Dimethylamine Dehydrogenase from *Hyphomicrobium* X: Purification and Some Properties of a New Enzyme that Oxidizes Secondary Amines

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Dimethylamine dehydrogenase was purified 15.6-fold from *Hyphomicrobium* X grown anaerobically on dimethylamine as sole carbon source by ammonium sulphate fractionation and chromatography on DEAE-cellulose. The preparation was free from trimethylamine dehydrogenase. The molecular weight of the enzyme was 176 000 and subunit analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis indicated that it consists of two, probably identical, subunits with molecular weights of 91 000. The absorption spectrum showed a maximum at 441 nm. Reduction of the enzyme with dimethylamine produced a new absorption maximum at 356 nm, while the absorption at 441 nm decreased. The pH optimum for the oxidation of dimethylamine was 8.1. In this reaction, stoichiometric amounts of methylamine and formaldehyde were formed as products. The enzyme showed absolute specificity towards secondary amines; dimethylamine, methylethylamine, diethylamine, methylpropylamine, ethylpropylamine and methylethanolamine were oxidized while primary and tertiary amines and quaternary ammonium salts were not. Apart from phenazine methosulphate, only phenazine ethosulphate, Wurster's blue and methylene blue served as artificial electron acceptors. The apparent K_m of the enzyme for dimethylamine at pH 7.7 was $15.6 \pm 1.6 \mu\text{M}$. Trimethylamine was a potent competitive inhibitor of dimethylamine oxidation with an apparent K_i of $7.1 \mu\text{M}$. This inhibition of dimethylamine dehydrogenase by trimethylamine probably explains the observed accumulation of dimethylamine during anaerobic growth of *Hyphomicrobium* X on trimethylamine.

INTRODUCTION

Earlier reports on the oxidation of dimethylamine in aerobic methylotrophic microorganisms have implicated a secondary amine mono-oxygenase (EC 1.14.99.–) as a key enzyme. This enzyme was first reported in *Pseudomonas aminovorans* by Eady & Large (1969) and subsequently detected in other organisms capable of growth on methylated amines (Myers & Zatman, 1971; Colby & Zatman, 1973). Among the hyphomicrobia that can utilize tertiary and secondary methylated amines, *Hyphomicrobium vulgare* NQ (Eady *et al.*, 1971) and *H. vulgare* ZV (Loginova *et al.*, 1976) were found to contain dimethylamine mono-oxygenase.

Recently, it was shown that during anaerobic growth of *Hyphomicrobium* X on trimethylamine and dimethylamine in the presence of nitrate, dimethylamine is oxidized by a dimethylamine dehydrogenase (Meiberg & Harder, 1978). The evidence indicated that this was a new enzyme. There have been previous reports on dimethylamine dehydrogenase activity, but these refer to the ability of purified trimethylamine dehydrogenase to oxidize the substrate at low rates (Colby & Zatman, 1974; Steenkamp *et al.*, 1978*d*; Meiberg & Harder, 1978).

In the present paper, the general properties of a partially purified dimethylamine dehydrogenase from *Hyphomicrobium* X are described. Special attention is paid to those kinetic parameters which might explain the rather complicated pattern of substrate utilization which occurs during anaerobic growth of this organism on trimethylamine (Meiberg & Harder, 1978).

METHODS

Maintenance and growth of the organism. These have been described previously (Meiberg & Harder, 1978).

Purification of dimethylamine dehydrogenase. This procedure was essentially as described by Meiberg & Harder (1978), but with some modifications to improve the separation of dimethylamine dehydrogenase from the contaminating trimethylamine dehydrogenase. The active ammonium sulphate fraction (50 to 80 % saturation) was dialysed and then applied to a column of DEAE-cellulose (Whatman, DE52). Protein was eluted with a linear salt gradient of 0 to 0.3 M-NaCl in 20 mM-sodium phosphate buffer, pH 7.5 (a total volume of 400 ml); the flow rate was 10 ml h⁻¹. The fractions containing dimethylamine dehydrogenase activity without any trimethylamine dehydrogenase were pooled and concentrated by ultrafiltration.

