Microbial oxidation of amines

Distribution, purification and properties of two primary-amine oxidases from the yeast *Candida boidinii* grown on amines as sole nitrogen source

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1. The yeast *Candida boidinii* was grown on glucose as carbon source with a range of amines and amino acids as nitrogen sources. Cells grown on amines contained elevated activities of catalase. If the amines contained *N*-methyl groups, formaldehyde dehydrogenase, formate dehydrogenase and *S*-formylglutathione hydrolase were also elevated in activity compared with cells grown on $(NH_4)_2SO_4$. 2. Cells grown on all the amines tested, but not those grown on urea or amino acids, contained an oxidase attacking primary amines, which is referred to as methylamine oxidase. In addition, cells grown on some amines contained a second amine oxidase, which is referred to as benzylamine oxidase. 3. Both amine oxidases were purified to near homogeneity. 4. Benzylamine oxidase was considerably more stable at 45 and 50°C than was methylamine oxidase. 5. Both enzymes had a pH optimum in the region of 7.0, and had a considerable number of substrates in common. There were, however, significant differences in the substrate specificity of the two enzymes. The ratio $V/K_m^{app.}$ increased with increasing n-alkyl carbon chain length for benzylamine oxidase, but decreased for methylamine oxidase. 6. Both enzymes showed similar sensitivity to carbonyl-group reagents, copper-chelating agents and other typical 'diamine oxidase inhibitors'. 7. The stoicheiometry for the reaction catalysed by each enzyme was established. 8. The kinetics of methylamine oxidase were examined by varying the methylamine and oxygen concentrations in turn. A non-Ping Pong kinetic pattern with intersecting double-reciprocal plots was obtained, giving K_m values of $10\,\mu M$ for O_2 and $198\,\mu M$ for methylamine. The significance of this unusual kinetic behaviour is discussed. Similar experiments were not possible with the benzylamine oxidase, because it seemed to have an even lower K_m for O_2. 9. Both enzymes had similar subunit M_r values of about 80000, but the benzylamine oxidase behaved as if it were usually a dimer, M_r 136000, which under certain conditions aggregated to a tetramer, M_r 288000. Methylamine oxidase was mainly in the form of an octamer, M_r 510000, which gave rise quite readily to dimers of M_r 150000, and on gel filtration behaved as if the M_r was 286000.

The metabolism of the methylated amines mono-methylamine, dimethylamine and trimethylamine has been extensively investigated in methylotrophic bacteria (Trotsenko & Loginova, 1978; Large, 1981). With one or two possible exceptions (Wolf & Hanson, 1979), yeasts seem to be incapable of growth on such compounds as sole carbon source (Van Dijken & Harder, 1974; Van Dijken *et al.*, 1981). Nonetheless they are readily used by yeasts as a nitrogen source when an alternative carbon

source such as glucose is supplied (Van Dijken *et al.*, 1979, 1980). There is also evidence that the inability of yeasts to use amines as carbon source is not confined to methylated amines (Van Dijken & Bos, 1981). Studies have shown that the ability to use both methylated amines and other amines as nitrogen source is widespread in most genera of yeasts (Van Dijken *et al.*, 1980; Van Dijken & Bos, 1981).

The enzymic basis of the utilization of primary amines as nitrogen source by yeasts and moulds is the possession of an amine oxidase (Yamada *et al.*, 1965a, 1966, 1976), and a number of these have

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been purified from both yeasts (Yamada *et al.*, 1966) and moulds (Yamada *et al.*, 1965*b*). The enzymic mechanism by which these organisms can use secondary and tertiary amines is not so well worked out, since extracts oxidizing such amines have not yet been obtained (Yamada *et al.*, 1966, 1976).

Previous work has shown (Van Dijken *et al.*, 1979; Zwart *et al.*, 1980) that the yeasts *Candida utilis* and *Hansenula polymorpha* when grown on methylamine as nitrogen source contain elevated activities of catalase, formaldehyde dehydrogenase and formate dehydrogenase compared with cells grown on ammonium salts, and contain in addition an amine oxidase attacking methylamine. We confirm these observations here for *C. boidinii* and moreover show that, when grown on glucose as carbon source and various amines as nitrogen source, this yeast contains two different amine oxidases, both oxidizing primary amines. We have investigated the relative occurrence of these two oxidases and report their purification and partial characterization. Preliminary reports of part of this work have been made to the Society for General Microbiology (Van Dijken *et al.*, 1979; Large *et al.*, 1980, 1981).

Materials and methods

Materials

Horseradish peroxidase (type P8125), the di-ammonium salt of ABTS, 5-aminopentyl-agarose, 5,5'-dithiobis-(-2-nitrobenzoic acid), glyoxylase II, benzylamine, benzaldehyde, thioglycollic acid, glutathione, bovine γ-globulin and the molecular-weight standard proteins were obtained from Sigma. Lilly 18947 and 53225 were gifts from Eli Lilly International, Indianapolis, IN, U.S.A. *p*-Dimethylaminomethylbenzylamine was a gift from Dr. W. G. Bardsley, University of Manchester, U.K.

S-Formylglutathione was prepared and standardized as described by Uotila (1973). Before use as a substrate, contaminating glutathione was removed with *N*-ethylmaleimide as described by Uotila (1973). The sodium salt of ABTS was prepared by passing the ammonium salt down a column of Zeo-Karb 225 (Na^+ form) and standardized by using a millimolar absorption coefficient of $431 \cdot mmol^{-1} \cdot cm^{-1}$ at 343 nm (Werner *et al.*, 1970). Methylamine dehydrogenase from *Pseudomonas* AM1 was purified as described by Boulton & Large (1979).

Other chemicals were obtained from Sigma or Fisons. All amines were used in aqueous solution adjusted to pH 5.5 with HCl.

Organisms and growth conditions

C. boidinii CBS 5777 was grown on the following medium (per litre): glucose, 10 g; $MgSO_4,7H_2O$,

0.2 g; KH_2PO_4, 3 g; nitrogen source (see below); vitamin solution, 10 ml, giving final concentrations of the vitamins as in Lodder (1970); trace-element solution (Vishniac & Santer, 1957), 0.2 ml. The pH of the medium was adjusted to 6.0 with NaOH, and it was then sterilized by autoclaving, except that the glucose and vitamins were sterilized by filtration (Sartorius 0.45 μm membrane filters) and added aseptically to the rest of the medium when it was cool. The concentration of nitrogen source was 30 mM for methylated amines, 15 mM for $(NH_4)_2SO_4$ and n-butylamine and 10 mM for all the others. Flasks were grown with aeration on a New Brunswick Gyrotory shaker at 30°C. Growth was followed by measurement of absorbance at 663 nm. Cells were harvested by centrifuging for 20 min at 4°C at 10000 g, and washed with 50 mM-potassium phosphate buffer, pH 7.0, before storage at −15°C.

Preparation of cell-free extracts

Cell material was suspended in 2 vol. of 50 mM-potassium phosphate, pH 7.0, and passed once through a French pressure cell at 8.6 MPa. The material was then centrifuged for 15 min at 50000 g at 4°C.

Enzyme assays

Methylamine oxidase. Two methods were used.

(1) *Spectrophotometric assay.* The H_2O_2 formed with methylamine as substrate was coupled to the oxidation of the sodium salt of ABTS by using horseradish peroxidase (Crabbe *et al.*, 1976) and following the increase in absorbance at 405 nm (millimolar absorption coefficient of the radical-cation product is $18.41 \cdot mmol^{-1} \cdot cm^{-1}$; Werner *et al.*, 1970). Polystyrene cuvettes (light-path 1 cm) contained in a total volume of 3 ml: 10 μmol of methylamine hydrochloride, 2.5 μmol of Na_2ABTS, 50 μmol of potassium phosphate buffer, pH 7.0, 8 units (approx. 100 μg) of horseradish peroxidase (Sigma type I) and enzyme. The reaction was started by addition of methylamine and the reaction was followed at 25°C against a blank containing all components except substrate. One unit of enzyme is the amount required to catalyse the formation of ABTS radical cation at a rate of 1 mM/min under these conditions. This assay gives a transient time of 0.001 s and a steady-state H_2O_2 concentration of 20 nM (Easterby, 1973). Catalase does not interfere with this assay until a concentration of more than 3×10^4 units of catalase/unit of methylamine oxidase is reached.

