Microbial Oxidation of Amines

PARTIAL PURIFICATION OF A TRIMETHYLAMINE MONO-OXYGENASE FROM *PSEUDOMONAS AMINOVORANS* AND ITS ROLE IN GROWTH ON TRIMETHYLAMINE

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1. A mono-oxygenase, which oxidizes trimethylamine and other tertiary amines bearing methyl or ethyl groups, was partially purified sixfold from *Pseudomonas aminovorans* grown on trimethylamine as sole carbon source. 2. The preferred electron donor was NADPH. The enzyme had a pH optimum of 8.0–9.4 for trimethylamine oxidation, and 8.8–9.2 for dimethylamine oxidation. 3. The oxidation product of trimethylamine was shown to be trimethylamine *N*-oxide. Other tertiary amines were probably also converted into *N*-oxides. 4. The enzyme also oxidized secondary amines. 5. The oxidation of trimethylamine was only slightly inhibited by CO and not at all by KCN or proadifen hydrochloride (SKF 525-A), but was inhibited by trimethylsulphonium chloride, tetramethylammonium chloride, 2,4-dichloro-6-phenylphenoxyethylamine (Lilly 53325) and its *NN*-diethyl derivative (Lilly 18947). 6. The oxidation of dimethylamine showed a similar response to inhibitors and a parallel loss in activity on heating at 35°C. 7. The activities of the trimethylamine mono-oxygenase, trimethylamine *N*-oxide demethylase and the secondary-amine mono-oxygenase increased severalfold during adaptation of succinate-grown bacteria to growth on trimethylamine, and the trimethylamine mono-oxygenase was the first enzyme to show an increase in activity. It is concluded that all three enzymes are involved in growth on trimethylamine by this organism.

Pseudomonas aminovorans (den Dooren de Jong, 1927) grows on a wide range of compounds as sole carbon source. As its name implies, the bacterium utilizes a considerable number of amines as carbon source, including mono-, di- and tri-methylamine and trimethylamine *N*-oxide. It is thus a typical facultative methylotroph (Colby & Zatman, 1972). We have previously identified enzymes in this organism that catalyse (a) NAD(P)H-dependent oxidation of secondary amines to primary amines and aldehydes, and (b) the non-oxidative cleavage of trimethylamine *N*-oxide to dimethylamine and formaldehyde (Eady *et al.*, 1971; Large, 1971a).

In the present paper we describe some properties of an enzyme catalysing the oxygenation of trimethylamine and other tertiary amines bearing methyl or ethyl groups to their corresponding *N*-oxides (eqn. 1):

We propose the name trimethylamine mono-oxygenase for this enzyme. It is present in high activity in extracts of *Ps. aminovorans* grown on trimethylamine

as sole carbon source, but is barely detectable in cells grown on trimethylamine *N*-oxide, dimethylamine, methylamine or succinate (Jarman & Large, 1972). The activity is unstable, with a half-life of 11 min at 35°C (Jarman & Large, 1972), and its purification results in major losses in activity. The present paper describes a number of properties of the partially purified enzyme, and shows that it also catalyses the oxidation of secondary amines. We demonstrate the identity of the oxidation product of trimethylamine, and present evidence for the involvement of the enzyme in growth on trimethylamine. The enzyme shows some resemblances to the dimethylaniline mono-oxygenase (*N*-oxide-forming) of pig liver microsomal preparations (EC 1.14.13.8) studied by Ziegler and co-workers (Ziegler & Mitchell, 1972). A preliminary report of some of this work has been published (Large *et al.*, 1972).

Materials and Methods

Maintenance and growth of the organism

Stock cultures of *Ps. aminovorans* N.C.I.B. 9039 were maintained as described previously, with mineral-salt medium B used for growth in liquid culture (Eady *et al.*, 1971). The carbon source was

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trimethylamine hydrochloride, prepared by neutralizing a 25% (w/v) solution of the free base (Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.) with 11M-HCl and diluting to give a concentration in the culture medium of 0.25% (w/v) (expressed in terms of the free base). Other conditions of growth and harvesting of cells were as described previously by Eady *et al.* (1971). Cell material was stored in liquid N₂.

Measurement of bacterial growth

Growth was determined by measurement of turbidity at 570nm in a Unicam SP.600 spectrophotometer, previously calibrated in terms of dry wt. of cells/ml of suspension.

Preparation of cell-free extracts

Ultrasonic extracts of trimethylamine-grown cells were prepared as described by Eady *et al.* (1971).

Chemical determinations

(a) *Protein*. This was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (fraction V) as standard.

(b) *Trimethylamine*. This was determined chemically by a modification (Sass *et al.*, 1958) of Cromwell's (1950) *cis*-aconitic anhydride method, with standards of authentic trimethylamine hydrochloride. More usually, however, an enzymic method was used, similar in principle to that used by Large *et al.* (1969) for primary amines. The enzyme preparation was from *Hyphomicrobium vulgare* strain NQ-521 (N.C.I.B. 11052), which has trimethylamine dehydrogenase activity (Colby & Zatman, 1973). Crude ultrasonic extracts of trimethylamine-grown *H. vulgare* in 40mM-sodium-potassium phosphate buffer, pH7.5, were heated at 65°C for 20min. The precipitated protein was removed by centrifugation, and the supernatant was used as the source of trimethylamine dehydrogenase without further purification. The enzyme assay and amine determinations were done in 67mM-phosphate buffer, pH7.5, with other reactants as described by Colby & Zatman (1973). The extent of reduction of the dye (2,6-dichlorophenol-indophenol) was measured (1nmol of trimethylamine reduces 2nmol of dye). This method can be used for either trimethylamine or dimethylamine determination (in which case 1nmol of dimethylamine reduces 1nmol of dye); but the method cannot be applied to mixtures.