Enzyme assays. Dimethylamine dehydrogenase was measured with an anaerobic spectrophotometric assay (Meiberg & Harder, 1978) which was slightly modified for optimal results. The reaction mixture contained (in 1 ml) 75 µmol sodium pyrophosphate buffer pH 7.7, 0.10 µmol 2,6-dichlorophenolindophenol (DCPIP), 2.5 µmol phenazine methosulphate (PMS) and purified enzyme; the reaction was started by adding 3 µmol dimethylamine. In the kinetic experiments, the concentration of one reactant was changed, while the concentrations of the other reactants were kept constant.

Oxygen-uptake was measured with a Clark-type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co., U.S.A.). The air-saturated assay mixture was similar to that described before, except that the total volume in the electrode chamber was 3 ml and DCPIP was omitted from the reaction mixture.

Trimethylamine dehydrogenase (EC 1.5.99.7) was measured spectrophotometrically in a reaction mixture similar to that used for the dimethylamine dehydrogenase, but with 3 mM-trimethylamine as the substrate.

Catalase (EC 1.11.1.6) was measured according to the procedure of Lück (1963).

Malate dehydrogenase (EC 1.1.1.37) was assayed as described by Bergmeyer (1974).

Molar absorption coefficients of electron acceptors. These were taken as: DCPIP at 600 nm and at pH 7.7, 21.69×10^3 M⁻¹ cm⁻¹ (Armstrong, 1964); reduced cytochrome *c* at 550 nm, 21.0×10^3 M⁻¹ cm⁻¹, NADH and NADPH at 340 nm, 6.22×10^3 M⁻¹ cm⁻¹, and K₃Fe(CN)₆ at 400 nm, 1.02×10^3 M⁻¹ cm⁻¹ (Colby & Zatman, 1974); Wurster's blue at 612 nm, 8.29×10^3 M⁻¹ cm⁻¹.

Molecular weight determination. The molecular weight of the dimethylamine dehydrogenase was estimated by gel filtration on Sephadex G-200 (Andrews, 1970). Ferritin (540 000 mol. wt), catalase (240 000), bovine serum albumin dimer (136 000) and monomer (68 000), malate dehydrogenase (70 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and cytochrome *c* (12 500) were used as standards.

Polyacrylamide gel electrophoresis. Electrophoresis was performed on 7.0 % (w/v) acrylamide gels in Tris/HCl buffer pH 8.9 according to the procedure of Davis (1964). Electrophoresis was conducted at room temperature at 4 mA per tube for 1.5 h. Proteins were stained in 0.25 % (w/v) Coomassie blue in 45.4 % (v/v) methanol/0.92 % (v/v) acetic acid for 1 h and destained in 5 % (v/v) methanol/7.5 % (v/v) acetic acid.

Enzyme activity in the gels was detected by coupling the oxidation of substrates with the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). The reaction mixture contained 0.5 ml 0.15 M-sodium pyrophosphate buffer pH 7.7, 1 ml 0.1 % (w/v) MTT, 0.1 ml 150 mM-dimethylamine or trimethylamine, gel and water up to 5 ml. Incubation was at room temperature for 5 to 15 min in the dark.

Sodium dodecyl sulphate (SDS)-gel electrophoresis was performed using 7.5 % (w/v) acrylamide gels in the presence of 0.1 % (w/v) SDS according to the procedure of Weber *et al.* (1972). The enzyme preparation and other protein samples which were used as molecular weight markers were incubated in 0.01 M-sodium phosphate buffer pH 7.0, containing 1 % (w/v) SDS and 1 % (w/v) 2-mercaptoethanol at 100 °C for 2 min prior to electrophoresis. The subunit molecular weights of the marker proteins were: cytochrome *c*, 12 500; aldolase, 40 000; catalase, 58 000; bovine serum albumin, 68 000; β-galactosidase (*Escherichia coli*), 130 000.

Absorption spectra. These were measured in an Aminco-Chance model DW-2 spectrophotometer, operating in the split-beam mode. The enzyme preparation was dissolved in air-saturated 75 mM-sodium pyrophosphate buffer pH 7.7. Additional oxidation of the preparation was allowed by saturating the enzyme solution with air. Subsequent reduction of the enzyme was performed by adding dimethylamine or sodium dithionite.

Estimation of methylated amines. These were measured as described previously (Meiberg & Harder, 1978) with the following modifications: (i) a Packard gas chromatograph model 427 was used; (ii) a soda-lime

pre-column was fitted in the injection block at a temperature of 180 °C in order to release the free amines from their salts.