(2) *Polarographic assay.* Oxygen uptake was measured with a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) coupled to a Servogor 210 recorder. The reaction volume was 3 ml, containing 50 μmol of potassium phosphate

buffer, pH 7.0, 10 μmol of methylamine hydrochloride and enzyme. The reaction was started by addition of enzyme and oxygen consumption was followed at 25°C. One unit of enzyme is the amount required to catalyse the consumption of 0.5 μmol of $O_2 \cdot min^{-1} \cdot ml^{-1}$ [this is the same magnitude as the unit in assay method (1)].

Benzylamine oxidase. This was assayed by either of the above two methods with methylamine replaced by either 0.5 μmol of benzylamine hydrochloride or by 3 μmol of isobutylamine hydrochloride. In most experiments (except those for the stoicheiometry) isobutylamine was used as substrate because it shows no substrate inhibition at higher concentrations. Additionally, assay method (3) was used, in which the formation of benzaldehyde is followed spectrophotometrically at 250 mm as described by Tabor *et al.* (1954). The temperature was 25°C.

Formaldehyde dehydrogenase (EC 1.2.1.1) and formate dehydrogenase (EC 1.2.1.2). These were assayed as described by Van Dijken *et al.* (1976), but the temperature was 25°C. The rates of absorbance change were corrected for NADH oxidase activity at the appropriate pH value.

Catalase (EC 1.11.1.6). This was assayed by the addition of 0.1 ml of a diluted enzyme sample to 2.9 ml of a solution containing 0.1 ml of 30% (v/v) H_2O_2 in 50 ml of 50 mM-phosphate buffer, pH 7.0. The decrease in absorbance at 240 nm was followed and the time taken for the A_{240} to fall from 0.45 to 0.40 was determined at 25°C. This corresponds to the decomposition of 3.45 μmol of H_2O_2 in the 3 ml solution. Activity is expressed in Sigma units = 3.45/[time (min) required].

S-Formylglutathione hydrolase (EC 3.1.2.12). This was assayed as follows. Polystyrene cuvettes contained, in a total volume of 1 ml: 100 μmol of Tris/HCl buffer, pH 7.4, 0.15 μmol of 5,5'-dithio-bis-(2-nitrobenzoic acid) and 0.2 μmol of S-formylglutathione. After measurement of non-enzymic deacylase activity, the reaction was started by addition of enzyme and the reaction was followed at 412 nm and 25°C against a blank containing water. One unit of enzyme is that required to catalyse the formation of 3-carboxy-4-nitrothiophenol at a rate of 1 mM/min under these conditions (see Ellman, 1959).

Peroxidase (EC 1.11.1.7). This was assayed spectrophotometrically at 25°C and 405 nm by using the amine oxidase assay (assay 1) in which the methylamine and amine oxidase were replaced by various concentrations of H_2O_2.

Isocitrate dehydrogenase (EC 1.1.1.42). This was assayed as described by Bergmeyer (1974).

Variation of oxygen concentration in assay mixtures

This was performed by varying the flow rate of oxygen (or air) and nitrogen as described previously (Brook & Large, 1976). The dissolved oxygen concentration at each gas-flow rate was measured by using a Rank oxygen electrode calibrated by the method of Robinson & Cooper (1970).

Conductivity measurements

Phosphate concentration in column eluates was measured by using an Electrolytic Conductivity measuring set (Electronic Switchgear, Hitchin, Herts., U.K.) calibrated with standard solutions of potassium phosphate, pH 7.0.

Purification of methylamine oxidase

All steps were performed at 4°C. A typical purification is shown in Table 1.

Steps 1 and 2: preparation of extract and $(NH_4)_2SO_4$ treatment. Frozen *C. boidinii* grown on methylamine (or dimethylamine) as nitrogen source

Table 1. *Purification of methylamine oxidase from C. boidinii*
Cells grown on dimethylamine (28 g wet wt.) were used.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification factor	Yield (%)
1. Crude extract	91	203	1074	0.188	1	100
2. 35–70%-satn.-$(NH_4)_2SO_4$ precipitate	34	167	741	0.225	1.2	82.3
3. Sepharose 4B chromatography (combined fractions)	155	157	270	0.580	3.1	77.7
4. 5-Aminopentyl-agarose chromatography (combined fractions)	84	130	99	1.314	7.0	64.0
5. Hydroxyapatite chromatography (combined, after concentration)	14.6	94.3	44	2.15	11.5	46.5
6. DEAE-Sepharose CL-6B chromatography (combined, after concentration)	8.5	59	15	3.95	21.0	28.9
7. 5-Aminopentyl-agarose chromatography (homogeneous fractions)	11.6	13	3.8	3.39	18.1	6.4

Table 2. *Purification of benzylamine oxidase from C. boidinii*
Cells grown on n-butylamine (20 g wet wt.) were used

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification factor	Yield (%)
1. Crude extract	67	28.4	992	0.029	1	100
2. 45–85%-satn.-$(NH_4)_2SO_4$ precipitate	15	16.0	390	0.041	1.41	56.3
3. Sepharose 6B chromatography (combined fractions)	83	15.9	101	0.157	5.41	56.0
4. DEAE-Sepharose CL-6B chromatography (combined fractions)	28	14.8	18.2	0.810	27.9	52.1
5. DEAE-cellulose chromatography (combined, after concentration)	6.9	7.1	4.6	1.52	52.4	24.9
6. Hydroxyapatite chromatography (combined, after concentration)	4.5	4.8	1.08	4.46	153.8	17.0

was suspended in 2 vol. of 50 mM-potassium phosphate, pH 7.0, and disrupted as described above. The pellet obtained was resuspended in 15 ml of the same buffer and passed a second time through the pressure cell. After centrifuging for 20 min at 50 000 g, the supernatant of this was combined with the previous one. The material was filtered through a Whatman GF/C filter, which partially removes lipid. The combined filtered supernatants were adjusted to 35% satn. by addition of solid $(NH_4)_2SO_4$, and the precipitated protein was discarded. The supernatant was adjusted to 70% satn. and centrifuged at 50 000 g for 20 min. The precipitate was collected.

Step 3: gel filtration. The precipitate from step 2 was dissolved in 7.5 ml of 10 mM-potassium phosphate buffer, pH 7.0, and applied to a column (1 m × 2.5 cm diam.) of Sepharose 4B equilibrated with the same buffer. The column was washed overnight with the same buffer and 4 ml fractions were collected.

Step 4: 5-aminopentyl-agarose chromatography. The combined active fractions from step 3 were applied to a column (10 cm × 2.5 cm diam.) of 5-aminopentyl-agarose and washed with 10 mM-potassium phosphate, pH 7.0. A linear gradient of 10–250 mM-potassium phosphate (in 300 ml) was then applied. The major peak of activity was eluted at 80 mM-phosphate. Sometimes two peaks were observed, one emerging slightly earlier at 55 mM-phosphate. The most active fractions were combined, and concentrated by using an Amicon concentration cell with a Diaflo PM30 membrane to about one-tenth of the original volume.

