Subcellular Localization and Properties of Partially Purified Dimethylamine and Trimethylamine Mono-oxygenase Activities in *Candida utilis*

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By techniques involving differential centrifugation and specific precipitation with CaCl_2 , it was shown that dimethylamine and trimethylamine mono-oxygenase activities co-sediment with NADPH-cytochrome *c* reductase activity in sphaeroplast lysates of *Candida utilis* grown on trimethylamine as sole nitrogen source. Since the active fraction also contained low levels of cytochromes P-450 and P-420, it was concluded that the two amine mono-oxygenases are located in the smooth endoplasmic reticulum and thus end up in the microsomal fraction on cell fractionation. Ten to twenty-fold enrichment of mono-oxygenase specific activity could be achieved by separation of activity from soluble protein by centrifugation or gel filtration. Cell-free extracts prepared in the absence of FAD showed only very low mono-oxygenase activity for either substrate. Some activity could be restored by addition of flavin nucleotides: there was a fivefold stimulation by FAD and a fourfold stimulation by FMN. All trimethylamine mono-oxygenase activity was lost when a partially purified preparation containing both activities was incubated for more than 24 h at 0 °C, suggesting that separate enzymes are responsible for the oxidation of secondary and tertiary amines. The enzyme preparation oxidized a wide range of secondary alkylamines up to dibutylamine and tertiary alkylamines up to tributylamine. Primary amines, choline, di- and triethanolamine, spermine, spermidine and substituted anilines were not oxidized. NADH had a lower apparent K_m value and higher $V_{\max}$ value than NADPH. Secondary and tertiary alkylamines containing more than one kind of alkyl group gave more than one kind of aldehyde on oxidation. Stoichiometry determinations showed a consumption of 1 mol NAD(P)H and 1 mol O_2 per mol aldehyde formed. Carbon monoxide, cyanide, proadifen hydrochloride (SKF 525-A), mercurials and mercaptoethanol all inhibited both activities.

INTRODUCTION

In contrast to methylotrophic bacteria, where the diverse enzyme systems involved in the oxidation of di- and trimethylamine have been fairly well characterized (Large, 1981; Anthony, 1982), very little is known about the oxidation of these two compounds in yeasts. Many yeasts are able to use them (and also monomethylamine) as sole nitrogen source, though not as carbon source (Van Dijken & Bos, 1981), but there have been few studies on the enzymic mechanisms involved. Micro-organisms able to use these amines as sole nitrogen source are said to be *methazotrophic* (Zatman, 1981). Yamada *et al.* (1976) showed that whole cells of the methazotrophic yeast *Candida* sp. WY-3 converted trimethylamine into dimethylamine and formaldehyde and *n*-butylmethylamine into *n*-butylamine and formaldehyde, but they were unable to find activity in cell-free extracts. We have shown that the presence of a low concentration of FAD is necessary in the buffer used to prepare the extract before mono-oxygenase activity for dimethylamine and trimethylamine can be demonstrated in *Candida boidinii*, *C. utilis* and several other yeast species (Green & Large, 1983a).

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Although the key enzymes for the oxidation of monomethylamine – methylamine oxidase and catalase – are derepressed under nitrogen-limiting conditions even in the absence of amines (Zwart & Harder, 1983; Green & Large, 1984), the expression of mono-oxygenase activity for dimethylamine and trimethylamine requires the presence of a secondary or tertiary alkylamine in the growth medium (Green & Large, 1984). This is in contrast to the observations of Yamada *et al.* (1976) who found that trimethylamine could be oxidized by whole cells of *Candida* sp. WY-3 grown on several different primary amines as sole nitrogen source. Mono-oxygenase activity for trimethylamine (and other tertiary alkylamines) is also induced in cells grown on secondary alkylamines, although there is evidence (see Green & Large, 1983*a* and below) that separate enzymes are responsible for the oxidation of secondary and tertiary amines.

In the present paper, we show that the carbon monoxide-sensitive systems catalysing the oxidation of dimethylamine and trimethylamine in cell-free extracts of *C. utilis* are located in the microsomal cell fraction. We describe further properties of the enzymes and their separation from soluble protein. *Candida utilis* has recently been renamed *Hansenula jadinii* (Barnett *et al.*, 1983), of which it is the asexual state. The original name will be used in this paper. Preliminary accounts of parts of these results have been published (Green & Large, 1983*b*; Large & Green, 1984).

METHODS

Materials. 2,4-Dichloro-6-phenylphenoxyethylamine (Lilly 53325) and its *N,N*-diethyl derivative (Lilly 18947) were gifts from Eli Lilly International, Indianapolis, USA; proadifen hydrochloride (SKF 525-A) was a gift of Smith, Kline and French, Welwyn Garden City, UK. Zymolyase 5000 was from Miles Laboratories, Stoke Poges, Slough, UK. Sepharose 2B and 4B were obtained from Pharmacia. Methylamine dehydrogenase (EC 1.4.99.3) was prepared from *Pseudomonas* AM1 as described by Boulton & Large (1979). 4-Aminobutyl-Sepharose was prepared as described by Shaltiel & Er-El (1973). Secondary amines were obtained from the sources listed in Eady *et al.* (1971) and tertiary amines from the sources in Boulton *et al.* (1974). Carbon monoxide came from BDH. All other chemicals were from Sigma or Fisons Scientific Apparatus.