(c) *Oxygen*. This was measured with a Beckman model 777 Oxygen Analyser as described by Eady *et al.* (1971).

(d) *Formaldehyde*. This was determined by the acetylacetone method of Nash (1953).

(e) *Trimethylamine N-oxide*. This was determined by the method of Mitchell & Ziegler (1969), with standards of authentic material. In this method the *N*-oxide is converted into formaldehyde by treatment with SO₂ at 0°C. When it was necessary to determine trimethylamine *N*-oxide and formaldehyde in the same sample, the part of the sample used for formaldehyde determination was treated in an identical manner with the sample for *N*-oxide determination, except that the SO₂ treatment was omitted. It was found that the addition of the Mitchell & Ziegler (1969) reagents did not affect colour development in the Nash (1953) method, so that the trimethylamine *N*-oxide concentration could be obtained from the difference in the extinction at 412nm of the two samples.

(f) *Aliphatic aldehydes*. These were determined by the anthranilaldehyde method of Albrecht *et al.* (1962).

(g) *Dimethylamine*. This was determined by the method of Dubin (1960).

(h) *Methylamine*. This was determined by the enzymic method of Large *et al.* (1969).

Thin-layer and ion-exchange chromatography

Trimethylamine and trimethylamine *N*-oxide were separated by t.l.c. on cellulose (Camag DF-O, obtained from Griffin and George Ltd., Wythenshaw, Manchester M23 9NP, U.K.) with markers of authentic amines. The solvents used were butan-1-ol-formic acid (99%, w/v)-water (77:10:13, by vol.) and butan-1-ol saturated with aq. 25% (v/v) acetic acid (Baker & Chaykin, 1962). The *R_F* values in the first solvent were 0.56 for the *N*-oxide and 0.43 for trimethylamine, and in the second solvent 0.61 and 0.46 respectively. Spots were detected by spraying with 0.05% Bromocresol Green in ethanol (Dawson *et al.*, 1969). Separation by ion-exchange on Zeo-Karb 226 in citrate-phosphate buffer, pH5.0, was performed as described by Blau (1961).

Enzyme assays

The activity of the trimethylamine mono-oxygenase was measured by two methods.

(a) *Standard spectrophotometric assay*. Silica cuvettes (1.5ml capacity, 1cm light-path) contained: 100μmol of phosphate buffer, pH7.5 (or in some cases 8.0), 0.25μmol of NADPH, 1.5μmol of KCN, enzyme and water to a final volume of 1.5ml. The reaction was started by the addition of 1μmol of trimethylamine hydrochloride, and the reference cuvette contained all components except trimethylamine and NADPH. The oxidation of NADPH was measured at 340nm and 25°C in a Unicam SP. 1800 recording spectrophotometer; 1 unit of enzyme is the amount required to catalyse the oxidation of 1μmol of

NADPH/min under these conditions. KCN inhibits trimethylamine *N*-oxide demethylase and dimethylamine mono-oxygenase and was omitted when the purified trimethylamine mono-oxygenase was used. Cyanide-insensitive dimethylamine-oxidizing activity of the enzyme was measured at pH 8.0 with sodium-potassium phosphate buffer or at pH 8.5 with sodium pyrophosphate buffer and 10 μ mol of dimethylamine hydrochloride. The other components were identical with the trimethylamine-oxidizing assay, and cyanide was not omitted when the purified enzyme was used.

(b) *Formation of trimethylamine N-oxide.* The reaction mixture consisted of 2 μ mol of trimethylamine hydrochloride, 100 μ mol of phosphate or glycine-NaOH buffer (pH 7.5 or 8.0), 1 μ mol of NADPH (or 0.5 μ mol of NADP⁺ and an NADPH-generating system; see below), enzyme and water to 2 ml. The reaction was started by addition of trimethylamine, incubated at 25°C and stopped after various times with 0.2 ml of 2.5 M-HClO₄. Samples in duplicate were taken for trimethylamine *N*-oxide determination.

The CO- and cyanide-sensitive secondary-amine mono-oxygenase was assayed as described by Eady *et al.* (1971), except that the rates were usually corrected for CO-resistant activity at pH 6.8 by subtracting the rate measured in a separate cuvette, which had been bubbled vigorously for 15 s with CO after addition of extract and before addition of NADPH. In trimethylamine-grown extracts, 75% of the activity measured at pH 6.8 under these conditions was CO-sensitive.

Trimethylamine *N*-oxide demethylase activity was measured as described by Large (1971a).

NADPH-generating system

Where this was used, it consisted of 20 mM-glucose 6-phosphate (disodium salt), 0.25 mM-NADP⁺ and 0.75 unit of glucose 6-phosphate dehydrogenase/ml.

