial and peroxisomal enzyme activities of *Candida boidinii* by sucrose-gradient centrifugation. (a) ●, Sucrose concentration; □, protein concentration. (b) ○, Cytochrome oxidase; △, NADH dehydrogenase. (c) ●, Polyamine oxidase; ▲, catalase; ■, NAD-linked glycerol-3-phosphate dehydrogenase (broken line).

Table 4. Distribution of enzymes of polyamine and nitrogen metabolism in various yeast species grown on spermidine

Yeast	Specific activity [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$; except catalase, $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]									
	Methyl-amine oxidase	Benzyl-amine oxidase	1-Pyrroline oxidase	Spermine oxidase		Putrescine oxidase	Putrescine amino-transferase	Glutamate dehydrogenase		Catalase
				pH 7.0	pH 10.0			NAD	NADP	
<i>Candida boidinii</i>	0	5	20	0	514	0	3	178	138	67
<i>C. insectalensis</i>	0	14	34	0	1	0	—	14	47	378
<i>C. nagoyaensis</i>	19	91	2	0	10	1	—	0	570	386
<i>C. steatolytica</i>	0	21	0	16	0	116	—	28	1	174
<i>Hansenula minuta</i>	0	8	1	0	0	0	—	9	170	97
<i>H. polymorpha</i>	34	28	1	0	12	0	—	0	340	60
<i>Pichia pastoris</i>	37	7	—	17	0	47	1	87	143	104
<i>P. pinus</i>	17	39	1	1	2	1	—	98	326	246
<i>Sporopachydermia cereana</i>	48	10	11	15	0	39	—	0	0	285
<i>Trichosporon melibiosaceum</i>	0	0	1	0	90	0	—	0	2140	91

—, No data available.

DISCUSSION

The role of polyamine oxidase in the dissimilation of spermidine is clearly demonstrated by its induction before growth on spermidine begins (Fig. 1). The properties of the enzyme (e.g. pH optimum, products formed) resemble those of liver polyamine oxidase (Hölttä, 1977). It seems likely that despite the difference of stability, yeast polyamine oxidase is, like the liver and mould enzymes (Isobe *et al.*, 1980*a*; Kobayashi & Horikoshi, 1982), a flavoprotein. We have shown that polyamine oxidase is peroxisomal, like the liver enzyme. Growth on spermidine is also accompanied by elevated activities of catalase (Fig. 1 and Table 3), which is also a peroxisomal enzyme (Tolbert, 1981) and necessary for the removal of H_2O_2 formed by polyamine oxidase. Yeast polyamine oxidase differs from that found in liver by the fact that it is not stimulated by aldehydes (Hölttä, 1977). Several workers have recently described polyamine oxidases from fungi, both intracellular (Yamada *et al.*, 1980*a*; Isobe *et al.*, 1980*a,b,c*) and extracellular (Kobayashi & Horikoshi, 1981, 1982), but the yeast enzyme differs from that found in moulds by being significantly less stable and in failing to show activity in the pH region from 4 to 6 (Isobe *et al.*, 1980*a*; Kobayashi & Horikoshi, 1982). The apparently narrow substrate specificity observed with the mould enzymes is probably due to failure of the experimenters to test long chain secondary amines, which we found to be fairly good substrates. Yeast polyamine oxidase also differs from putrescine oxidase from *Micrococcus rubens* (EC 1.4.3.10) (Yamada *et al.*, 1965; Swain & DeSa, 1976; Okada *et al.*, 1979) in its total failure to oxidize putrescine or cadaverine. No polyamine oxidase seems to have been tested for activity with dialkylamines in the way in which Okada *et al.* (1979) tested putrescine oxidase. This is undoubtedly due to the fact that most of the analogues studied by Okada *et al.* (1979) had to be synthesized. However, using only commercially available secondary amines we have been able to show that unlike putrescine oxidase (for which the essential structural feature for a substrate to show activity is $NH_2(CH_2)_4NH-$), our enzyme is active with dialkylamines of structure $RNHR^1$ where R must be at least C_4 and R^1 at least C_3 . The presence of a primary amino group is not essential for activity. This is also borne out by the other active substrates (Table 1), whose range resembles that of the rat liver enzyme (Hölttä, 1977; Bolkenius & Seiler, 1981; Suzuki *et al.*, 1981) rather than the mould enzyme (Kobayashi *et al.*, 1983). *N*¹-Acetylspermidine seems to be the best substrate for the enzyme, and one wonders whether acetylation might precede spermidine oxidation, as probably occurs in mammals (Seiler *et al.*, 1980; Bolkenius & Seiler, 1981; Matsui *et al.*, 1983). In the case of the extracellular *Penicillium* enzyme (Kobayashi *et al.*, 1983), *N*¹-acetylspermidine was a much poorer substrate than spermidine, and this was true for acetylated polyamines in general.