Growth and maintenance of yeast cultures. *Candida utilis* NCYC 321 was grown and maintained as described by Haywood & Large (1981), the carbon source being 55 mM-glucose and the nitrogen source 30 mM-trimethylamine hydrochloride. Growth was followed by measurement of optical density at 663 nm. Cells were harvested by centrifuging at 10000 *g* for 20 min at 4 °C, washed with 50 mM-potassium phosphate buffer, pH 7.0, and used immediately.

Preparation of cell-free extracts. Cells were suspended in an equal volume of 50 mM-potassium phosphate buffer, pH 7.0, containing 20 µM-FAD, and passed twice through a French pressure cell operated at 7.85 MPa. Unbroken cells were removed by centrifugation at 20000 *g* for 15 min at 4 °C.

Enzyme assays. Dimethylamine and trimethylamine mono-oxygenase activity were measured by two methods. (a) The spectrophotometric method involved following decrease of absorbance at 340 nm and 25 °C in polystyrene cuvettes (1 cm light path) containing 67 µmol potassium phosphate buffer pH 7.0, 0.4 µmol NAD(P)H, 5 µmol dimethylamine or 10 µmol trimethylamine hydrochloride and enzyme in a total volume of 3 ml. The reaction was started by addition of the amine after measuring any endogenous NAD(P)H oxidation. One unit of enzyme is defined as the amount required to catalyse the amine-dependent oxidation of 1 µmol NAD(P)H min⁻¹ under these conditions. (b) The formaldehyde-accumulation assay involved measuring formaldehyde formation from di- or trimethylamine. The assay mixture was the same as (a) except that incubation was performed in test tubes and 1 µmol of NAD(P)H was used. Reaction was started by addition of enzyme and stopped by adding 0.2 ml of 2.5 M-HClO₄. After neutralization and centrifugation, the formaldehyde was determined. One enzyme unit is the amount required to catalyse the formation of 1 µmol of formaldehyde min⁻¹. Catalase (EC 1.11.1.6), methylamine oxidase and NADP-linked isocitrate dehydrogenase (EC 1.1.1.42) were assayed as described by Haywood & Large (1981). Malate dehydrogenase (EC 1.1.1.37) was assayed by measuring the rate of decrease in absorbance at 340 nm when 200 µmol phosphate buffer pH 7.5, 0.4 µmol NADH and 15 µmol oxaloacetate were incubated with enzyme (reaction volume 3 ml). NADPH-cytochrome *c* reductase (EC 1.6.2.4) and cytochrome oxidase (EC 1.9.3.1) were assayed as described by Delaissé *et al.* (1981). Alkaline phosphatase (EC 3.1.3.1) was assayed by measuring the rate of increase in absorbance at 412 nm when 200 µmol Tris/HCl buffer pH 8.0 and 14.5 µmol 4-nitrophenyl phosphate were incubated with enzyme in a total volume of 3 ml. All assays were performed at 25 °C, and are averages of two determinations.

Preparation and lysis of sphaeroplasts. Exponential phase yeast cultures (OD₆₆₃ = 1.0) grown with trimethylamine as sole nitrogen source were harvested and washed in 70 mM-potassium phosphate buffer pH 7.5. Cells (5 g wet weight) were suspended in buffer A (70 mM-potassium phosphate, pH 7.5; MgCl₂ 1 mM, EDTA 1 mM) containing 2 M-sorbitol. After 10 min osmotic stabilization at room temperature the cells were centrifuged

at 10000 *g* for 10 min. The pellet thus obtained was resuspended in buffer A containing 2 M-sorbitol and 1 mg Zymolyase 5000 ml⁻¹ to a concentration of 20 mg wet weight of cells ml⁻¹ (Osumi *et al.*, 1975). Mercaptoethanol was omitted because it inhibits amine oxidase activity (Zwart *et al.*, 1980). The cells were incubated at 30 °C for about 1 h, the formation of sphaeroplasts being followed under a phase contrast microscope. When sphaeroplast formation was complete, an equal volume of buffer A was added, and the mixture left to stand in ice for 10 min. The Zymolyase was then removed by two washes in buffer A containing 1 M-sorbitol. The sphaeroplasts were then lysed by diluting the sorbitol concentration of the suspension to 0.3 M with buffer A, followed by gentle shaking. Unbroken sphaeroplasts and debris were removed by centrifuging for 5 min at 1000 *g* and 4 °C.