Purification of the trimethylamine mono-oxygenase

To a crude ultrasonic extract, prepared as described above, was added 0.2 vol. of ice-cold 10% (w/v) streptomycin sulphate with stirring. After 15 min at 0°C, the precipitate was removed (by centrifugation at 4°C, 20 min at 30000g) and the volume of the supernatant adjusted with 20 mM-sodium-potassium phosphate, pH 6.8, to 1.3–1.6 units/ml. Solid (NH₄)₂SO₄ was then added to a final concentration of 40% saturation at 0°C (Dawson *et al.*, 1969). After the suspension had been left for 15 min at 0°C, the precipitate was centrifuged (20 min, 30000g at 4°C) and discarded and further solid (NH₄)₂SO₄ added to give 50% saturation. The precipitate contained the cyanide-sensitive secondary-amine mono-oxygenase of Eady *et al.* (1971). The supernatant was then adjusted to 65% saturation with (NH₄)₂SO₄, and the precipitate

centrifuged as described above. The trimethylamine mono-oxygenase was present in this precipitate, which was redissolved in 5 ml of 50 mM-phosphate buffer, pH 6.8, and applied to a column (2.5 cm × 40 cm) of Sephadex G-150 equilibrated with 50 mM-phosphate buffer and eluted with the same buffer. The combined active fractions were applied to a column (1 cm × 10 cm) of hydroxyapatite (Bio-Rad Bio-Gel HTP) equilibrated with the same buffer. After washing with this buffer, the column was eluted stepwise with successive applications of 6 ml of phosphate buffer, pH 6.8, of increasing concentration (100 mM, 150 mM and 200 mM). Most of the activity was eluted in the 100 mM-phosphate fraction, with a little in the following 150 mM-phosphate fraction. The 100 mM-phosphate fractions were combined and stored in liquid N₂.

Purification of trimethylamine N-oxide demethylase

This was performed as described by Large (1971a), any residual traces of trimethylamine mono-oxygenase and secondary-amine mono-oxygenase being removed by heating the preparation for 15 min at 35°C (Jarman & Large, 1972).

Adaptation of succinate-grown Ps. aminovorans to growth on trimethylamine

Ps. aminovorans was taken through 15 serial subcultures at 30°C on 19 mM-sodium succinate as sole carbon and energy source. When the last culture (1500 ml) had reached late exponential phase it was divided into two equal volumes and the cells from each were harvested aseptically by centrifugation at 20°C. After being washed in sterile 50 mM-phosphate buffer, pH 7.5, the pellets were resuspended in 500 ml of sterile mineral-salts medium containing either 19 mM-sodium succinate or 25 mM-trimethylamine hydrochloride as sole carbon source. These suspensions were used to inoculate 1700 ml of the same medium contained in 4-litre flasks (fitted with an outlet for aseptic removal of samples), which were then shaken at 30°C. The initial cell density was 375 μ g dry wt./ml. Samples were withdrawn at intervals from the two cultures for measurement of growth and specific activity of the tertiary-amine- and secondary-amine-oxidizing enzymes and trimethylamine *N*-oxide demethylase. Cells for enzyme-activity determinations were centrifuged at 20000g for 1 min at 20°C, washed in 50 ml of cold 50 mM-sodium-potassium phosphate buffer, pH 7.5, centrifuged again and the pellets stored in liquid N₂ until extracts were made for assay.

Buffers

Sodium-potassium phosphate, sodium hydrogen maleate, NaHCO₃, glycine-HCl, glycine-NaOH and

sodium acetate-acetic acid buffers were prepared as described by Dawson *et al.* (1969). Sodium pyrophosphate was adjusted to the required pH value with 2M-HCl.

Special chemicals

Trimethylamine hydrochloride, bovine serum albumin, crystalline glucose 6-phosphate dehydrogenase (type XI, from *Torula*), streptomycin sulphate, glucose 6-phosphate, NADP and NADPH were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames KT2 7BH, U.K. Ethyldimethylamine, diethylmethylamine, trimethylsulphonium chloride and triethylamine *N*-oxide were obtained from K & K Laboratories, Plainview, N.Y., U.S.A. 2-Chloroethyldimethylamine, *NN*-dimethylaminoethanol, *NN*-dimethyldiaminoethane, *NN*-dimethylaminopropan-1-ol and -2-ol, *NN*-dimethyl-1,3-diaminopropane and benzyldimethylamine were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Secondary amines were obtained from sources described by Eady *et al.* (1971), and CO was from Matheson Gas Products, East Rutherford, N.J., U.S.A. Proadifen hydrochloride (SKF 525-A) was a gift from Smith, Kline and French Laboratories Ltd., Welwyn Garden City, Herts., U.K. All other chemicals were from Fisons Scientific Apparatus Ltd.

Results

Variables in the spectrophotometric assay system for trimethylamine mono-oxygenase

Effect of pH on enzyme activity. The pH-activity profile of the crude extract showed a sharp optimum at pH 7.5–7.7 for trimethylamine oxidation, and in consequence a pH of 7.5 was adopted for the standard assay during enzyme purification. The pH-activity curve for the oxidation of trimethylamine and dimethylamine by the partially purified enzyme, however (Fig. 1), showed a much broader optimum, shifted to higher pH values. Over the range pH 8.5–9.5, it was essentially similar for both substrates. With the partially purified enzyme most assays were performed at pH 8.0 for trimethylamine and 8.5 for dimethylamine.

Trimethylamine and dimethylamine concentration. The enzyme showed inhibition by trimethylamine concentrations higher than 1 mM. A standard concentration of 0.67 mM was adopted for the spectrophotometric assay. Similar inhibition was observed with all the tertiary amines tested, but not with dimethylamine, and this was used in the spectrophotometric assay at a final concentration of 6.7 mM.