Smith (1972) has classified the various ways in which enzymic attack on polyamines can occur. We have shown that benzylamine/putrescine oxidase of *Pichia pastoris* attacks the two primary amino groups of both spermine and spermidine (Green *et al.*, 1983). Spermine is thus oxidized by *P. pastoris* in the same way (mechanism 6 of Smith, 1972) as by plasma amine oxidase (Tabor *et al.*, 1964). Spermidine was oxidized by *P. pastoris* at both primary amino groups, a new mechanism. With the present polyamine oxidase however, the secondary amino group(s) of spermidine and spermine are cleaved to yield putrescine and one or two molecules of what is probably 3-aminopropionaldehyde, which are mechanisms 1 and 2 of Smith (1972), in the same way as the enzymes from liver (Hölttä, 1977) and *Penicillium* (Yamada *et al.*, 1980*a*; Kobayashi & Horikoshi, 1982). Despite the apparent formation of traces of ammonia from spermidine, we believe that the yeast polyamine oxidase is specific for secondary amino groups.

The data in Table 4 suggest that at least two different mechanisms for sperm(id)ine degradation occur in yeasts, an enzymic attack on the primary amino groups as in *P. pastoris*, *C. steatolytica* and *Sporopachydermia cereana*, or an enzymic attack on the secondary amino groups as in *C. boidinii*, *C. nagoyaensis*, *Hansenula polymorpha* and *Trichosporon melibiosaceum*. The enzyme responsible for oxidizing the primary amino groups is maximally active at pH 7.0 and is probably the same as the benzylamine/putrescine oxidase already characterized in *P. pastoris* (Green *et al.*, 1983). The enzyme responsible for oxidizing secondary amino groups is maximally active at pH 10.0 and is, we believe, polyamine oxidase. These two modes of attack do not seem to occur together in one species.

Fig. 4. Suggested pathway for the breakdown of spermidine by *Candida boidinii*. The enzymes involved are: (1), polyamine oxidase; (2), diamine aminotransferase; (3), benzylamine oxidase; (4), catalase; (5), unknown. The possibility of an additional step involving the N^1 -acetylation of spermidine preceding reaction (1) cannot at this stage be excluded.

We suggest that spermidine and putrescine are degraded by *C. boidinii* according to Fig. 4. It is possible that acetylation of the spermidine might precede oxidation, as is thought to occur in mammals (Matsui *et al.*, 1983). Although only low levels of benzylamine oxidase have been found in spermidine-grown cells (Table 4), its behaviour during growth is consistent with its playing a role in spermidine metabolism (Fig. 1*a*), while the pattern of methylamine oxidase activity probably is not. Both oxidases have been found to oxidize 1-pyrroline (4-aminobutyraldehyde) (G. W. Haywood, unpublished results). The conversion of putrescine to 4-aminobutyraldehyde (reaction 2 in Fig. 4) may be a transamination (Tables 3 and 4), because *C. boidinii*, unlike some of the species in Table 4, has no putrescine oxidase activity. In the experiment presented in Figs 1 and 2, putrescine transaminase was elevated in the cells grown on spermidine (results not shown). The fate of the C_3 product (probably 3-aminopropionaldehyde) is not certain, but it may be metabolized via either malonaldehyde or β -alanine. Attempts to show the accumulation of succinaldehyde during growth on spermidine have been unsuccessful. Thus the fate of both the carbon moieties of spermidine remains obscure. It is clear from Fig. 1*a* that NAD-linked glutamate dehydrogenase (believed to be involved in yeasts in glutamate degradation: Roon & Even, 1973; Holzer *et al.*, 1965), may play a significant role in

the degradation of spermidine, implying that glutamate may be an intermediate in the process, as suggested in Fig. 4. This suggestion is also supported by the data in Table 3.

We previously believed that 4-aminobutyraldehyde (1-pyrroline) was not a substrate for benzylamine oxidase of *P. pastoris* (Green *et al.*, 1983). This conclusion was based on measurements of putrescine oxidation made with benzylamine/putrescine oxidase of *P. pastoris* at concentrations far below the K_m of the enzyme for 1-pyrroline. The enzyme behaved as if only one amino group of putrescine was oxidized. Now that it is clear that at appropriate concentrations 1-pyrroline is indeed a substrate we have an explanation for our observation (Green *et al.*, 1983) that at high putrescine concentrations, more than 1 mol of ammonia is formed by the *Pichia pastoris* enzyme per mol putrescine oxidized. 1-Pyrroline is also a substrate for benzylamine oxidase from *C. boidinii*, which, unlike those of *C. utilis* and *P. pastoris*, fails to oxidize putrescine. The postulated roles of benzylamine oxidase (and not of methylamine oxidase) and diamine aminotransferase in the breakdown of spermidine are also supported by the data of Table 3.

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