Step 5: hydroxyapatite chromatography. The concentrated material from step 4 was diluted to a final concentration of 20 mM-phosphate with distilled water and applied to a column (12 cm × 1 cm diam.) of hydroxyapatite (Bio-Rad Bio-Gel HTP) equilibrated in 20 mM-potassium phosphate, pH 7.0. After washing with the same buffer, the enzyme was

eluted with a linear gradient of 20–250 mM-potassium phosphate, pH 7.0 (total volume 150 ml). The peak of activity was eluted at 62 mM-phosphate. The most active fractions were combined and concentrated as in step 4.

Step 6: ion-exchange chromatography on DEAE-Sepharose CL-6B. Pooled concentrated fractions from step 5 were diluted with distilled water to a conductivity corresponding to that of 20 mM-phosphate. The material was applied to a column (25 cm × 2.0 cm diam.) of DEAE-Sepharose CL-6B and washed with 20 mM-potassium phosphate buffer, pH 7.0. The enzyme was then eluted with a linear gradient of 20–300 mM-phosphate, pH 7.0 (in 300 ml). The peak fractions were combined and concentrated before the protein concentration was determined.

Step 7: 5-aminopentyl-agarose chromatography. To obtain a homogeneous sample it was often necessary to dilute the material from step 6 to 10 mM in phosphate and repeat step 4. The fractions were carefully checked for homogeneity before combination, and yields at this stage were low because many fractions contained contaminants and had to be discarded.

Purification of benzylamine oxidase

A typical purification is shown in Table 2. All steps were carried out at 4°C.

Steps 1–3: preparation of extract, $(NH_4)_2SO_4$ treatment and gel filtration. Extracts were made from C. boidinii grown on n-butylamine as nitrogen source as described above for methylamine oxidase. After filtration on GF/C filter pads the material was adjusted to 45% satn. with $(NH_4)_2SO_4$, and the precipitated protein was discarded. The supernatant was adjusted to 85% satn. and the precipitate was collected. It was dissolved in 7 ml of 20 mM-potassium phosphate buffer, pH 7.0, and applied as described above to Sepharose 6B.

Step 4: chromatography on DEAE-Sepharose CL-6B. The combined active fractions from step 3 were applied to a column (25 cm × 2 cm diam.) of DEAE-Sepharose CL-6B and washed with 20 mM-potassium phosphate buffer, pH 7.0. The enzyme was then eluted with linear gradient of 20–300 mM-phosphate, pH 7.0 (in 300 ml). This brought about a nearly complete separation of benzylamine oxidase, which was eluted at approx. 105 mM-phosphate, from methylamine oxidase, which was eluted (often as a double peak) between 55 and 85 mM-phosphate (Fig. 1). The peak fractions were combined (no fraction was included which had less than 50% of the peak activity) and concentrated.

Step 5: chromatography on DEAE-cellulose. The conductivity of material from step 4 was measured; it was then diluted with water to 20 mM-phosphate and applied to a column of DEAE-cellulose (Whatman DE-11) previously equilibrated in 20 mM-phosphate, pH 7.0. After washing with the same buffer, the column was eluted with a linear gradient of 20–250 mM-phosphate (total volume 300 ml). The peak of enzyme activity was eluted at 70 mM-phosphate. Fractions containing more than 50% of the peak activity were combined and concentrated.

Step 6: chromatography on hydroxyapatite. The material from step 5 was diluted to 20 mM-phosphate with water and applied to a column (15 cm × 1 cm diam.) of hydroxyapatite equilibrated in 20 mM-phosphate, pH 7.0. After washing with the same buffer, the enzyme w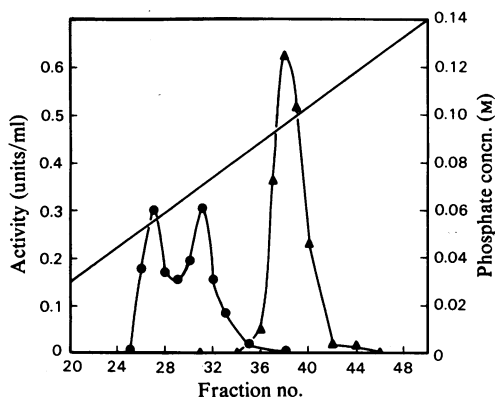as eluted with a linear gradient of 20–300 mM-phosphate (total volume 200 ml). The peak of activity emerged at 130 mM-phosphate. The fractions containing more than 50% of the peak activity were combined and concentrated.

Data processing of kinetic results

This was performed as described previously (Brook & Large, 1976).

Chemical determinations

Protein was determined by the dye-binding method of Bradford (1976) with dried bovine γ-globulin as standard. Formaldehyde was determined by the Nash (1953) method, and ammonia by the indophenol method (Chaney & Marbach, 1962) by using standards containing equimolar concentrations of formaldehyde to correct for the lower colour yield in the presence of formaldehyde. Methylamine was measured by the enzymic method of Large *et al.* (1969), benzylamine by the method of Obata *et al.* (1971) and benzaldehyde from absorbance measurements at 250 nm ($\Delta\varepsilon = 11.31 \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$; Buffoni & Blaschko, 1971).

Polyacrylamide-gel electrophoresis

Routine work was done at pH 8.3 with the gel system of Davis (1964), but for the Ferguson plots, the gel system used was the pH 7.3 system of Hedrick & Smith (1968), without spacer gels in each case. The retardation coefficients (slope of the line obtained by plotting the logarithm of the distance migrated relative to Bromophenol Blue against the total acrylamide concentration in the gel) of a series of high-purity protein standards (having isoelectric points below pH 7.0) were used to construct a calibration curve for the molecular-weight determination (see legend to Fig. 4).

Standards for peptide-subunit molecular-weight determination were from the Boehringer Combithek kit (cat. no. 161365), and sodium dodecyl sulphate/polyacrylamide gels were prepared in accordance with the instructions therein.

Gels were stained with Amido Black or Coomassie Blue and destained electrophoretically. Enzyme activity was detected by incubating gels at room temperature in the following mixture: 0.5 ml of 0.1 M-potassium phosphate, pH 7.0, 7 mg of 3,3′-diaminobenzidine hydrochloride in 2 ml of 0.25 M-sodium acetate, 200 µg of horseradish peroxidase and 3 µmol of methylamine or benzylamine, plus water to 3 ml. A brown band denoted enzyme activity and was not observed if the amine was omitted from the reaction mixture.

Determination of molecular weights by gel filtration

The method of Andrews (1970) was used, except that the gels used were Sepharose 4B or Sepharose

Fig. 1. *Separation of methylamine oxidase and benzylamine oxidase on DEAE-Sepharose CL-6B*
Partially purified benzylamine oxidase from step 3 (Table 2) was applied to the column and washed with 20 mM-potassium phosphate, pH 7.0. A linear gradient of phosphate (———) at the same pH was then used to elute the enzymes. ●, Methylamine oxidase activity [assay method (1)]; ▲, benzylamine (isobutylamine) oxidase activity [assay method (1)].

6B in 10 mM-potassium phosphate, pH 7.0. The molecular-weight marker proteins used were as described previously (Boulton et al., 1980) and they were detected by assay as therein described. Additional markers used were pig heart malate dehydrogenase (M_r 70000), rabbit muscle phosphorylase a (370000) and rabbit muscle aldolase (158000), assayed as described in Bergmeyer (1974).

Stoicheiometry of methylamine oxidation

(1) *Polarographic experiment.* Approx. 0.7 μmol of methylamine hydrochloride was incubated with 100 μg of enzyme and 3700 units of bovine liver catalase in phosphate buffer, pH 7.0, in the chamber of an oxygen electrode. When oxygen consumption was complete, 0.3 ml of 2.5 M-HClO$_4$ was added to stop the reaction. After neutralization and centrifuging, ammonia, formaldehyde and residual methylamine were determined in the test sample and in the control in which previously boiled enzyme had been used. The amount of reactant consumed or product formed was the difference between values for the test and the control.