NADPH or NADH concentration. From double-reciprocal plots obtained by using crude extract, the

Fig. 1. pH optimum for dimethylamine and trimethylamine oxidation by the purified enzyme preparation

The standard spectrophotometric assay (a) was used as described in the Materials and Methods section, except that three different buffers were used: sodium-potassium phosphate (●, ○), glycine-NaOH (▲, △) and carbonate (■, □). Solid symbols denote trimethylamine oxidation, open symbols dimethylamine oxidation.

apparent K_m for NADPH was found to be $14\mu\text{M}$. Concentrations of NADPH above $167\mu\text{M}$ were inhibitory. The concentration adopted in the standard spectrophotometric assay was $167\mu\text{M}$. Activity with NADH was very much lower and the errors of the determination were very much increased by the presence of substrate-independent NADH oxidation even in the presence of cyanide, but the apparent K_m for NADH was about 2 mM. These determinations were done with 0.67 mM-trimethylamine as substrate.

Enzyme concentration. The rate was linear with increasing enzyme concentration up to a rate of change of extinction at 340 nm of 0.2/min (corresponding to about 200 μg of protein for the partially purified enzyme).

Oxygen concentration. The K_m for oxygen was very low, and attempts to measure it were not successful. All K_m values for other substrates are apparent values, being determined at an oxygen concentration of 0.24 mM [the concentration in equilibrium with 0.021 MPa (0.21 atm) of O_2 at 25°C].

Changes in the specific activity of amine-oxidizing enzymes during adaptation of succinate-grown *Ps. aminovorans* to growth on trimethylamine

To investigate the roles during growth on trimethylamine of the trimethylamine mono-oxygenase, the secondary-amine mono-oxygenase (Eady *et al.*, 1971; Jarman & Large, 1972) and the trimethylamine *N*-oxide demethylase (Large, 1971a), the appearance of

Fig. 2. Induction of amine-metabolizing enzymes during growth of a succinate-grown inoculum of *Ps. aminovorans* on succinate and on trimethylamine

Cells of succinate-grown *Ps. aminovorans* were washed and resuspended in growth medium containing either 19mM-sodium succinate or 25mM-trimethylamine hydrochloride as sole carbon source. Subsequent growth on succinate (○) and trimethylamine (●) was followed turbidimetrically and cells were removed at intervals for determination of the specific activities of the three enzymes. (a) Specific activity of trimethylamine mono-oxygenase in cells growing on succinate (△) and trimethylamine (▲). Units of activity are μmol of NADPH oxidized/min. (b) Specific activity of trimethylamine *N*-oxide demethylase in cells growing on succinate (□) and trimethylamine (■). Units of activity are μmol of formaldehyde formed/min. (c) Specific activity of secondary-amine mono-oxygenase in cells growing on succinate (▽) and on trimethylamine (▼). Units of activity are μmol of NADPH oxidized/min. Further details will be found in the Materials and Methods section.

enzyme activities during adaptation of the cells from growth on succinate to growth on trimethylamine was followed. After 15 serial subcultures on 17mM-sodium succinate, all three enzymes had very low specific activities (in the region of 1–8nmol of substrate transformed/min per mg of protein). Such cells were resuspended in either succinate or trimethylamine medium (approximately equal concentrations of substrate in g-atoms of carbon/litre) and samples

were taken at intervals to measure growth and the activity of the three enzymes in cell-free extracts. The results are shown in Fig. 2. Cells transferred to succinate grew without lag and the concentrations of the three enzymes remained very low. Cells transferred from succinate to trimethylamine grew after a lag of 26h. During this period before growth began there was a rapid increase in the specific activity of the three enzymes. Increased trimethylamine mono-oxygenase activity was detectable after 6h, and by the time growth had begun it had risen from 8munits/mg of protein to 52munits/mg of protein, which was about 60% of the maximum specific activity detected. Increased activity of the other two enzymes was not detectable until 18h after transfer. The increase was from about 15munits/mg of protein for trimethylamine *N*-oxide demethylase and 3munits/mg of protein for secondary-amine mono-oxygenase to 40 and 26 respectively by the time growth had begun. By 7h after growth on trimethylamine could be detected, the specific activities of these two enzymes reached a maximum and then decreased, although their final specific activities towards the end of the exponential phase of growth on trimethylamine were still about twofold and tenfold respectively above the initial specific activities (and sixfold and 30-fold above the specific activities in succinate-grown cells).

Stability and attempted stabilization of the trimethylamine mono-oxygenase

The enzyme had a half-life in crude extracts of 11 min at 35°C (Jarman & Large, 1972). The partially purified enzyme had a half-life at 4°C of about 50h. This was independent of protein concentration over the range tested (0.5–10mg of protein/ml). This meant that during a purification lasting 4 days, 75% of the activity was lost, not taking into account any of the losses during fractionation procedures. Therefore attempts were made to stabilize the enzyme in the hope of improving the purification procedure. Addition of the following agents, which have been reported to stabilize various enzymes, failed to protect the partially purified enzyme against loss of trimethylamine-oxidizing activity when incubated with it over time-periods of up to 100h at 4°C, samples being removed at intervals for assay: 10% (v/v) ethanol, 10% (v/v) glycerol, 0.1mM-dithiothreitol, 10mM-2-mercaptoethanol, 10% (v/v) dimethyl sulphoxide, 2mM-trimethylamine hydrochloride, 10mM-EDTA, 0.7M-sucrose and 0.1mM-FMN. 2-Mercaptoethanol accelerated the loss of activity. NADH at 3mM was an effective stabilizer over a limited period of time, but lost effectiveness after a few hours. NADPH was not tested. Ethanol and anaerobic conditions, which were very successful in protecting the secondary-amine mono-oxygenase of *Ps. aminovorans* (Eady *et al.*, 1971; Jarman, 1973),

were not effective with the trimethylamine mono-oxygenase.