Estimation of formaldehyde. It was difficult to estimate formaldehyde in the dimethylamine dehydrogenase reaction mixture in studies on the stoichiometry of the reaction because of interference by PMS or insensitivity of the various methods. For these reasons, neither the colorimetric method of Nash (1953) nor enzymic assays with alcohol oxidase from *Candida boidinii* or alcohol dehydrogenase from yeast could be used. However, the colorimetric method described by Colby & Zatman (1974) gave reproducible results provided that the time of colour development was kept constant.

Chemicals. *N*-Methylethylamine hydrochloride was obtained from Eastman Kodak Co., U.S.A. *N,N*-Diethylmethylamine and *N*-ethylmethylamine were from Fluka, Buchs, Switzerland. *N*-Methyl-*n*-propylamine, *N*-ethyl-*n*-propylamine and *N*-methyl-*n*-butylamine were gifts from Dr P. J. Large, University of Hull. *N,N*-Dimethylethanolamine and unsymmetrical dimethylethylenediamine were from Aldrich Europe, Belgium. All other amine compounds were from Merck. The purity of the substrates was checked in the gas chromatograph under the conditions described previously. In all cases, except methylethylamine, the amount of contaminating substrates present was negligible and could not account for the activities recorded; methylethylamine contained significant amounts of non-oxidizable ethylamine. Biochemicals and enzymes were from Boehringer, Wurster's blue, prepared as described by Michaelis & Granick (1943), was a gift from Dr J. A. Duine, Technische Hogeschool, Delft, The Netherlands.

RESULTS

Partial purification of dimethylamine dehydrogenase

Recently, Meiberg & Harder (1978) reported the partial purification of trimethylamine and dimethylamine dehydrogenases from *Hyphomicrobium X*. However, complete separation of the two enzymes was not obtained. An improved procedure for the purification of the dimethylamine dehydrogenase, as described in Methods, was developed and resulted in an enzyme preparation which was devoid of trimethylamine oxidizing activity. A summary of the enzyme purification is presented in Table 1. A 14-fold purification was achieved by ammonium sulphate fractionation followed by DEAE-cellulose column chromatography. When the concentrated enzyme preparation was kept at 0 °C for 14 d, a protein precipitate developed which was discarded. The final enzyme preparation, which was purified 15.6-fold, had a yellow-brown colour. The recovery was 43 %.

The purity of the enzyme was checked by polyacrylamide disc gel electrophoresis, using 7% acrylamide in Tris/HCl buffer pH 8.9. The partially purified enzyme (80 µg protein) gave one major and three minor bands. Only the major band showed dimethylamine dehydrogenase activity; the R_f value of the enzyme was 0.51. On the basis of the above polyacrylamide gel electrophoresis, impurities in the preparation were estimated to account for less than 10% of the total protein applied to the gel. The preparation was essentially free from trimethylamine dehydrogenase, methanol dehydrogenase and catalase and was therefore suitable for stoichiometric and kinetic studies.

Properties of dimethylamine dehydrogenase

Effect of freezing on stability. Freezing of the enzyme preparation in liquid nitrogen resulted in a complete loss of activity. Attempts to stabilize the enzyme in 20% (w/v) glycerol or 80% ammonium sulphate were unsuccessful. After freezing once, the activity of the enzyme was decreased to 40% of its initial activity. Storage at 0 °C for 3 months resulted in the loss of 50% of the initial activity. However, similar to the observations made with trimethylamine dehydrogenase (Steenkamp & Mallinson, 1976), dimethylamine dehydrogenase was stabilized by ethylene glycol. Enzyme preparations kept at -10 °C in the presence of 20% (v/v) ethylene glycol retained full activity for at least 3 months.

Molecular weight and subunit molecular weight. The molecular weight of the dimethylamine dehydrogenase, as determined by gel filtration, was 176000. When the enzyme was treated with 1% SDS in 0.01 M-sodium phosphate buffer pH 7.0 and subjected to SDS-polyacrylamide gel electrophoresis, a single protein band was observed. The relative electrophoretic mobility of the protein corresponded to a subunit with a molecular weight of

Table 1. *Purification of dimethylamine dehydrogenase from Hyphomicrobium X*

Total activities are expressed as nmol substrate transformed min⁻¹ and specific activities as nmol substrate transformed min⁻¹ (mg protein)⁻¹.