Cell-fractionation. The lysed sphaeroplasts were fractionated by differential centrifugation at 4 °C as described by Delaissé *et al.* (1981). For details of centrifugation times and speeds, see legend to Table 1. Mitochondria and peroxisomes in fraction P₂ were separated by the method of Bartels & Jensen (1979). All the pellets were washed in 0.3 M-sorbitol in 10 mM-potassium phosphate, pH 7.0, containing 10 µM-FAD and resuspended in the same mixture before enzyme assays were performed.

Determination of kinetic parameters. K_m^{app} and V_{max} values were obtained by varying the amine (or electron donor) and its concentration in the spectrophotometric assay and plotting reciprocal rates against reciprocal concentrations. Lines were fitted by eye. One preparation was used for secondary amines, another for tertiary amines. Variation in the values obtained occurred from one preparation to another, but values were within one order of magnitude. Representative data like those in Table 2 are to allow comparison between substrates.

Chemical determinations. Formaldehyde was determined by the method of Nash (1953), protein by the method of Bradford (1976), dimethylamine by the method of Dubin (1960), methylamine by the enzymic method of Large *et al.* (1969), NAD(P)H spectrophotometrically at 340 nm, and oxygen polarographically using a Rank oxygen electrode calibrated by the method of Robinson & Cooper (1970). Trimethylamine was determined by the enzymic method of Large & McDougall (1975), and total aldehyde by the method of Sawicki *et al.* (1961).

Determination of reaction stoichiometry. (a) Spectrophotometric experiment. A normal spectrophotometric reaction mixture was set up except that approximately 0.1 µmol dimethylamine or trimethylamine was used as substrate. When the reaction had gone to completion and the amount of NADH oxidized had been measured, 0.3 ml of 2.5 M-HClO₄ was added. After neutralization and centrifugation, formaldehyde, trimethylamine, dimethylamine and methylamine were estimated. Two control reactions were run, the first containing boiled enzyme, the other containing no tri- or dimethylamine to monitor any substrate-independent NADH oxidation. (b) Polarographic experiment. About 0.1 µmol tri- or dimethylamine was incubated with 0.6 µmol NADH in the chamber of a calibrated oxygen electrode. The reaction was started by addition of enzyme and oxygen consumption followed to completion. Then 0.3 ml of 2.5 M-HClO₄ was added, and after neutralization and centrifugation the formaldehyde formed was measured. Similar controls were performed as those in (a). The results in Table 4 are the differences between the test and the control. Experiments with standards suggested that the analytical measurements were accurate to about 10%.

Inhibition experiments. Inhibitors were preincubated with enzyme in the assay mixture for 5 min in the absence of substrate before the reaction was started by substrate addition to a final concentration of 2 mM. In the case of dimethylamine mono-oxygenase the formaldehyde accumulation assay was used, whereas in the case of trimethylamine mono-oxygenase the spectrophotometric assay was used to eliminate effects of the inhibitors on dimethylamine mono-oxygenase activity.

Difference spectra. These were determined on a Pye-Unicam SP1800 recording spectrophotometer at 25 °C. Samples were reduced with dithionite or NADH and oxidized with ferricyanide.

RESULTS

Subcellular fractionation of *C. utilis*

The isolation of the subcellular components of *C. utilis*, grown with trimethylamine as the sole source of nitrogen, was achieved by differential centrifugation of osmotically lysed sphaeroplasts, following the general scheme of Delaissé *et al.* (1981). The marker enzymes were chosen on the basis of the results obtained by Delaissé *et al.* (1981). The results are shown in Table 1.

About half the total malate dehydrogenase activity sedimented, mainly in fraction P₂ (material sedimenting after 5 min at 3500 *g*), and this same fraction contained 94% of the total cytochrome *c* oxidase activity. Malate dehydrogenase and cytochrome oxidase both had increased relative specific activities in the P₂ fraction (Table 1). Although cytochrome oxidase is a classical mitochondrial marker enzyme (Lloyd, 1974), malate dehydrogenase in yeasts can under some growth conditions occur in both mitochondria and peroxisomes (microbodies) (Zwart *et al.*, 1983). As a result of the lysis procedure used, 75% of the catalase activity appeared

Table 1. *Distribution of marker enzymes in subcellular fractions prepared by differential centrifugation of lysed sphaeroplasts of Candida utilis*

Relative amounts of each enzyme are the (amount in fraction) $\times$ (total amount recovered in all fractions)⁻¹ $\times$ 100. Relative specific activities (in parentheses) are the (percentage of total recovered activity) $\times$ (percentage of total recovered protein)⁻¹. Recoveries are presented as a percentage relative to the sphaeroplast lysate, S₁. P₂ represents the pellet obtained by centrifuging the lysate S₁ for 5 min at 3500 g. P₃ represents the pellet from centrifuging the supernatant from P₂ for 10 min at 12000 g. Similarly P₄ and P₅ represent the pellets from centrifugation for 20 min at 25000 g and 70 min at 50000 g, respectively. S₅ is the final supernatant.