The enzyme also lost activity on dialysis. Dialysis of partially purified enzyme at 4°C against 5mM-sodium-potassium phosphate buffer, pH7.5, decreased the half-life to 12h as compared with 50h for an undialysed sample in the same buffer at 4°C.

The effect of pH on stability was examined by incubating samples of partially purified enzyme for 30min at 0°C or at 25°C in the presence of buffers (25mM) in the pH range 2–11. The samples were then brought back to pH7.5 with an excess of 0.2M-sodium-potassium phosphate buffer, pH7.5, and assayed with trimethylamine as substrate. There was no detectable irreversible loss of enzyme activity in this short period of time over the pH range 4–9 at 0°C or over the range pH6–8 at 25°C. Outside these extremes, activity was irreversibly lost.

Purification of the trimethylamine mono-oxygenase

The problems encountered during purification were due in part to the instability of the enzyme and in part to the problem of separating the activity from the enzymes catalysing the further metabolism of the product. Careful $(\text{NH}_4)_2\text{SO}_4$ fractionation was necessary to separate the trimethylamine mono-oxygenase from the secondary-amine mono-oxygenase. This was followed by gel filtration on Sephadex G-150 and adsorption on, and elution from, a hydroxyapatite column, as described in detail in the Materials and Methods section. The losses in activity at each step were considerable. An overall purification of sixfold was achieved at the expense of a recovery of only 0.5% of the starting activity. The partially purified material, which had a specific activity of about

0.6 unit/mg of protein, was used for the remaining experiments in this section.

Properties of the partially purified enzyme

Identity of the product of trimethylamine oxidation. It seemed likely that the oxidation product of trimethylamine was trimethylamine *N*-oxide. This was confirmed by the observation that formaldehyde was formed in the presence (but not in the absence) of partially purified trimethylamine *N*-oxide demethylase, an enzyme that so far as is known is specific for *N*-oxides (Large, 1971a) (Table 1). This process was inhibited by 1mM-KCN, a known inhibitor of the demethylase (Large, 1971a). The product of the reaction in the absence of demethylase had an R_F value identical with that of authentic trimethylamine *N*-oxide on cellulose t.l.c. in two different solvents. It also behaved identically with authentic trimethylamine *N*-oxide on ion-exchange chromatography on Zeo-Karb 226 in citrate-phosphate buffer, pH5.0 (Blau, 1961). Trimethylamine *N*-oxide, which is a very weak base, binds only very slightly to the column, whereas trimethylamine is eluted later. The enzymic product separated in this way gave rise to formaldehyde on incubation with trimethylamine *N*-oxide demethylase. The corresponding fractions from the control incubation (without mono-oxygenase) contained less than 5% of the product detected in the complete system and gave no formaldehyde on treatment with the demethylase. Final confirmation of the identity of the product is its conversion into formaldehyde on treatment with SO_2 (Mitchell & Ziegler, 1969), and this was used to determine the product either in crude mixtures (Table 1) or after ion-exchange separation.

Table 1. *Products of the oxidation of trimethylamine by partially purified trimethylamine mono-oxygenase in the presence and absence of trimethylamine N-oxide demethylase*

The complete system contained 100 μmol of phosphate buffer, pH7.5, 1 μmol of NADPH, 2 μmol of trimethylamine hydrochloride and 0.5mg of partially purified trimethylamine mono-oxygenase in a final volume of 1 ml. Other additions or omissions are indicated below. After 30min at 25°C, samples (0.1 ml) were removed for NADPH determination (at 340nm) and 0.1 ml of 2.5M-HClO₄ was added to each tube. Formaldehyde and trimethylamine *N*-oxide were then determined as described in the Materials and Methods section.

Tube no.	Omissions	Additions	NADPH consumed (μmol)	Trimethylamine <i>N</i> -oxide formed (μmol)	Formaldehyde formed (μmol)
1	None	None	0.70	1.26	0
2	Trimethylamine	None	0	0.20	0
3	NADPH	None	—	0.09	0.03
4	Enzyme	Boiled enzyme	0.27	0.15	0.01
5	Trimethylamine	Trimethylamine <i>N</i> -oxide (2mM)	0.08	—	0.04
6	None	Demethylase (1 unit)	0.59	0	0.58
7	None	Demethylase (1 unit), KCN (1mM)	0.82	1.13	0
8	Enzyme	Demethylase (1 unit)	0.29	0.24	0
9	Enzyme, trimethylamine	Demethylase (1 unit), trimethylamine <i>N</i> -oxide (2mM)	0.13	—	0.26

Fig. 3. Substrate consumption and product formation by the partially purified trimethylamine mono-oxygenase