Fraction	Vol. (ml)	Total protein (mg)	Total activity	Specific activity	Recovery (%)	Purification factor
Crude cell-free extract	25	245.5	5739.8	23.4	100	1
Ammonium sulphate fraction 50 to 80 % saturation	3.7	125.7	4741.4	37.7	82.6	1.6
DEAE-cellulose eluate*	9.95	8.0	2616.0	327.0	45.6	14.0
Concentrated DEAE-cellulose eluate after 14 d at 0 °C	9.8	6.8	2473.8	363.8	43.1	15.6

* Assayed after concentration by ultrafiltration.

Fig. 1. Absorption spectrum of dimethylamine dehydrogenase. The enzyme concentration was 0.16 mg protein ml⁻¹ in 75 mM-sodium pyrophosphate buffer pH 7.7: curve A, oxidized enzyme; curve B, after addition of 3 mM-dimethylamine; curve C, after addition of a crystal of solid sodium dithionite to B.

91 000. This value is half the molecular weight of the native enzyme, suggesting that the dimethylamine dehydrogenase consists of two subunits of equal size.

Absorption spectrum. The visible spectrum of dimethylamine dehydrogenase in air-saturated buffers is shown in Fig. 1. The oxidized enzyme had an absorption maximum at 441 nm (curve A). Addition of dimethylamine to the purified enzyme caused a decrease in the absorption at 441 nm and produced an absorption peak at 356 nm (curve B). The peak at 441 nm was bleached completely by the addition of solid sodium dithionite (curve C).

Effect of pH on activity. The pH optimum curve for enzyme activity exhibited a symmetrical pattern. The optimum pH in 75 mM-sodium pyrophosphate buffer was 8.1. The behaviour of the enzyme in other buffer systems was not tested. At pH values above 7.7, the non-enzymic reduction of DCPIP became substantial and made the measurement of initial rates difficult. For this reason, subsequent experiments were done at a suboptimal pH of 7.7. For the calculation of the enzyme activity, the dependence of the molar absorption coefficient of DCPIP on pH (Armstrong, 1964) was taken into account.

Influence of PMS and DCPIP on enzyme activity. PMS could serve as an artificial primary electron acceptor for the dimethylamine dehydrogenase. The initial velocity of the enzyme reaction was maximal at 2.4 mM-PMS; above this concentration, PMS inhibited enzyme

Fig. 2. Substrate consumption and product formation by dimethylamine dehydrogenase. The complete reaction mixture (total vol. 0.4 ml) contained 30 μ mol sodium pyrophosphate buffer pH 7.7, 1 μ mol PMS, 28 μ g enzyme protein and 0.8 μ mol dimethylamine. $\circ$, Dimethylamine; $\bullet$, methylamine; $\square$, formaldehyde.

activity. The apparent K_m for PMS was 1.18 mM at pH 7.7. At the optimum PMS concentration, the enzyme activity at various DCPIP concentrations showed a saturation curve with maximum activity at 100 μ M. This concentration was used for further studies.

Product identification. Incubation of dimethylamine dehydrogenase with dimethylamine as substrate and PMS as electron acceptor resulted in a disappearance of dimethylamine with concurrent production of methylamine and formaldehyde (Fig. 2). Dimethylamine (0.80 μ mol) was quantitatively converted into 0.81 μ mol methylamine and 0.84 μ mol formaldehyde. In oxygen-uptake assays (performed in the presence of catalase) the oxidation of 0.6 μ mol dimethylamine corresponded to an oxygen consumption of 0.29 μ mol. These results are consistent with the following stoichiometry of dimethylamine oxidation by dimethylamine dehydrogenase:

Substrate specificity. The purified dimethylamine dehydrogenase was highly specific for secondary amines as substrate (Table 2). Neither primary nor tertiary amines nor quaternary ammonium compounds were oxidized. Oxidation of secondary methylalkylamines was limited to dimethyl-, methylethyl- and methylpropylamine. Increased chain length of the alkyl group in methylalkylamines caused a decrease in activity. The highest activity was observed with dimethylamine, the lowest with methylpropylamine, whereas methylbutylamine was not oxidized at all. The gradual decrease in activity with increase in chain length suggests an increasing steric hindrance for the enzyme to oxidize methyl groups. The enzyme showed strong preference for oxidation of methyl groups because oxidation of methylethylamine yielded ethylamine; formation of methylamine as a result of the oxidation of the ethyl group of methylethylamine was not observed. However, since the enzyme showed a high activity with diethylamine as a substrate it is clearly capable of oxidizing ethyl groups. The enzyme reacted slowly with ethylpropylamine. Dipropylamine did not serve as a substrate for the enzyme, indicating that dimethylamine dehydrogenase may be restricted to oxidative demethylation or de-ethylation of secondary amines.