Constituent	Absolute amount in fraction S ₁ (units of enzyme activity or mg of protein)	Relative amounts in each fraction (relative specific activities in parentheses)						Recovery (%)
		S ₁	P ₂	P ₃	P ₄	P ₅	S ₅	
Catalase	12420	(0.82)	18.4 (2.97)	3.7 (1.34)	2.3 (0.56)	2.95 (0.21)	72.5 (1.00)	95.6
Methylamine oxidase	4.95	(0.83)	53.1 (8.56)	29.5 (7.07)	0 (0)	0 (0)	27.5 (0.38)	97.1
Cytochrome c oxidase	8.62	(1.06)	94.2 (15.2)	5.8 (2.10)	0 (0)	0 (0)	0 (0)	125.0
Malate dehydrogenase	44.8	(1.00)	39.7 (6.40)	4.76 (1.72)	3.3 (0.80)	5.53 (0.39)	46.7 (0.65)	118.0
NADPH-cytochrome c reductase	1.98	(0.74)	0 (0)	0 (0)	0 (0)	23.7 (1.68)	76.3 (1.06)	87.4
Trimethylamine mono-oxygenase	0.17	(1.07)	0 (0)	0 (0)	31.1 (7.59)	68.9 (4.89)	0 (0)	125.0
Dimethylamine mono-oxygenase	4.52	(0.79)	0 (0)	0 (0)	17.7 (4.32)	74.5 (5.28)	7.8 (0.11)	92.7
Alkaline phosphatase	5.60	(0.90)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1.39)	105.0
Isocitrate dehydrogenase (NADP-linked)	5.02	(0.76)	0 (0)	0 (0)	0 (0)	13.3 (0.94)	86.7 (1.24)	89.1
Protein	109		6.2	2.76	4.1	14.1	72.0	117.3

in the S₅ (soluble) fraction, but of the 25% which sedimented, most was found in fractions P₂ and P₃ (the latter sedimented in 10 min at 12000 g), with enhanced specific activity in both fractions. The majority of methylamine oxidase activity (73%) was also sedimented in these two fractions, again with increased relative specific activity. Catalase is a marker for yeast peroxisomes and 80% of the catalase activity of *C. tropicalis* is known to be peroxisomal (Yamada *et al.*, 1982). Since cytoplasmic catalase has not been observed in *C. utilis* (Veenhuis *et al.*, 1983) it seems likely that, as in the case of cell fractionation of *Saccharomycopsis lipolytica* (Delaissé *et al.*, 1981) and *C. stellatoidea* (Jenkins *et al.*, 1983), catalase is leaking from the peroxisomes. A similar explanation might also apply to all or some of the malate dehydrogenase found in the soluble fraction S₅.

Clearly fraction P₂ and to a lesser extent P₃ contained both mitochondrial and peroxisomal material. A separation was achieved by the procedure of Bartels & Jensen (1979), in which resuspended P₂ material was layered over 50% (w/v) sucrose and centrifuged for 30 min at 50000 g. The peroxisomes are sedimented by this method while the mitochondria remain at the buffer-sucrose interface. It was found that 98% of the methylamine oxidase of fraction P₂ and 99% of the catalase activity sedimented, while 99% of the cytochrome oxidase remained at the interface. This confirms the peroxisomal location of methylamine oxidase as already established in *C. utilis* by cytochemical staining techniques (Zwart *et al.*, 1980).

NADPH-cytochrome c reductase as well as trimethylamine and dimethylamine mono-oxygenases were found in the P₅ fraction (sedimenting after 70 min at 50000 g) with increased relative specific activity (Table 1). A small (0.004) cytochrome P-450 peak was observed in this fraction, as measured by (reduced-plus-CO minus reduced) difference spectra. Similar spectra

showed a peak at 422 nm in fractions P_4 (0.016), P_5 (0.024) and S_5 (0.010), where the figures in parentheses give the absorbance at 422 nm (mg protein)⁻¹. The distribution of these components suggests that trimethylamine and dimethylamine mono-oxygenases are located in the smooth endoplasmic reticulum of the cell (Delaissé *et al.*, 1981) for which cytochrome P-450 and NADPH-cytochrome *c* reductase are enzymic markers. This proposal is supported by the specific precipitation of the microsomal fraction of *C. utilis* with calcium chloride by the procedure of Käppeli *et al.* (1982). Of the activities in sonically lysed sphaeroplast extracts, 90% of the dimethylamine mono-oxygenase, 100% of the trimethylamine mono-oxygenase and 88% of the NADPH-cytochrome *c* reductase activity could be precipitated by 16 mM-calcium chloride.

Although 76% of the NADPH-cytochrome *c* reductase activity appeared in the 'soluble' S_5 fraction (Table 1), its relative specific activity was not increased and about 30% of the activity could be sedimented by further centrifuging for 1 h at 100 000 *g*, while no alkaline phosphatase or NADP-linked isocitrate dehydrogenase activity was sedimented by this procedure. This suggests that the NADPH-cytochrome *c* reductase is associated with very finely broken-up fragments of membrane.