The results of three separate experiments with different enzyme preparations are presented together for comparison. Symbols: ▲, trimethylamine; ●, trimethylamine *N*-oxide; ■, formaldehyde; ▼, dimethylamine; ○, methylamine. Trimethylamine was determined in each case by the enzymic method (see the Materials and Methods section). (a) Conversion of trimethylamine into trimethylamine *N*-oxide with NADPH as electron donor. The reaction mixture contained 4.5 μ mol of NADPH, 4.5 μ mol of trimethylamine hydrochloride, 180 μ mol of sodium-potassium phosphate buffer pH 8.0, and 1 mg of trimethylamine mono-oxygenase in a total volume of 9 ml. The incubation was at 25°C and at intervals samples were removed for analysis after deproteinization with HClO_4 (final concentration 0.25 M). (b) Effect of the addition of trimethylamine *N*-oxide demethylase to the trimethylamine mono-oxygenase reaction mixture. This contained 96 μ mol of trimethylamine-HCl, 960 μ mol of maleate buffer, pH 7.0 (a pH value midway between the optima of the two enzymes), 20 mg of trimethylamine mono-oxygenase, 960 μ mol of glucose 6-phosphate, 24 μ mol of NADP^+ and 70 units of glucose 6-phosphate dehydrogenase in a total volume of 48 ml. Incubation conditions were as in (a). At the point indicated by the arrow 0.2 ml (4 mg) of partially purified trimethylamine *N*-oxide demethylase was added. (c) Conversion of dimethylamine into formaldehyde. The reaction mixture contained 20 μ mol of dimethylamine-HCl, 200 μ mol of sodium-potassium phosphate buffer, pH 8.0, 2 mg of trimethylamine mono-oxygenase, 200 μ mol of glucose 6-phosphate, 2.5 μ mol of NADP^+ and 6 units of glucose 6-phosphate dehydrogenase in a total volume of 10 ml. Incubation conditions were as in (a).

Contaminating activities. Although the best preparations of the partially purified enzyme were only sixfold purified, they were free of cyanide-sensitive secondary-amine mono-oxygenase activity, which is removed by the $(\text{NH}_4)_2\text{SO}_4$ fractionation described in the Materials and Methods section. Any traces of activity not removed by this procedure were removed

by the Sephadex G-150 step. Trimethylamine *N*-oxide demethylase activity was also absent from the purified preparation (Table 1 and Fig. 3b).

Time-course and stoichiometry of the reaction. The reaction was followed by measuring the formation of trimethylamine *N*-oxide as a function of time. When NADPH was used as electron donor, the reaction was

linear with time for 10min, and there was a parallel disappearance of trimethylamine (Fig. 3a). No formaldehyde was detected in the reaction mixture unless purified trimethylamine *N*-oxide demethylase was added (Fig. 3b).

The results of an experiment in which the disappearance of reactants and the formation of product was followed are shown in Table 2. The results confirm the stoichiometry of eqn. (1) above.

Reaction products from other tertiary and secondary amines. Difficulty was encountered in identifying the oxidation products of other tertiary amines, since no authentic *N*-oxides except for those of trimethylamine and triethylamine were available. However, it was found that the oxidation products of ethyldimethylamine and diethylmethylamine would react with both SO₂ and purified trimethylamine *N*-oxide demethylase to give formaldehyde. It has not been possible to establish whether an *N*-oxide is formed from triethylamine because triethylamine *N*-oxide is not a substrate for trimethylamine *N*-oxide demethylase (Large, 1971a). Moreover we have not been able to detect the formation of acetaldehyde from authentic triethylamine *N*-oxide on treatment with SO₂, although this ought to occur (Mitchell & Ziegler, 1969; Culvenor, 1953).

Dimethylamine was oxidized to formaldehyde (Fig. 3c). The identity of the other product has not been established; however, it is not methylamine, because it does not react with purified primary-amine dehydrogenase from *Pseudomonas* AM1 (Large *et al.*, 1969). The low concentration of methylamine detected is probably an impurity in the incubation mixture. When concentrations of methylamine similar to those of the formaldehyde formed (50 μ M) were incubated with the enzyme, no disappearance of methylamine was detected.

Effect of cations. No metal-ion activation was demonstrated. 1mM-Co²⁺, 0.33mM-Cu²⁺ and 0.33mM-Zn²⁺ were inhibitory. Other cations tested at 1mM in glycine-NaOH buffer, pH8.0, were without effect. These included Mn²⁺, Mg²⁺, Ca²⁺, K⁺ and NH₄⁺.

Effect of various possible inhibitors. With trimethylamine as substrate, no inhibition was detected at pH7.5 with the following compounds (the highest concentration tested appearing in parentheses): diethyldithiocarbamate (3mM), EDTA (10mM), 2,2'-bipyridyl (2mM), KCN (5mM), proadifen hydrochloride (SKF 525-A; 2-diethylaminoethyl 2',2'-diphenylvalerate hydrochloride) (0.33mM), 2-mercaptoethylamine hydrochloride (cysteamine) (1mM). These compounds were tested after a 15min preincubation with enzyme. KCN (5mM) did not inhibit the oxidation of dimethylamine by the preparation.

The partially purified trimethylamine mono-oxygenase showed a slight inhibition by high concentrations of CO. In an incubation similar to that of assay

method (a), bubbling of the reaction mixture with CO for 5min followed by the addition of 0.6 μ mol of NADPH, caused a 19% inhibition of NADPH oxidation at pH9.5, but no inhibition at pH7.5, over an incubation period of 5min in air. The formation of trimethylamine *N*-oxide in the same system was inhibited by 25% at pH9.5. Under a defined atmosphere of CO+air (1:1), 15% inhibition of trimethylamine *N*-oxide formation was observed at pH9.5 and no inhibition at pH7.5. In a similar experiment with dimethylamine, no inhibition by CO was detected at pH8.5 or 9.5. It is doubtful whether this extent of inhibition is significant, particularly in comparison with the secondary-amine mono-oxygenase of this organism, where an atmosphere of CO+air (1:9) produced 96% inhibition of the conversion of dimethylamine into formaldehyde (Eady *et al.*, 1971). Other compounds inactive as inhibitors included trimethylamine *N*-oxide, triphenylammonium iodide, ethyleneimine and 1-(1-naphthyl)-2-thiourea (Ziegler & Mitchell, 1972). Primary amines and ammonia were not inhibitory. In a few preparations they showed a stimulatory effect.