(2) *Spectrophotometric experiment.* The normal spectrophotometric assay was performed, by using 50 μg of purified enzyme, except that only approx. 0.25 μmol of methylamine hydrochloride was added and the reaction was followed at 660 nm (where the molar absorbance of the ABTS radical cation is lower than at 405 nm, 9.11·mmol^{-1}·cm^{-1}; Werner et al., 1970). In the determination of residual methylamine at the end of the reaction, advantage was taken of the fact that the radical cation of ABTS is an electron acceptor for the methylamine dehydrogenase of *Pseudomonas* AM1 (S. J. Rigby & P. J. Large, unpublished work), which could thus be added to measure methylamine. In the control containing boiled amine oxidase, after an incubation lasting as long as the reaction in the test cuvette took to go to completion, 0.6 μmol of chemically oxidized ABTS (prepared as described by Duine et al., 1978) was added, followed by methylamine dehydrogenase to determine the unchanged methylamine. The colour of the ABTS radical cation precluded measurement of formaldehyde and ammonia.

Stoicheiometry of benzylamine oxidation

This was performed essentially as in the polarographic experiment in methylamine oxidation. To measure H$_2$O$_2$ formation, a spectrophotometric experiment was performed in two cuvettes each containing phosphate buffer, enzyme, peroxidase and about 0.25 μmol of benzylamine. In addition one contained 2.5 μmol of ABTS. The formation of radical cation was followed in this cuvette at 660 nm until reaction was complete. Both cuvettes were then acidified with 0.3 ml of 2.5 M-HClO$_4$ and the one

without ABTS was used to measure benzaldehyde, ammonia and residual benzylamine. The initial benzylamine concentration was measured on a sample from a third cuvette without enzyme.

Results

Distribution of amine oxidases and other key enzyme activities in C. boidinii grown on different amines as nitrogen source

C. boidinii was grown on glucose with a number of different compounds as nitrogen source. Cells were harvested early in the exponential phase (A_{663} of the cultures = 1.0) and crude extracts were assayed for methylamine oxidase and benzylamine oxidase activities, and for catalase and various enzymes of formaldehyde oxidation: formaldehyde dehydrogenase, formate dehydrogenase and S-formylglutathione hydrolase (Table 3). Isocitrate dehydrogenase, which would not be expected to change much in specific activity, was also assayed. Except in methylamine-grown cells, benzylamine oxidase was assayed with isobutylamine as substrate, which is inactive with methylamine oxidase (Table 4). Methylamine oxidase was present in cells grown on all the amines tested (Table 3), but benzylamine (isobutylamine) oxidase was only detectable in cells grown on primary amines, and, for methylamine and ethylamine, only at a low activity. Neither oxidase was present in cells grown on amino acids or ammonia. Under no growth conditions was benzylamine oxidase found without methylamine oxidase also being present. It is noteworthy that methylamine oxidase was gratuitously induced at high activity in cells grown on isobutylamine and 1,6-diaminohexane, which are not substrates for the enzyme. Catalase was present at much elevated activities in the cells grown on every amine, but not in cells grown on amino acids, except for glycine (no glycine oxidase activity was detected). Amines containing methyl groups induced high activities of the enzymes involved in formaldehyde oxidation. In some experiments low benzylamine oxidase activity (5–10 munits/mg of protein) was detectable in cells grown on trimethylamine (J. Green, unpublished work).

Amine oxidase activities in crude extracts

Electrophoretic examination of crude extracts of *C. boidinii* grown on either methylamine or n-butylamine gave four active bands when either methylamine or benzylamine was used as substrate in the activity stain. These bands, which only appeared very slowly over a period of up to 24 h, had R_m values of 0.09, 0.18, 0.26 and 0.34 (A. J. Ramsey, unpublished work). When a crude extract of cells grown on n-butylamine was heated at 45°C, the methylamine oxidase and benzylamine (iso-

Table 3. *Specific activities of various oxidative enzymes in crude extracts of C. boidinii grown on various nitrogen sources*

Units are munits/mg of protein except for catalase (units/mg of protein)

Nitrogen source for growth	Specific activity of:						
	Benzylamine oxidase	Methylamine oxidase†	Catalase	Formaldehyde dehydrogenase	S-Formylglutathione hydrolase	Formate dehydrogenase	Isocitrate dehydrogenase
Methylamine	8.02*	100.0	83	120	282	17.4	40.6
Dimethylamine	0	85.5	111	360	578	19.7	48.7
Trimethylamine	0	64.4	199	530	760	60.0	71.1
Trimethylamine N-oxide	1.74	128.0	66	260	—§	81.0	110.0
Choline	0	110.0	135	580	1480	45.8	76.0
Ethylamine	6.13	60.5	106	122	87	Low‡	35.5
Butylamine	85.2	63.0	358	190	215	Low	117.0
Isobutylamine	31.8	38.0	162	92	126	Low	35.8
1,6-Diaminohexane	13.0	11.0	130	26	—	14.0	32.0
Benzylamine	95.0	38.0	209	49	—	Low	96.4
Lysine	0	0	20	15	—	11.0	49.0
Glycine	0	0	110	18	—	Low	57.0
Glutamate	0	0	13	23	—	Low	48.0
Urea	0	0	27	17	—	12.0	43.0
$(NH_4)_2SO_4$	0	0	13	34	58	Low	24.3

* Assay method (3). Remaining values in this column used assay method (1) with isobutylamine as substrate.
† Assay method (1).
‡ 'Low' means that the rate was below that of NADH oxidase, and so no activity could be detected.
§ '—' means no data available.

Table 4. *Kinetic parameters for the two amine oxidases of C. boidinii*

These were measured with purified enzyme by assay method (1) (peroxidase-coupled assay) at 25°C. For other active substrates, see the text.

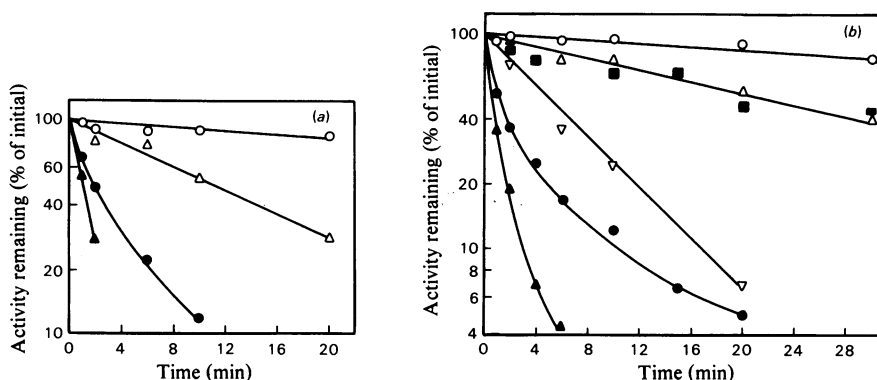|	Methylamine oxidase			Benzylamine oxidase		
Substrate	$K_m^{app.}$ (mM)	V (mM-ABTS oxidized/min per mg of protein)	$\dfrac{V}{K_m}$	$K_m^{app.}$ (mM)	V (mM-ABTS oxidized/min per mg of protein)	$\dfrac{V}{K_m}$
Methylamine	0.227	2.41	10.62	1.83	0.132	0.07
Ethylamine	0.77	5.05	6.56	16.74	4.51	0.27
n-Propylamine	1.54	5.68	3.69	2.04	5.30	2.59
n-Butylamine	0.238	2.45	10.29	0.182	5.40	29.67
Isobutylamine		0	0	1.13	2.80	2.48
n-Pentylamine	1.00	3.77	3.77	0.31	4.18	13.48
n-Hexylamine	2.50	4.55	1.82	0.044	3.41	77.50
n-Heptylamine	3.57	0.636	0.18	0.012	2.25	187.50
n-Octylamine	0.952	0.060	0.06	0.0097	3.14	323.71
n-Nonylamine	0.172	0.060	0.35	0.0069	2.88	417.39
n-Decylamine	0.20	0.095	0.48	0.0077	2.86	371.42
1,6-Diaminohexane		0	0	0.50	1.52	3.04
Benzylamine		0	0	0.0025	0.79	316.0
β-Phenylethylamine	0.11	0.815	7.41	0.044	3.13	71.13