The S_5 fraction contained more than 70% of the S_1 total of alkaline phosphatase, isocitrate dehydrogenase (NADP-linked) and protein, together with 47% of the malate dehydrogenase activity, the two former enzymes having somewhat increased relative specific activity (Table 1). The NADP-linked isocitrate dehydrogenase may be considered to mark the soluble fraction of the cell. Although alkaline phosphatase is found in the vacuoles of *Saccharomyces cerevisiae* (Holley & Kidby, 1973) and *C. utilis* (Veenhuis *et al.*, 1980), vacuoles are fragile structures and their contents may have been released during lysis of the sphaeroplasts.

Partial purification of dimethylamine and trimethylamine mono-oxygenase activity

Mono-oxygenase activity for both dimethylamine and trimethylamine was enriched by approximately 10 to 20-fold by either of two procedures. In one, a crude extract (previously centrifuged at 20 000 *g* for 15 min) was then centrifuged at 150 000 *g* for 60 min at 4 °C to yield a pellet containing all the dimethylamine and trimethylamine mono-oxygenase activity. After resuspension in 10 mM-potassium phosphate, pH 7.0, containing 10 µM-FAD and recentrifuging, the pellet was free of methanol oxidase, alcohol dehydrogenase and NADH-formaldehyde reductase activity (which was probably similar to that observed by Hou *et al.*, 1982). Alternatively, the crude extract was applied to a column (10 cm × 2 cm diam.) of Sepharose 2B, equilibrated in 10 mM-potassium phosphate, pH 7.0, containing 10 µM-FAD, and eluted with the same buffer. The excluded fractions containing the activity were immediately applied to a column (6 cm × 1 cm diam.) of 4-aminobutyl-Sepharose 4B in the same buffer, to which the activity did not bind. The combined active fractions from this column typically had a specific activity of 0.22 units (mg protein)⁻¹ and could be stabilized by adding bovine serum albumin to a concentration of 5 mg ml⁻¹. Purification factors of up to 20-fold were achieved. On polyacrylamide gel electrophoresis of 100 µg of protein on 4% or 7% gels, no proteins entered the gel. This fact, taken together with the exclusion from Sepharose 2B and the sedimentation at 150 000 *g*, suggests that the activity was membrane-bound. Attempts to purify the system further were unsuccessful, and further purification will clearly require prior solubilization of the enzyme activity.

The enzyme preparation had a pH optimum of 7.0 for the oxidation of both dimethylamine and trimethylamine. The activity was stable at 4 °C for 40 min within the pH range 4 to 10 (dimethylamine) or 5 to 9 (trimethylamine). Beyond these limits, activity was rapidly lost.

Effect of addition of flavin nucleotides

Partially purified dimethylamine mono-oxygenase was prepared in the absence of FAD and showed a much reduced capacity to degrade dimethylamine. In the spectrophotometric assay, FAD stimulated the activity of the enzyme more than fivefold and FMN almost fourfold (Fig. 1). No synergistic effect was observed when the concentration of FAD was increased in the presence of 0.76 µM-FMN. With trimethylamine mono-oxygenase activity, 40 nM-FAD

Fig. 1

Fig. 1. Effect of flavin nucleotides on the rate of dimethylamine oxidation by partially purified dimethylamine mono-oxygenase of *Candida utilis* prepared in the absence of FAD. The effect of adding FAD or FMN to the enzyme was measured in the spectrophotometric assay. ●, Effect of FAD; ▲, effect of FMN (Sigma grade I). The protein concentration of the enzyme was 1.22 mg ml^{-1} .

Fig. 2

Fig. 2. Relative stabilities of dimethylamine and trimethylamine mono-oxygenases of *Candida utilis*. Partially purified enzyme ($3.1 \text{ mg protein ml}^{-1}$) was incubated at 22.5°C and samples were removed at intervals, and the activity measured in the spectrophotometric assay with dimethylamine (●) or trimethylamine (▲) as substrate; 100% activity was about $0.1 \text{ unit (mg protein)}^{-1}$ for trimethylamine mono-oxygenase and $0.2 \text{ unit (mg protein)}^{-1}$ for dimethylamine mono-oxygenase.

stimulated activity threefold and $2.8 \mu\text{M}$ -FMN stimulated activity twofold (at a protein concentration of 3.1 mg ml^{-1}). The activity reconstituted in this way was still significantly lower than in extracts prepared by breaking open cells suspended in buffer containing FAD.

Are the dimethylamine and trimethylamine mono-oxygenases separate enzymes?

Although many of the properties of dimethylamine mono-oxygenase and trimethylamine mono-oxygenase were very similar, the two amine oxidation systems showed a 90-fold difference in sensitivity to inhibition by carbon monoxide in whole cells of *C. boidinii* (Green & Large, 1983a), trimethylamine mono-oxygenase being the more sensitive. They also showed a significant difference in stability. When a partially purified preparation containing both activities (which co-purified) was incubated for several hours at 22.5°C , the trimethylamine mono-oxygenase activity proved to be much less stable (Fig. 2), and at 35°C the former enzyme had a half-life of 1.7 min (protein concentration 1.5 mg ml^{-1}), while at 37°C the latter had a half-life of only 0.4 min (protein concentration 5.7 mg ml^{-1}). A preparation which had been left in ice for more than 24 h effectively lost all trimethylamine mono-oxygenase activity. Thus it was possible to obtain dimethylamine mono-oxygenase free of trimethylamine mono-oxygenase activity, but not vice versa.