The only compounds tested which inhibited the enzyme were semicarbazide (45% inhibition at 17mM), iodoacetamide (71% inhibition at 17mM), cuprizone (70% inhibition at 0.5mM), tetramethylammonium chloride (50% inhibition at 0.2mM), 2,4-dichloro-6-phenylphenoxyethylamine (Lilly

Table 2. *Stoichiometry of the oxidation of trimethylamine by the trimethylamine mono-oxygenase*

The complete system contained in a final volume of 3.3 ml: 200 μ mol of sodium-potassium phosphate buffer, pH8.0; 2 μ mol of NADPH; 1 μ mol of trimethylamine hydrochloride; 3 μ mol of KCN; partially purified mono-oxygenase preparation and water. The reaction was started by addition of the NADPH and trimethylamine together. Oxygen uptake was followed at 25°C to completion with a Beckman oxygen analyser. At the end of the reaction, samples (0.1 ml) were removed to measure the extinction at 340nm, and the mixture was then deproteinized by addition of 0.1 ml of 2.5M-HClO₄. Denatured protein was removed by centrifugation and the supernatant was analysed for trimethylamine (by the method of Sass *et al.*, 1958) and trimethylamine *N*-oxide, as described in the Materials and Methods section. The results are compared with a control which contained all the reactants except enzyme.

Reactants consumed (μ mol)	Complete system	No enzyme	Difference
NADPH	1.80	1.18	0.62
Oxygen	0.67	0.12	0.55
Trimethylamine	0.79	0	0.79
Product formed (μ mol)			
Trimethylamine <i>N</i> -oxide	0.79	0	0.79

53325) (40% inhibition at 0.33mM), 2,4-dichloro-6-phenylphenoxyethyldiethylamine (Lilly 18947) (74% inhibition at 0.27mM) and trimethylsulphonium chloride, a substrate analogue (30% inhibition at 1mM). All these observations were made with 0.67mM-trimethylamine as substrate in assay method (a). With 6.7mM-dimethylamine as substrate, very similar results were obtained, except with tetramethylammonium chloride, which was much less effective as an inhibitor of dimethylamine oxidation.

Substrate specificity. The partially purified enzyme oxidized a wide range of secondary and tertiary amines. The following tertiary amines were oxidized: trimethylamine, ethyldimethylamine, diethylmethylamine, triethylamine, *NN*-dimethylaminoethanol, 2-chloroethyldimethylamine, *NN*-dimethyldiaminoethane, *NN*-dimethyl-1,3-diaminopropane, *NN*-dimethylaminopropan-1-ol, *NN*-dimethylaminopropan-2-ol, *NN*-dimethylaniline and benzyldimethylamine. The following secondary amines were oxidized: dimethylamine, ethylmethylamine, diethylamine, methyl-*n*-propylamine, ethyl-*n*-propylamine and methyl-*n*-octylamine. Apparent K_m values for these substrates were determined, and in almost all cases were less than 200 μ M for the tertiary amines and in the region 200–600 μ M for the secondary amines tested. Comparison of relative rates by means of V_{max} values was difficult because all the tertiary amines tested showed substrate inhibition at concentrations above about 1mM and gave hyperbolic double-reciprocal plots. The concentration of substrate that gave maximum enzyme activity varied with substrate and with the enzyme preparation. Extrapolated V_{max} values for trimethylamine, ethyldimethylamine, diethylmethylamine and triethylamine obtained with the same preparation were in the proportions 100:97:69:17. Under optimal conditions, the rate for dimethylamine was almost identical with that for trimethylamine, and V_{max} values for dimethylamine, diethylamine and methyl-*n*-propylamine were in the proportions 100:35:31. The following compounds were not substrates: tri-*n*-propylamine, triethanolamine, *NN*-dimethylglycine and nitrilotriacetic acid. All active substrates seem to bear at least two unsubstituted alkyl groups, and all active tertiary amines bear at least two methyl or ethyl groups.

Effect of heat on the partially purified enzyme. In an attempt to determine whether the two activities of trimethylamine mono-oxygenase (oxidation of secondary and of tertiary amines) were due to the same enzyme or to different ones, the effect of heating the partially purified material at 35°C was examined. Samples were removed at intervals, cooled to 0°C, and assayed for trimethylamine- and dimethylamine-oxidizing activity by assay method (a). Both activities were lost in a rather complex fashion; semilogarithmic plots of the two activities against time were non-

linear. Over the first 8min there was an initial rapid loss with half-lives of approx. 14min for trimethylamine-oxidizing activity and 8min for dimethylamine-oxidizing activity. The former value agrees reasonably well with the value for the half-life of trimethylamine-oxidizing activity previously observed at this temperature in crude extracts (Jarman & Large, 1972). After 8min at 35°C there was a transition to much slower rates of inactivation, which were about the same for the two activities. The differences are not sufficiently great for a conclusive decision to be reached at the present degree of purity of the enzyme as to the number of separate enzymes present in the preparation.