Fig. 2. *Effect of heat on the methylamine oxidase and benzylamine oxidase activities of crude extracts and on the purified enzymes*

(a) Crude extracts of *n*-butylamine-grown *C. boidinii* (5.1 mg of protein/ml) were heated at 45°C (circles) or 50°C (triangles) and samples were removed for assay at the times indicated. ○, △, benzylamine (isobutylamine) oxidase activity; ●, ▲, methylamine oxidase activity. (b) Effect of heat on the purified methylamine oxidase and benzylamine oxidase. Open symbols denote benzylamine (isobutylamine) oxidase (0.15 mg/ml), closed symbols methylamine oxidase (1 mg/ml): ○, ●, 45°C; △, ▲, 50°C; ■, 40°C; ▽, 52°C.

butylamine) oxidase activities showed a sharp difference in stability (Fig. 2a). The benzylamine oxidase lost only 12% of its activity in 10 min, whereas the methylamine oxidase lost 88%. The same effect was observed with the purified enzymes (see below).

Properties of the methylamine oxidase

Purity of the preparation, and molecular weight. Careful combination of fractions from the last three chromatographic steps, after monitoring the protein

pattern by polyacrylamide-gel electrophoresis, gave material with two major bands and one minor band. The latter was an insignificant impurity. The two major bands (R_M values 0.11 and 0.26 at pH 8.3 respectively; Bromophenol Blue = 1.0) both showed activity, although the slower-moving band was always the most prominent. Investigation of these bands by means of Ferguson (1964) plots, obtained by measuring the mobility at pH 7.3 at different acrylamide concentrations (Fig. 3), gave a converging pattern of lines, suggesting that the two
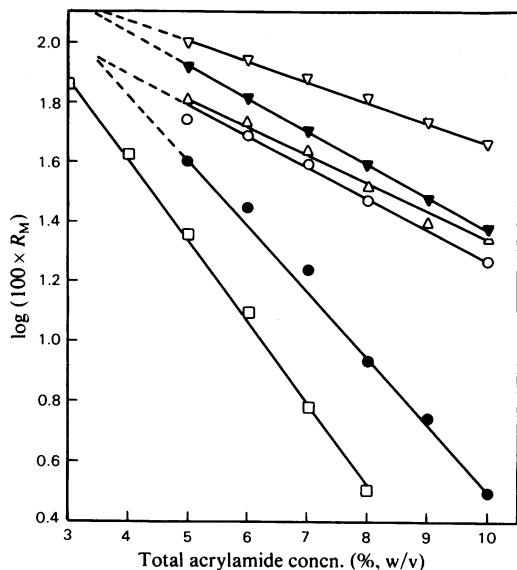
Fig. 3. *Ferguson plots of electrophoretic mobility of methylamine oxidase and benzylamine oxidase in poly-acrylamide gels*

Conditions were as described in the Materials and methods section. O, ●, denote respectively the faster- and slower-moving methylamine oxidase bands; △, benzylamine oxidase. For comparison, the plots for two of the standards shown in Fig. 4 are also given: ▽, bovine serum albumin monomer; ▼, bovine serum albumin dimer; □, thyroglobulin.
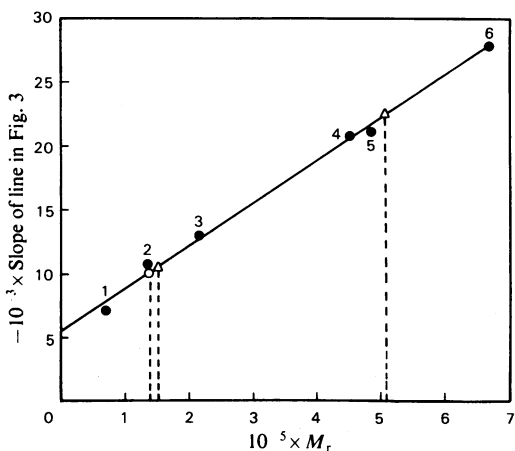

Fig. 4. *Relationship between retardation coefficient and relative molecular mass for the amine oxidases compared with various protein standards*

The retardation coefficient ($-10^3 \times$ slope of Fig. 3) was plotted against relative molecular mass for the following standards: 1, bovine serum albumin monomer; 2, bovine serum albumin dimer; 3, β-amylase (sweet potato); 4, apoferritin monomer; 5, urease (jack bean); 6, bovine thyroglobulin. The two bands of methylamine oxidase are denoted by △, and the benzylamine oxidase by O.

active bands are size isomers (cf. bovine serum albumin, Fig. 3). The molecular weights of the two bands were determined from a standard curve in which the retardation coefficients of a series of protein standards were plotted against their M_r values (Hedrick & Smith, 1968) (Fig. 4), which gave values of 150000 for the M_r of the faster-moving minor active band, and 510000 for that of the slower-moving major active band, suggesting that a tetramer is dissociating into monomers. Excision of the separated bands from unstained gels and re-running them separately on new gels showed that the high-M_r component would regenerate the faster running band, but not vice versa.

In contrast, determinations of M_r value by the Andrews (1970) gel-filtration method on Sepharose 4B gave a value of 286000. A second determination on Sepharose 6B gave $M_r = 257000$ for the methyl-amine oxidase and 288000 for the benzylamine oxidase (see below).

On sodium dodecyl sulphate/polyacrylamide gels, a single band was detected with M_r 81000, suggesting that the smallest active monomer actually consists of two subunits of identical size.

Substrate specificity. Table 4 lists the apparent K_m and V values (oxygen concentration 0.25mM) for a series of substrates of the enzyme compared with the corresponding data for benzylamine oxidase. Very significant quantitative differences in specificity can be seen (despite a considerable qualitative overlap), as shown by the different ratios of $V/K_m^{app.}$. The enzyme was also active with the following amines: 1,2-diaminoethane, ethanolamine and 3-methylbutylamine. The following were not active as substrates: 1,2- and 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopent-ane (cadaverine), 1,6-diaminohexane, spermine, spermidine, isopropylamine, isobutylamine, 2-aminobutane, 3-amino-3-methylpropane, histamine, benzylamine, noradrenaline, tyramine, dopamine, p-dimethylaminomethylbenzylamine, glycine, β-alan-ine, formamide, D-glucosamine, dimethylamine, tri-methylamine, tetramethylammonium chloride, di-ethylamine, triethylamine and triethanolamine.

Effect of temperature and pH. The enzyme had a pH optimum of 7.0–7.2 as determined with methyl-amine as substrate by both assay methods. The heat-stability of the purified enzyme shows it to be much more labile than benzylamine oxidase (Fig. 2b).

Inhibition. It was necessary to test inhibitors in assay method (2), so as to avoid any effects on peroxidase in the coupled assay. The enzyme was particularly sensitive to carbonyl-group reagents (Table 5) and to copper-chelating agents, although other amine oxidase inhibitors were also effective. No significant differences between the quantitative sensitivity of the two enzymes to the most effective

Table 5. *Effect of various inhibitors on methylamine oxidase and benzylamine oxidase*
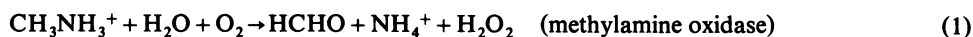
Inhibitors were tested in the oxygen electrode assay at 25°C, with 5 min preincubation in the complete assay system in the absence of substrate. Enzyme concentrations: methylamine oxidase, 120 μg of protein; benzylamine oxidase, 24 μg of protein.