Properties of the partially purified enzyme preparation

The enzyme preparation was specific for secondary and tertiary amines. Primary amines and quaternary ammonium derivatives (tetramethylammonium and choline) were not oxidized. Apparent K_m and V_{max} values for active substrates are given in Table 2. Other tertiary amine substrates additional to those in Table 2 included tripropylamine, tributylamine and the amines in Table 3 (see below). The following secondary and tertiary amines were not oxidized: diethanolamine, *N*-methylaniline, sarcosine, spermine, spermidine, *NN*-dimethylaniline, and triethanolamine.

With respect to electron donors, NADH and NADPH were both active. NADH was shown to be the more efficient donor with a V_{max} of $0.128 \text{ units (mg protein)}^{-1}$ and a K_m^{app} of $28.5 \mu\text{M}$ compared to a V_{max} of $0.03 \text{ units (mg protein)}^{-1}$ and a K_m^{app} of $166 \mu\text{M}$ for NADPH. The ratios V_{max}/K_m^{app} are thus 4.5 for NADH and 0.18 for NADPH (concentrations in mM). Accordingly, in

Table 2. Kinetic parameters for the secondary-amine mono-oxygenase of *Candida utilis*

These were measured with partially purified enzyme in the spectrophotometric assay with NADH as the electron donor, at pH 7.0 and 25 °C. A separate preparation was used for the tertiary amines. Double reciprocal plots of rate and concentration were used in the evaluation. Values are representative (see Methods).

Substrate	K_m^{app} (μ M)	V_{max} [Units (mg protein) ⁻¹]	V_{max}/K_m^{app} [Units (mg protein) ⁻¹ mM ⁻¹]
Secondary amines			
Dimethylamine	36.8	0.220	5.98
Diethylamine	21.7	0.225	10.37
Dipropylamine	303	0.114	0.376
Dibutylamine	28.3	0.058	2.05
Methylethylamine	93.0	0.310	3.33
Methylpropylamine	80.0	0.263	3.29
Methylisopropylamine	278	0.179	0.664
Methylbutylamine	29.4	0.080	2.72
Methyloctylamine	70.4	0.057	0.812
Methylethanolamine	654	0.270	0.413
Ethylpropylamine	833	0.222	0.267
Ethylbutylamine	125	0.340	2.72
Propylbutylamine	800	0.370	0.463
Tertiary amines			
Trimethylamine	1.18	0.026	0.022
Dimethylethylamine	4.0	0.314	0.079
Diethylmethylamine	0.833	0.087	0.105
Triethylamine	4.44	0.147	0.033

Table 3. Formation of aldehydes from secondary amines by the partially purified amine mono-oxygenases of *Candida utilis*

Total aldehyde formed was measured by the method of Sawicki *et al.* (1961) and formaldehyde separately by the acetylacetone method (Nash, 1953).

Substrate	Formaldehyde as % of the total aldehyde
Secondary amines	
Dimethylamine	95.1
Methylethylamine	44.6
Methylpropylamine	0
Methylisopropylamine	49.0
Methylbutylamine	78.9
Methyloctylamine	0
Methylbenzylamine	21.2
Tertiary amines	
Trimethylamine	101.8
Dimethylethylamine	95.7
Dimethyl-2-chloroethylamine	12.3
Dimethylaminoethanol	99.7
1-Dimethylamino-2-propanol	36.5
3-Dimethylamino-1-propanol	92.6
Dimethylbenzylamine	97.0
Dimethylcyclohexylamine	13.9
Dimethyldodecylamine	0
Diethylmethylamine	38.8
Dibutylmethylamine	4.4

most experiments NADH was used, except where the rate of substrate-independent NADH oxidation was too high to be acceptable.

The K_m for oxygen could not be determined, since it was below the level of accurate polarographic measurement.

Mono-oxygenase attack on secondary and tertiary amines containing more than one kind of alkyl group gave rise to more than one kind of aldehyde (Table 3). The other aldehydes formed in this experiment were not further investigated.

Table 4. *Stoichiometry of the dimethylamine and trimethylamine mono-oxygenase reactions*

Experiments 1 and 3 were spectrophotometric, experiments 2 and 4 were polarographic. For experimental details, see Methods section. The values represent substrate consumed or product formed (nmol).

Expt no. . . .	1	2	3	4
Substrate				
Dimethylamine	124	—	—	—
Trimethylamine	—	—	130	—
NADH	135	—	232	—
Oxygen	—	188	—	205
Product				
Formaldehyde	136	175	229	204
Dimethylamine	—	—	20	—
Methylamine	132	—	86	—

Table 5. *The effect of various inhibitors of the dimethylamine mono-oxygenase and trimethylamine mono-oxygenase of C. utilis*

Inhibitors were present for a 5 min pre-incubation period in the complete assay system in the absence of the amine substrate. Substrate concentration was 2 mM. Dimethylamine mono-oxygenase was measured by the formaldehyde accumulation assay, trimethylamine mono-oxygenase by the spectrophotometric method. ND, Not done.