Discussion

The activity of the partially purified trimethylamine mono-oxygenase with both secondary and tertiary amines may either represent a single enzyme of broad specificity, or two closely related enzymes that have not been separated. A conclusive answer to this problem must await the availability of a more highly purified enzyme, but we favour the alternative of a single enzyme with dual specificity because there is already in the organism a second enzyme that oxidizes secondary amines (Eady *et al.*, 1971). The tertiary-amine mono-oxygenase (EC 1.14.13.8) purified by Ziegler and co-workers from pig liver microsomal preparations (Ziegler *et al.*, 1969, 1971; Ziegler & Mitchell, 1972) also oxidizes both secondary and tertiary amines, although the range of amines attacked and the response to inhibitors are different. The microsomal enzyme converts tertiary amines into *N*-oxides and secondary amines into *NN*-dialkylhydroxylamines (eqn. 2) (Ziegler & Mitchell, 1972):

In acid solution, the secondary hydroxylamines decompose to give primary amines and aldehydes. We have not yet established whether substituted hydroxylamines are formed from secondary amines by the *Ps. aminovorans* enzyme: this requires a more highly purified preparation, but the evidence we have obtained does not support the formation of a primary amine as well as an aldehyde. It is conceivable that an *NN*-dialkylhydroxylamine is formed, which might be further oxidized if the preparation contains a mono-oxygenase similar to that recently described in microsomal preparations by Kadlubar *et al.* (1973) (eqn. 3). The nitron oxidation product of that enzyme decomposes in acid solution (eqn. 4) without the formation of a primary amine.

An enzyme catalysing an analogous reaction, with NADH rather than NADPH as electron donor, although as yet uncharacterized, is probably involved in the microbial degradation of nitrilotriacetic acid (Cripps & Noble, 1973; Tiedje *et al.*, 1973).

A comparison of the present enzyme with the other amine-oxidizing enzymes of *Ps. aminovorans* confirms the views previously expressed (Eady *et al.*, 1971; Large, 1971*b*; Jarman & Large, 1972) that it is different from either of the two previously recognized enzymes. This conclusion is based mainly on considerations of stability and of distribution in the cells grown on different substrates. This is now reinforced by observations on its inhibitor response (it is slightly inhibited by high concentrations of CO, but not by cyanide, whereas the secondary-amine mono-oxygenase is extremely sensitive to low concentrations of both CO and cyanide). It seems likely that the trimethylamine mono-oxygenase is the first step in the oxidative dissimilation of trimethylamine, since it forms the *N*-oxide, which can then be successively demethylated by trimethylamine *N*-oxide demethylase and the secondary-amine mono-oxygenase (Jarman & Large, 1972), to yield formaldehyde and methylamine. It is then probable (T. R. Jarman, unpublished work) that the methylamine so formed is oxidized to formaldehyde by the formation and subsequent oxidation of *N*-methylglutamate, as proposed by Hersh *et al.* (1971) (see also Bellion & Hersh, 1972) for methylamine oxidation in *Pseudomonas* sp. MA.

The data reported in the present paper show clearly that synthesis of the three amine-oxidizing enzymes occurs before growth begins when succinate-grown bacteria are adapting to growth on trimethylamine, and that the trimethylamine mono-oxygenase, the first enzyme in the oxidation pathway, appears first, clearly implicating it in growth on trimethylamine.

Trimethylamine mono-oxygenase was also found by Colby & Zatman (1973) in bacterial strains 1A1, 1A2, 1A3, 1B1, 2B2 and *Pseudomonas* 3A2 which they isolated from enrichment cultures in which trimethylamine was the sole carbon source. It is also present in the marine *Pseudomonas* sp. N.C.M.B. 1154 (Budd & Spencer, 1968; Budd, 1969) when grown on trimethylamine as sole nitrogen source. The enzyme from the marine bacterium shows slight differences from the *Ps. aminovorans* enzyme (C. A. Boulton & P. J. Large, unpublished work), but its presence in the organism explains many of the observations on trimethylamine metabolism by intact and macerated cell suspensions of *Pseudomonas* sp. N.C.M.B. 1154 made by Budd (1969). Our previous report (Large *et al.*, 1972) that trimethylamine mono-oxygenase is present in *H. vulgare* strain NQ-521 was erroneous. The enzyme is present in an organism which contaminates some cultures of *H. vulgare* NQ. Pure cultures of *H. vulgare* NQ contain trimethylamine dehydrogenase and not

the mono-oxygenase. Trimethylamine dehydrogenase was originally described by Colby & Zatman (1971, 1973) in two obligately methylotrophic bacteria. It utilizes various dyes as electron acceptor and catalyses the conversion of trimethylamine into formaldehyde and dimethylamine (eqn. 5), and also the oxidation of other tertiary amines. We have not detected it in *Ps. aminovorans*.

Our characterization of trimethylamine mono-oxygenase confirms beyond question the existence of two different enzymes oxidizing trimethylamine. Colby & Zatman (1973) suggested that two different pathways for the oxidation of trimethylamine exist, one involving oxygenation to give trimethylamine *N*-oxide, followed by non-oxidative cleavage of the latter, the other the direct dehydrogenation of trimethylamine to formaldehyde and dimethylamine. The same authors also suggested that these different pathways might be correlated with the conditions of facultative or obligate methylotrophy respectively. Our observation that pure cultures of *H. vulgare* NQ-521, which is not an obligate methylotroph (Hirsch & Conti, 1964), contain trimethylamine dehydrogenase does not support this idea. No organism, however, has yet been found in which both trimethylamine mono-oxygenase and trimethylamine dehydrogenase are present, which suggests that the two pathways of trimethylamine oxidation may be mutually exclusive.

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