Table 2. *Substrate specificity of dimethylamine dehydrogenase*

Substrate solutions were adjusted to pH 7.7 before addition. Each compound was tested at two concentrations (1 and 10 mM); the concentration given in parentheses gave the maximum rate. Activities are expressed as a percentage of the activity obtained with 1 mM-dimethylamine.

Substrate	Relative activity
Dimethylamine (1 mM), CH_3NHCH_3	100
Methylethylamine (1 mM), $\text{CH}_3\text{NHCH}_2\text{CH}_3$	92.1
Diethylamine (1 mM), $\text{CH}_3\text{CH}_2\text{NHCH}_2\text{CH}_3$	78.3
Methylpropylamine (1 mM), $\text{CH}_3\text{NHCH}_2\text{CH}_2\text{CH}_3$	12.9
Ethylpropylamine (10 mM), $\text{CH}_3\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_3$	6.8
Methylethanolamine (1 mM), $\text{CH}_3\text{NHCH}_2\text{CH}_2\text{OH}$	54.2

The following compounds were not oxidized: methylamine, trimethylamine, tetramethylammonium chloride, ethylamine, triethylamine, tetraethylammonium chloride, propylamine, dipropylamine, dibutylamine, dimethylethylamine, methyldiethylamine, methylbutylamine, ethanolamine, diethanolamine, triethanolamine, dimethylethanolamine, dimethylformamide.

Table 3. *Effect of alternative electron acceptors on dimethylamine dehydrogenase activity*

Reactions were recorded with the anaerobic spectrophotometric assay or the oxygen-uptake assay. Activities were calculated from the rate of reduction of the terminal electron acceptor and are expressed as a percentage of the activity obtained with PMS/DCPIP at 600 nm.

Acceptor	Concn (mM)	Relative activity
Spectrophotometric assay		
Wurster's blue	0.3	73.8
PMS (+0.1 mM-DCPIP)	2.5	100
PMS (+0.1 mM-cytochrome <i>c</i>)	2.5	77.6

The following electron acceptors were not active: potassium ferricyanide, cytochrome *c* (horse heart), cytochrome c_{550} (*Hyphomicrobium* X)*, DCPIP, NAD^+ and NADP^+ .

Oxygen-uptake assay		
PMS†	2.5	140.1
PES† (phenazine ethosulphate)	2.5	103.4
Methylene blue†	10	17.2

No enzyme-dependent oxygen consumption could be measured with O_2 (air saturation), menadione, FAD, benzyl viologen and methyl viologen.

* Cytochrome c_{550} was isolated from *Hyphomicrobium* X as described by Large *et al.* (1979).

† Catalase (13000 units) was included in the reaction mixture.

Specificity of dimethylamine dehydrogenase for electron acceptors. Several potential electron acceptors were tested in the spectrophotometric or oxygen-uptake assays (Table 3). Only PMS, PES, Wurster's blue and, to a lesser extent, methylene blue served as primary electron acceptors. Horse heart cytochrome *c* could replace DCPIP or oxygen as secondary electron acceptor. Cytochrome *c* alone (either from horse heart or from *Hyphomicrobium* X) was unable to oxidize the reduced enzyme (Large *et al.*, 1979). The apparent K_m for Wurster's blue was 0.11 mM.

Influence of trimethylamine and reaction products on dimethylamine oxidation by dimethylamine dehydrogenase. Trimethylamine is a potent inhibitor of dimethylamine dehydrogenase activity. Double-reciprocal plots of dimethylamine concentration against initial velocity with different trimethylamine concentrations in the reaction mixture showed competitive inhibition of dimethylamine dehydrogenase by trimethylamine (Fig. 3). An apparent K_i value for trimethylamine of $7.1 \pm 1.3 \mu\text{M}$ was calculated from the slopes of the different plots. The apparent K_m for dimethylamine at pH 7.7 in the absence of trimethylamine was estimated to be $15.6 \pm 1.6 \mu\text{M}$.