Inhibitor	Dimethylamine		Trimethylamine	
	Inhibitor concn	Percentage inhibition	Inhibitor concn	Percentage inhibition
Lilly 53325	0.5 mM	65.6	0.33 mM	28.3
Lilly 18947	ND		0.33 mM	50.0
Tetramethylammonium chloride	5 mM	20.7	6.0 mM	45.0
<i>trans</i> -2-Phenylcyclopropylamine	0.25 mM	60.6	3.3 mM	0
Harmaline hydrochloride	ND		0.66 mM	0
Pargyline hydrochloride	1.16 mM	15.7	0.66 mM	100
Aminoacetonitrile	0.1 mM	0	ND	
Disodium EDTA	2.5 mM	9.8	3.0 mM	0
Cuprizone	12.5 μ M	4.9	15 μ M	27.6
2,2'-Bipyridyl	0.5 mM	12.7	0.6 mM	0
Potassium cyanide	0.5 mM	78.7	ND	
Carbon monoxide	30 s bubbling	100	30 s bubbling	100
Hydroxylammonium chloride	0.5 mM	99	1.2 mM	100
Sodium azide	5 mM	14.6	ND	
Proadifen hydrochloride	0.5 mM	71.7	0.33 mM	53.0
Metrapone	1.0 mM	0	1.16 mM	52.2
Iodoacetamide	1.25 mM	0	ND	
<i>N</i> -Ethylmaleimide	1.25 mM	2	ND	
Sodium <i>p</i> -hydroxymercuribenzoate	0.2 mM	94.1	0.24 mM	97.4
Sodium mersalyl	0.5 mM	96.4	ND	
Mercaptoethanol	0.5 mM	67.3	ND	

Determination of the reactants consumed and products formed (Table 4) showed that the stoichiometry of the two oxidations is

Some differences were observed in the sensitivity of the two enzymes to different inhibitors (Table 5). In addition to the compounds listed, divalent cations at 0.1 mM (Co^{2+} , Fe^{2+} , Mn^{2+} and Zn^{2+} – all potent inhibitors of bacterial dimethylamine mono-oxygenase) failed to inhibit the enzyme. The only inhibitors to be examined kinetically were carbon monoxide and potassium cyanide. CO inhibited both dimethylamine and trimethylamine oxidation

uncompetitively with K_i values of respectively 7.5 μM and 0.35 μM . Cyanide inhibition was also uncompetitive with K_i values of 0.9 mM and 0.8 mM, respectively.

The (NADH-reduced minus oxidized) difference spectrum of the enzyme preparation showed absorption maxima at 444 nm and 426 nm. Addition of excess dimethylamine bleached these peaks after 5 min. The (dithionite-reduced-plus-CO minus reduced) difference spectrum had a peak at 422 nm and a shoulder at 446 nm.

DISCUSSION

Previous studies on yeasts capable of oxidizing secondary and tertiary amines (Yamada *et al.*, 1976) failed to detect any enzyme activity in cell-free extracts. The key to the discovery of the two amine mono-oxygenases was the addition of FAD to the cell-breakage buffer. FAD appeared to have two effects in that it partially stabilized the amine mono-oxygenase activities as well as stimulating them. Supplementation of dimethylamine mono-oxygenase with FAD never resulted in the same level of activity as that obtained when FAD was present in the cell-breakage buffer, indicating some irreversible inactivation of the 'apoenzyme'. FMN could replace FAD, but it was not as effective. The addition of FAD to a number of analogous systems such as the alkyl arylamine mono-oxygenase of pig-liver microsomes (Pettit *et al.*, 1964) and the ω -hydroxylase system of *Saccharomycopsis lipolytica* (Delaissé *et al.*, 1981) stimulates or reactivates their respective activities. These observations suggest that FAD (and/or FMN) are probably constituents of the secondary and tertiary amine mono-oxygenase systems of yeasts. Other enzymes capable of oxidizing trimethylamine or dimethylamine, such as dehydrogenases (Colby & Zatman, 1973; Meiberg & Harder, 1978) and oxidases (Tipton, 1980) were not detected in *C. utilis* or *C. boidinii*.

No significant differences have been found between the amine mono-oxygenase systems of *C. utilis* and the previously studied systems of *C. boidinii* (Green & Large, 1983*a*, 1984) except that there are higher levels of enzyme activity in *C. utilis* (Green & Large, 1983*a*). The data presented here show that the amine mono-oxygenases are almost certainly located in the smooth endoplasmic reticulum, as they are found in the microsomal fraction (P_5) along with NADPH-cytochrome *c* reductase on disruption of yeast sphaeroplasts.