Inhibitor	Concn. tested	Inhibition (%) of	
		Methylamine oxidase	Benzylamine oxidase
Carbonyl reagents			
Semicarbazide hydrochloride	3 mM	100	—
	30 μM	85	54
KCN	2.7 mM	100	100
	9.1 μM	54	—
	100 μM	—	38
Isoniazid	3 mM	100	100
	6.1 μM	32	—
	60 μM	—	62
Hydroxylamine hydrochloride	0.33 mM	100	100
Chelating agents			
Sodium diethyldithiocarbamate	15 mM	86	—
	3.3 mM	12	4
2,2'-Bipyridyl	3 mM	33	46
Cuprizone	0.3 mM	54	—
	0.1 mM	—	40
Disodium EDTA	33 mM	12	4
Substrate analogues			
Dimethylamine hydrochloride	3 mM	20	54
Tetramethylammonium chloride	30 mM	8	95
Lilly 53325	0.3 mM	0	60
Lilly 18947	0.3 mM	0	61
2-Bromoethylamine hydrochloride	10 μM	29	12
Amine oxidase inhibitors			
trans-2-Phenylcyclopropylamine	6.7 mM	90	62
Harmaline hydrochloride	3 mM	77	10
Pargyline hydrochloride	3 mM	35	57
Aminoacetonitrile	6.6 μM	51	74
Mercurials			
Sodum *p*-hydroxymercuribenzoate	0.3 mM	86	35
Sodium mersalyl	0.61 mM	97	100
	60 μM	33	55

inhibitors tested over a 10^6-fold concentration range (cuprizone, semicarbazide, aminoacetonitrile and 2-bromoethylamine) could be detected by either of the two assay methods.

Stoicheiometry of methylamine oxidation. Although the enzyme preparation exhibited no detectable catalase activity by spectrophotometric assay, measurements of oxygen uptake in the absence of peroxidase or catalase were always less than expected. Accordingly, to demonstrate the stoicheiometry of the reaction two experiments were necessary, one in which oxygen consumption was measured in the presence of catalase and one in which the presumed H_2O_2 product was converted quantitatively with peroxidase into the radical cation of ABTS. In the polarographic assay, 0.47 μmol of oxygen and 0.475 μmol of methylamine were consumed and 0.44 μmol of formaldehyde and 0.55 μmol of ammonia were formed. In the spectrophotometric assay 0.268 μmol of methylamine was consumed with the formation of 0.455 μmol of ABTS radical cation, equivalent to 0.227 μmol of H_2O_2. These results are in accordance with the stoicheiometry of eqn. (1):

$$CH_3NH_3^+ + H_2O + O_2 \rightarrow HCHO + NH_4^+ + H_2O_2 \quad \text{(methylamine oxidase)} \quad (1)$$

$$H_2O_2 + 2\,ABTS \rightarrow 2\,ABTS_{ox.} + 2H_2O \quad \text{(peroxidase)} \quad (2)$$

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2 \quad \text{(catalase)} \quad (3)$$

coupled with either eqn. (2) (spectrophotometric assay) or eqn. (3) (polarographic assay).

Kinetics of methylamine oxidase

A systematic study of the reaction kinetics was performed by varying the concentration of the two substrates in turn, methylamine in Fig. 5 and oxygen in Fig. 6. To prevent possible product inhibition, ABTS was used throughout this work as the sodium rather than the ammonium salt, which is the form in which it is supplied. In the coupled spectrophotometric assay (Figs. 5 and 6), the data showed a pattern of intersecting lines characteristic of a sequential mechanism rather than the parallel double-reciprocal lines characteristic of the Ping Pong or substitution mechanism observed for a number of amine oxidases (see references in the Discussion section). In order to rule out any possible influence of peroxidase on the coupled assay, the experiments were then repeated by using the polarographic assay method (2). This is an inherently less precise method than the spectrophotometric method at the very low oxygen concentrations used, and the results are not presented here. They gave, however, the same intersecting pattern of lines as in Figs. 5(a) and 6(a), and the K_m values obtained were 12 μM for oxygen and 0.16 mM for methylamine hydrochloride. The secondary plots for the spectrophotometric method gave the following kinetic parameters (Dalziel, 1957); from Fig. 5(b): $\phi_{MeNH_2} = 0.247$ mM·min, $\phi_{O_2·MeNH_2} = 0.0023$ mM^2·min, K_m for methylamine = 0.198 mM, $\phi_0 = 1.25$ min, $\phi_{O_2} = 0.0127$ mM·min, K_m for $O_2 = 0.010$ mM; from Fig. 6(b): $\phi_{MeNH_2} = 0.247$ mM·min, $\phi_{O_2·MeNH_2} = 0.0026$ mM^2·min, K_m for methylamine = 0.198 mM, $\phi_0 = 1.25$ min, $\phi_{O_2} = 0.0129$ mM·min, K_m for $O_2 = 0.010$ mM. The good agreement between the two different plotting sequences suggests that the results are reasonably reliable, despite limitations in the accuracy of the method.

Product-inhibition experiments. It should be possible to confirm the sequential nature of the reaction by product-inhibition studies, but several of the products at the concentrations necessary could only be tested in assay method (2). A clear-cut indication of the type of inhibition could not always be obtained, but with methylamine as the variable substrate NH_4^+ ions gave a non-competitive inhibition pattern when tested in assay method (1) (since peroxidase was not affected) with a K_i (slope) of 7.6 mM. With assay method (2), formaldehyde gave an uncompetitive pattern with K_i (intercept) 31 mM, and H_2O_2 [which could not be tested in assay method (1) because it is a substrate for peroxidase] showed nearly competitive inhibition with K_i (slope) 22.5 mM. Experiments varying the oxygen concentration were not performed because of the
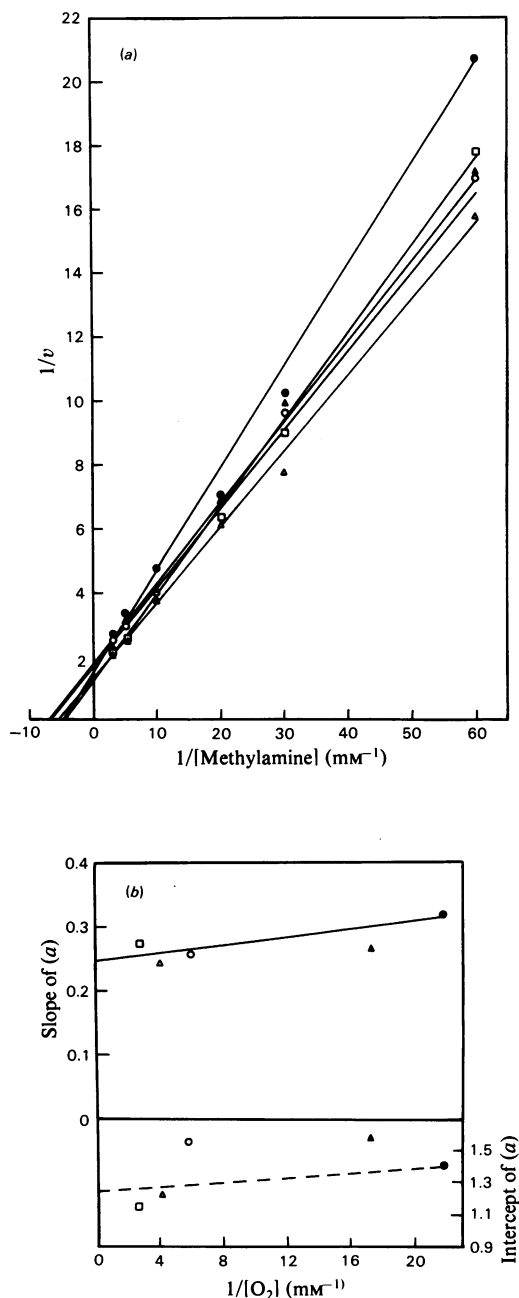

Fig. 5. *Double-reciprocal plots of rate and substrate (methylamine) concentration for methylamine oxidase of C. boidinii*

(a) Primary plots of reciprocal rate and reciprocal methylamine concentration at a series of fixed oxygen concentrations as follows: ●, 46 μM; ▲, 59 μM; ○, 178 μM; △, 253 μM; □, 432 μM. (b) Secondary plots of the slopes (solid line) and intercepts (broken line) of the lines in (a) against reciprocal oxygen concentration. Assay method (1) was used.

difficulties in measuring low inhibited rates at very low oxygen concentrations.