Dimethylamine at concentrations up to 3.0 mM was not inhibitory for the enzyme, but at 10 mM it caused 23 % inhibition. Freshly prepared formaldehyde did not inhibit dimethyl-

Fig. 3. Inhibition of dimethylamine dehydrogenase by trimethylamine. Reciprocal values of the velocity of the enzyme reaction were plotted against the reciprocal dimethylamine concentration at three concentrations of trimethylamine: ●, 30 μM ; □, 60 μM ; ■, 90 μM ; ○, no inhibitor.

amine dehydrogenase even at unphysiological concentrations of 20 mM. Methylamine at 1 mM did not alter the enzyme activity significantly; at higher concentrations (6 and 17.5 mM) it was slightly inhibitory.

DISCUSSION

Hyphomicrobium X is able to grow anaerobically on trimethylamine and dimethylamine in the presence of nitrate as the terminal electron acceptor. Under these conditions one would not expect the organism to have a functional secondary amine mono-oxygenase. We therefore attempted to detect an enzyme system responsible for the anaerobic conversion of dimethylamine to methylamine and formaldehyde. Indeed, using the assay system of Colby & Zatman (1973) for the estimation of trimethylamine dehydrogenase, we were able to detect a dimethylamine-dependent reduction of PMS/DCPIP (Meiberg & Harder, 1976, 1978), and showed that this activity was not due to a dual substrate specificity of the trimethylamine dehydrogenase. The results reported in this paper show that dimethylamine dehydrogenase is a new enzyme. It is not, however, the only dehydrogenase capable of oxidizing dimethylamine, since the trimethylamine dehydrogenases of organisms 4B6 and W3A1 (Colby & Zatman, 1974; Steenkamp *et al.*, 1978*d*) can also oxidize dimethylamine, albeit at a low rate. For instance, the enzyme from bacterium 4B6 exhibited a dimethylamine oxidation rate which was only 5% of the rate with trimethylamine. No pertinent data have been reported for trimethylamine dehydrogenase from bacterium W3A1, but it was stated that the rate of reduction of the enzyme with dimethylamine was much slower than with trimethylamine as the substrate (Steenkamp *et al.*, 1978*d*). The same, relatively poor, kinetic behaviour with dimethylamine was observed with a partially purified preparation of trimethylamine dehydrogenase from *Hyphomicrobium X* (Meiberg, 1979). This virtually ruled out a possible role of trimethylamine dehydrogenase in dimethylamine oxidation and it emphasizes the importance of dimethylamine dehydrogenase in *Hyphomicrobium X* during anaerobic growth on trimethylamine and dimethylamine.

Apart from the difference in kinetic properties, the enzyme resembles the trimethylamine dehydrogenases known so far in many respects. It catalyses the same type of reactions, namely the oxidative *N*-dealkylation (demethylation) of a secondary amine with the formation of stoichiometric amounts of free (form)aldehyde and primary amine (Fig. 2), and

its kinetics for PMS and DCPIP are similar to that of trimethylamine dehydrogenase (Colby & Zatman, 1974; Meiberg, 1979).