The stoichiometry of trimethylamine and dimethylamine oxidation is in accordance with that of a mono-oxygenase (mixed-function oxidase), equations (1) and (2). Of the products of the reactions, methylamine is oxidized by methylamine oxidase and formaldehyde is degraded by a glutathione-dependent formaldehyde dehydrogenase (Green & Large, 1984). The degradation products of trimethylamine and *N*-methylbutylamine were in agreement with the results obtained by Yamada *et al.* (1976) with whole-cell suspensions. Methylamine oxidation and the removal of the resulting H_2O_2 by catalase take place in the peroxisomes. It may be presumed that the oxidation of formaldehyde takes place in the cytosolic fraction of the cell (Sahm *et al.*, 1975; Tanaka *et al.*, 1976; Veenhuis *et al.*, 1983) and it is thus evident that the process of obtaining nitrogen from methylated amines must involve a good deal of shuttling of metabolites from one subcellular compartment to another (Large & Green, 1984). This is a common phenomenon where peroxisomes are involved in metabolic pathways (see Huang *et al.*, 1983).

The K_m for oxygen of the dimethylamine mono-oxygenase was too low to be measured. The very low K_m values for oxygen of the amine mono-oxygenases and methylamine oxidase (Haywood & Large, 1981) allow methazotrophic yeasts to utilize amines as nitrogen source in almost anaerobic conditions, in contrast to the situation of growth of yeasts on methanol, which is very slow at low oxygen concentrations (Veenhuis *et al.*, 1983).

The two main pieces of evidence indicating that two separate mono-oxygenases are required for the oxidation of trimethylamine and dimethylamine are (a) the different stability of the two activities (Fig. 2) and (b) their different sensitivity to inhibition by carbon monoxide, as shown by a 20-fold difference in K_i (see text) and a 90-fold difference in partition constant in whole cells (Green & Large, 1983*a*).

The substrate specificity observed by us agrees with the incomplete whole cell data of Yamada *et al.* (1976). Generally when we compare the yeast system with the dimethylamine mono-

oxygenase of *Pseudomonas aminovorans* (Eady *et al.*, 1971) and the trimethylamine mono-oxygenase of the same bacterium (Boulton *et al.*, 1974), we see a broader specificity in the yeast enzymes, which show activity with amines that contain two or three alkyl groups with a carbon chain length of C₃ or greater, and a lack of substrate inhibition which was found with both bacterial enzymes at amine concentrations above 1 mM. Bacterial trimethylamine mono-oxygenase is an N-hydroxylase rather than a C-hydroxylase (Bickel, 1971) and so forms trimethylamine N-oxide as product rather than dimethylamine and formaldehyde (Boulton *et al.*, 1974). Consequently the two trimethylamine mono-oxygenases show quite different properties, most importantly the insensitivity of the bacterial enzyme to carbon monoxide, cyanide and proadifen hydrochloride. Differences between the bacterial and yeast amine mono-oxygenases are further discussed by Large & Green (1984).

Since trimethylamine N-oxide is probably not an intermediate in trimethylamine degradation in yeasts, and since no enzyme (reductase or demethylase) has been detected which can catalyse its metabolism, even in yeasts grown on trimethylamine N-oxide as nitrogen source, its metabolism remains to be clarified. Similar negative results were obtained by Yamada *et al.* (1976). It seems probable that it must first be reduced to trimethylamine and then demethylated via the two mono-oxygenases described here. N-Oxide reductases are difficult enzymes to study, since they are often inhibited by oxygen at relatively low concentrations (Hlavica, 1982).

When secondary amines containing both methyl and other alkyl groups are oxidized by yeast dimethylamine mono-oxygenase, the aldehyde products arise from both alkyl groups, as observed also with the bacterial enzyme (Eady *et al.*, 1971). There is some evidence that the primary amine product may be matched to the substrate 'preferences' of methylamine oxidase.

The spectral studies, performed with partially purified dimethylamine mono-oxygenase from *C. utilis* should be treated cautiously, since there are a number of other microsomal activities present in the preparations. The absorption peaks (at 426 nm and 444 nm) in the (reduced minus oxidized) difference spectrum and in the (reduced-plus-CO minus reduced) difference spectrum (at 422 nm) provided little evidence for the presence of a cytochrome P-450 component. However, the extreme CO sensitivity of the system suggests that such an entity may be involved. The failure to detect cytochrome P-450 in *C. utilis* is not new (Kärenlampi *et al.*, 1980) and it may be that a cytochrome P-420, similar to that found in the bacterial secondary amine mono-oxygenase (Eady *et al.*, 1971), is in fact an active cytochrome and not merely denatured cytochrome P-450, as has been widely accepted. The shift and enhancement of the 426 nm peak to 422 nm in the spectrum of the reduced carbon monoxide-treated sample is very similar to observations made with the secondary-amine mono-oxygenase of *Pseudomonas aminovorans* (Eady *et al.*, 1971), where evidence of an active P-420 cytochrome is very good (Brook & Large, 1975).

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