Properties of the benzylamine oxidase

n-Butylamine-grown cells were selected as starting material for the purification of the benzylamine (isobutylamine) oxidase, because they contained high activities of this enzyme (Table 3) and better cell yields were obtained than from benzylamine. In the preparation described in Table 2, the crude
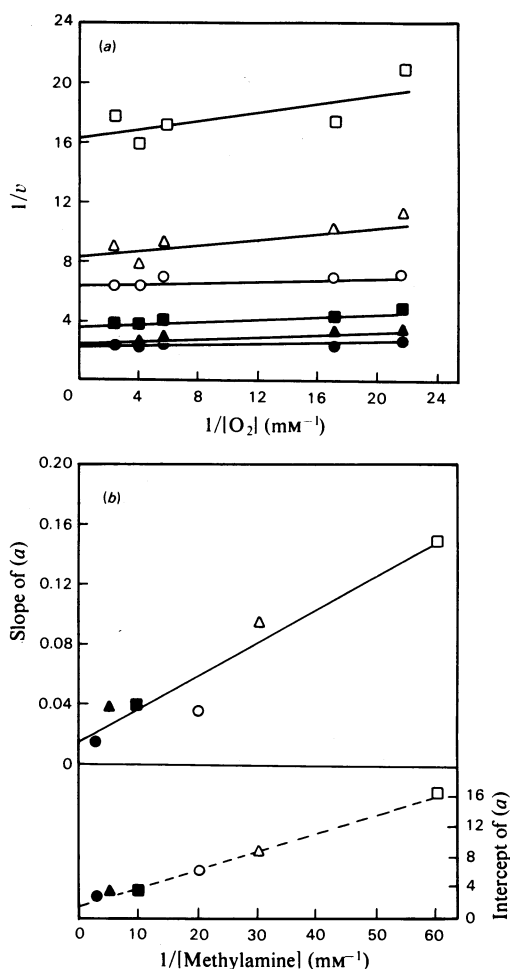

Fig. 6. *Double-reciprocal plots of rate and substrate (oxygen) concentration for methylamine oxidase of C. boidinii*

(a) Primary plots of reciprocal rate and reciprocal oxygen concentration at a series of fixed methylamine concentrations as follows: □, 16.7 μM; △, 33 μM; ○, 50 μM; ■, 100 μM; ▲, 200 μM; ●, 333 μM. (b) Secondary plots of the slopes (solid line) and intercepts (broken line) of the lines in (a) against reciprocal methylamine concentration. Assay method (1) was used.

extract, which contained 28.4 units of isobutyl-amine-oxidizing activity, also contained 52.7 units of methylamine-oxidizing activity. The methylamine oxidase was substantially removed in steps 2, 3 and 4 of the benzylamine oxidase purification (see Fig. 1).

Purity of the preparation and molecular weight. Polyacrylamide-gel electrophoresis at pH 8.3 of enzyme from step 6 showed three very minor impurities and a major band (R_M 0.31, estimated to be 98% of the protein) which was active when stained for activity with benzylamine or isobutyl-amine as substrate. Usually a second very minor active band at R_M 0.18 was also detectable. The R_M-0.31 band gave a M_r of 136 000 for benzylamine oxidase when the retardation coefficient was compared with those of proteins of known M_r (Fig. 4). This contrasts with the value of 288 000 obtained by the Andrews (1970) method on Sepharose 6B.

Determination of the subunit molecular weight by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave a single band with M_r 79 000. This suggests that the active forms of benzylamine oxidase described above consist of two or four polypeptide subunits of identical size.

Substrate specificity. This is summarized in Table 4. In addition to the compounds listed there, the following amines were also substrates: histamine, 1,5-diaminopentane, tyramine, p-dimethylamino-methylbenzylamine, ethanolamine and 3-methyl-butylamine. The following amines were inactive as substrates: isopropylamine, 2-aminobutane, 3-amino-3-methylbutane, 1,2-diaminoethane, 1,3-di-aminopropane, 1,4-diaminobutane (putrescine), spermine, spermidine, glycine, β-alanine, form-amide, D-glucosamine, dimethylamine, trimethyl-amine, tetramethylammonium chloride, diethyl-amine, triethylamine and triethanolamine.

Effect of temperature and pH. The enzyme had a broader pH optimum (with benzylamine as substrate) than did the methylamine oxidase, showing full activity over the pH range 6.2–pH 7.2. It was significantly more stable to heat than was the methylamine oxidase (Fig. 2b), even when tested at a lower protein concentration.

Inhibition. The enzyme was sensitive to essentially the same range of inhibitors as was the methylamine oxidase (Table 5), with the exception of substrate analogues, of which aromatic amines such as 2,4-dichloro-6-phenylphenoxyethylamine (Lilly 53325) and 2,4-dichloro-6-phenylphenoxyethyldi-ethylamine (Lilly 18947) were effective inhibitors of benzylamine oxidase but not of methylamine oxidase.

Stoicheiometry of benzylamine oxidation. In the polarographic assay 0.64 μmol of oxygen and 0.61 μmol of benzylamine were consumed and 0.68 μmol of benzaldehyde and 0.64 μmol of am-

monia were formed. In the spectrophotometric assay, the disappearance of $0.235\,\mu\text{mol}$ of benzylamine was accompanied by the formation of $0.288\,\mu\text{mol}$ of ammonia, $0.30\,\mu\text{mol}$ of benzaldehyde and $0.588\,\mu\text{mol}$ of ABTS radical cation (equivalent to $0.279\,\mu\text{mol}$ of H_2O_2). These also support the stoicheiometry of reactions (1)–(3) above (in which methylamine is replaced by benzylamine).

Kinetics of benzylamine oxidase

Kinetic measurements of the type described for methylamine oxidase proved impossible to perform. At an oxygen concentration of $46\,\mu\text{M}$, the lowest that could be attained with our system, there was no significant decrease in rate, as measured by either assay method. This suggests that the affinity of the enzyme for oxygen is extremely high, and the K_m for oxygen must lie in a concentration region where measurements of the present type cannot be made.

Discussion

Both the enzymes described here have properties resembling those of the copper-containing 'diamine oxidases' (EC 1.4.3.6). Both seem also to have been described previously. The methylamine oxidase closely resembles the enzyme described in a *Trichosporon* sp. grown on glucose with trimethylamine as nitrogen source by Yamada *et al.* (1966), as far as heat-stability, substrate specificity and sensitivity to inhibitors are concerned. Our values for the molecular weight do not agree well with the value of 165000 reported for the *Trichosporon* enzyme, unless the latter enzyme was perhaps fully dissociated under the conditions used. Our growth conditions produce considerably higher activity of the enzyme in the cell compared with those of Yamada *et al.* (1966) (3–5% of the soluble protein in *C. boidinii* compared with 1.4% in *Trichosporon*). Methylamine oxidase is also almost certainly the same enzyme as that identified by Zwart *et al.* (1980) in two other yeast species, *C. utilis* and *H. polymorpha*, grown on glucose with monomethylamine as nitrogen source. Zwart *et al.* (1980) present evidence that the enzyme is located in the peroxisomes, which explains the elevated activities of catalase accompanying the enzyme (Table 3), and explains how the toxic product of the reaction, H_2O_2, may be removed *in vivo*.