The absorption spectrum of the dimethylamine dehydrogenase is identical to the spectrum of the trimethylamine dehydrogenase of bacterium W3A1 (Steenkamp & Mallinson, 1976) and the trimethylamine dehydrogenase of *Hyphomicrobium* X (Meiberg, 1979). The spectral changes that occur after reduction of the W3A1 enzyme by trimethylamine or dithionite have been studied extensively by Steenkamp and co-workers. They found that the enzyme contains an unusual covalently bound flavin, 6-*S*-cysteinyl FMN (Steenkamp *et al.*, 1978*a, b*) and a single tetrameric iron-sulphur centre (Hill *et al.*, 1977). On the basis of our kinetic studies we cannot draw conclusions about the reaction mechanism, but it is tempting to consider the possibility that the reaction mechanism of dimethylamine dehydrogenase is similar to that described for trimethylamine dehydrogenase by Steenkamp *et al.* (1978*c, d*). It is thus possible that the enzyme is representative of the group of dissimilatory enzymes, common among methylotrophic micro-organisms, which contain unusual cofactors (Anthony & Zatman, 1967; Eady & Large, 1968; Duine *et al.*, 1978; Steenkamp *et al.*, 1978*b*). Of the artificial electron acceptors tested, only PMS, PES, methylene blue and Wurster's blue were active (Table 3). Wurster's blue has also been reported as a one-electron acceptor for the methanol dehydrogenase of *Hyphomicrobium* X (Duine *et al.*, 1978) and the mono-methylamine dehydrogenase of *Pseudomonas* AM1 (Eady & Large, 1968). It is the stable oxidized free-radical form of tetramethyl-*p*-phenylenediamine and has a redox potential of +0.263 V (Michaelis & Hill, 1933) at pH 7.0. Dimethylamine dehydrogenase had a relatively low apparent K_m for this compound as compared to PMS. The dye inhibited enzyme activity at concentrations higher than 0.4 mM. Except for Wurster's blue, the E'_0 values of the other active electron acceptors are in the range of +0.011 to +0.08 V. These data agree with the results of Colby & Zatman (1974) with purified trimethylamine dehydrogenase from bacterium 4B6. These authors argue that 'the natural electron acceptor has an E'_0 of about or just above zero and, although FAD and FMN were not active as primary electron acceptors, it is quite possible that the natural acceptor is a flavoprotein, because some flavoproteins have E'_0 values of about zero'. Indeed, very recently Steenkamp & Gallup (1978) reported the isolation and partial characterization of a flavoprotein which probably functions as the electron acceptor *in vivo* for the trimethylamine dehydrogenase of bacterium W3A1. At present it is not known whether such a flavoprotein also acts as the natural electron acceptor for dimethylamine dehydrogenase. In this connection it is of interest to note that cytochrome c_{co} is unable to accept the electrons from the oxidation of dimethylamine (Large *et al.*, 1979).

Dimethylamine dehydrogenase has an absolute specificity for secondary amines as substrates (Table 2). This behaviour is similar to that of the secondary amine mono-oxygenase of *Pseudomonas aminovorans* (Eady *et al.*, 1971) which oxidizes the same range of substrates. Kinetic experiments showed a potent competitive inhibition of dimethylamine oxidation by trimethylamine (Fig. 3). This suggests that the enzyme, although not capable of oxidizing trimethylamine, has a high affinity for this compound, a phenomenon analogous to the inhibition of trimethylamine oxidation by tetramethylammonium chloride (Colby & Zatman, 1974). This inhibition by trimethylamine most probably explains the accumulation of dimethylamine during anaerobic growth of *Hyphomicrobium* X on trimethylamine. During growth of the organism under denitrifying conditions an almost stoichiometric conversion of trimethylamine into dimethylamine was observed, despite the presence of dimethylamine dehydrogenase in the cells (Meiberg & Harder, 1978). This is probably a result of the initial high trimethylamine concentration in the culture completely inhibiting the oxidation of dimethylamine formed by the action of trimethylamine dehydrogenase. As growth proceeds, the simultaneous decrease in trimethylamine concentration and increase in dimethylamine concentration may then release the inhibition of dimethylamine dehydrogenase by trimethylamine and dimethylamine oxidation starts.

This hypothesis clearly hinges on the assumption that the dimethylamine dehydrogenase *in vivo* is in fact exposed to trimethylamine concentrations sufficiently high to cause inhibition. For several reasons (Meiberg, 1979) it appears unlikely that this condition can be reconciled with the possibility that both dehydrogenases are located in the cytoplasm. Although upon extraction by sonication both enzymes were found in the soluble fraction, preliminary results of cytochemical staining experiments suggested that they were membrane-bound. Not only the cytoplasmic membrane but also membranes which are dispersed throughout the cell's interior were shown to contain trimethylamine and dimethylamine dehydrogenase activities. It is almost certain that these membranes are invaginations of the cytoplasmic membrane (Meiberg, 1979). Similar cellular membrane invaginations have been found in *Hyphomicrobium B522* (Conti & Hirsch, 1965). However, in order to explain conclusively the *in vivo* substrate utilization during growth of *Hyphomicrobium X* on trimethylamine under anaerobic conditions by applying the observed inhibition kinetics, it remains to be elucidated whether the dimethylamine dehydrogenase is located in the outside or the inside of the membranes.

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