The oxidation of methylated amines generates a further potentially toxic metabolite, formaldehyde (Van Dijken & Bos, 1981), and the cells grown on these compounds have markedly elevated activities of formaldehyde dehydrogenase and *S*-formylglutathione hydrolase required for its removal by oxidation (Table 3). The reason for the low activity of formate dehydrogenase in such cells is the stage at which the cells were harvested. Formate dehydro-

genase does not increase in activity until the stationary phase of growth, when the activity of the two preceding enzymes leads to the accumulation of high concentrations of formate in the growth medium (Zwart *et al.*, 1980; Large *et al.*, 1981).

The K_m value for oxygen of methylamine oxidase is very low at $10\,\mu\text{M}$, and seems to be even lower for benzylamine oxidase. These values are lower than values in the literature for pig kidney diamine oxidase ($43\,\mu\text{M}$; Bardsley *et al.*, 1973), human placental amine oxidase ($190\,\mu\text{M}$; Bardsley *et al.*, 1974), pea seedling diamine oxidase (about $96\,\mu\text{M}$; Yamasaki *et al.*, 1970) and much lower than recorded values for monoamine oxidase ($234\,\mu\text{M}$; Tipton, 1968). Tipton (1980) suggests that the Ping Pong mechanism of monoamine oxidase arose through evolutionary pressures to ensure that the enzyme is relatively insensitive to fluctuations in oxygen concentration round the relatively high K_m value for oxygen if the concentrations of the amine substrates are relatively low, thus minimizing the effects of any transitory falls in the local concentrations of oxygen. The fact that methylamine oxidase of *C. boidinii* has a much lower K_m for oxygen, combined with its totally different cellular role (in supplying nitrogen for biosynthetic purposes rather than the removal of biogenic amines), could be possible reasons why it does not show a Ping Pong mechanism but a sequential mechanism. All other amine oxidases so far studied have a Ping Pong mechanism (Finazzi-Agro *et al.*, 1969; Oi *et al.*, 1970; Yamasaki *et al.*, 1970; Houslay & Tipton, 1973; Bardsley *et al.*, 1974). The intersecting nature of the primary double-reciprocal plots (Figs. 5 and 6) was demonstrated by two different methods, one not involving the use of a coupling enzyme. It must be admitted that there are inherent difficulties in arriving at accurate kinetic parameters at these low oxygen concentrations, and the mechanism proposed is in conflict with well-established data for other amine oxidases. It will be important to confirm our observations by using more sensitive methods of oxygen measurement, such as bacterial luminescence (Schindler, 1967). The product-inhibition data are not conclusive, but tentatively indicate an ordered Bi Ter reaction (Cleland, 1963). No systematic two-substrate kinetic studies seem to have been published on yeast or mould amine oxidases with a similar physiological role, such as those from *Trichosporon* or *Aspergillus niger*, which might confirm our suggestions. This may be due to the difficulties emphasized above.

The benzylamine oxidase resembles in many respects that of *A. niger* grown on glucose and n-butylamine (Yamada *et al.*, 1965a,b,c,d), certainly as regards its heat-stability (Yamada *et al.*, 1965c), sensitivity to carbonyl reagents (Yamada

et al., 1965*d*), molecular weight (Yamada *et al.*, 1965*c*; Kumagai *et al.*, 1978), and activity of the enzyme in crude extracts (Adachi & Yamada, 1969). The same enzyme occurs in a number of other moulds (Yamada *et al.*, 1965*a*). The substrate specificity, though in broad agreement (Yamada *et al.*, 1965*c*), does show a few quantitative differences in that the *C. boidinii* benzylamine oxidase is relatively more active with aliphatic 1-aminoalkanes and α,ω-diaminoalkanes of chain length longer than C_5. The peroxisomal location of benzylamine oxidase in yeasts has not yet been established, but its association with high activities of catalase (Table 3) makes this seem very probable. Amine oxidase in *A. niger* has been shown to be located in the peroxisomes (Van Dijken & Veenhuis, 1980).

The presence on polyacrylamide gels from crude extracts of *C. boidinii* of four enzymically active bands with methylamine as substrate, is satisfactorily explained by the presence of two enzymes, each of which is known to give two active bands.

It is interesting that *C. boidinii* grown on n-butylamine contains two amine oxidases, whereas only one has been reported in *A. niger*. There is a report, however, of two different benzylamine oxidases in the mould *Phycomyces blakesleeanus* grown on glucose and asparagine (Hofmann & Hilgenberg, 1979), of which only one oxidase could oxidize tryptamine. A possible reason for the existence of the two enzymes in *C. boidinii* may lie in their substrate specificity (Table 4). Methylamine oxidase is really a short-chain-aliphatic primary-amine oxidase, whereas benzylamine oxidase is an aromatic and long-chain-aliphatic primary-amine oxidase. Spermine, spermidine and α,ω-diaminoalkanes are inactive or poorly active substrates for both enzymes: this may explain why some organisms possess putrescine oxidase, which is a totally different kind of enzyme with an FAD prosthetic group (Adachi *et al.*, 1966). The presence of two amine oxidases enables *C. boidinii* to use amine nitrogen sources at maximum efficiency, especially at very low concentration. A more detailed study of the purified enzymes will be necessary to establish precisely where their differences lie, since the inhibition data suggest a close similarity in the active sites, and the two oxidases have polypeptide subunits of approximately the same size.

The methylamine oxidase consists of a single type of polypeptide subunit of M_r 81000. The Ferguson plots suggest that as isolated the enzyme is an octomer of M_r about 510000, dissociating into active dimers of M_r 150000, but several gel-filtration experiments on both Sepharose 4B and 6B gave an M_r value in the region of 257000, suggesting a tetramer, although no evidence for such tetramers was obtained on polyacrylamide-gel electrophoresis. The benzylamine oxidase, in contrast, was less consistent in its behaviour. It had a subunit molecular weight of 79000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Relatively highly purified material behaved on gel filtration on Sepharose 6B as if it had a higher M_r value (288000, corresponding to a tetramer) than methylamine oxidase (257000). In step (3) of the purification, however, the peak of benzylamine oxidase activity emerged from the Sepharose 6B column later than methylamine oxidase, at a volume corresponding to M_r 133400 (methylamine oxidase under these conditions, 226500). This would correspond to an active dimer, in agreement with the data from the Ferguson plots. We suggest that benzylamine oxidase dimer (M_r 133000) itself tends to dimerize (giving the 288000-M_r species) during pressure concentration of the enzyme, which is used in several later steps of the purification. Yamada *et al.* (1965*c*) report a molecular weight of 252000 for *A. niger* benzylamine oxidase, determined in the ultracentrifuge, and 255000 by gel filtration (Kumagai *et al.*, 1978). Kumagai *et al.* (1978) obtained a value for the subunit molecular weight of the *A. niger* enzyme of 125000 and suggested that the active enzyme is a dimer. Association–dissociation phenomena have been reported previously with pig plasma amine oxidase (Achee *et al.*, 1968) and pig kidney diamine oxidase (Pionetti, 1974).

The fact that the two amine oxidases of *C. boidinii* have similarly sized polypeptide subunits may indicate the possibility that they have evolved from duplicated genes (Clarke, 1978), and have undergone considerable changes in their quaternary structure as well as in their substrate specificity.

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