Microbial Oxidation of Amines

PARTIAL PURIFICATION OF A MIXED-FUNCTION SECONDARY-AMINE OXIDASE SYSTEM FROM *PSEUDOMONAS AMINOVORANS* THAT CONTAINS AN ENZYMICALLY ACTIVE CYTOCHROME-P-420-TYPE HAEMOPROTEIN

By R. R. EADY,* T. R. JARMAN AND P. J. LARGE

Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.

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1. Crude extracts of *Pseudomonas aminovorans* grown on methylamine, dimethylamine, trimethylamine or trimethylamine *N*-oxide contain an enzyme or enzyme system catalysing the NADH- or NADPH- and oxygen-dependent oxidation of dimethylamine to methylamine and formaldehyde. 2. The enzyme has been partially purified about five-fold. It is unstable, but can be stabilized by addition of 5% (v/v) ethanol. 3. The partially purified enzyme will utilize either NADH (K_m 6.5 μ M) or NADPH (K_m 13.2 μ M). The following secondary amines have been shown to be substrates: dimethylamine, ethylmethylamine, diethylamine, methyl-*n*-propylamine, ethyl-*n*-propylamine, *n*-butylmethylamine and *N*-methylethanolamine. The K_m values and comparative reaction rates for each substrate have been determined. Where the alkyl groups are different, the aldehyde products are derived from both groups. 4. The enzyme system has a pH optimum of 6.8 and is inhibited by mercurials, thiol compounds, cyanide and carbon monoxide. 5. The partially purified preparation had a spectral maximum at 412 nm with shoulders at 427 and 550 nm. Reduction with dithionite or NAD(P)H bleached the 412 nm peak, and the shoulder at 427 nm became a peak. Additional peaks appeared at 550 and 580–588 nm. Reduction of a preparation bubbled with carbon monoxide enhanced and sharpened the Soret peak and caused it to shift to 422 nm. 6. Analysis of the preparation showed the presence of flavin, acid-extractable iron and non-acid-extractable iron in the proportion 1.1:1.9:1. On reduction with dithionite or NADPH the preparation showed an electron-paramagnetic-resonance signal at around $g = 1.946$.

Pseudomonas aminovorans (den Dooren de Jong, 1927) will grow on a variety of amines as sole carbon source. In the present paper we describe some of the properties of a mixed-function secondary-amine oxidase system that is present when the organism is grown on methylamine, dimethylamine, trimethylamine or trimethylamine *N*-oxide as sole carbon source, but absent when it is grown on succinate. The enzyme is different both in properties and in specificity from the microsomal mixed-function amine oxidase studied by Ziegler, Mitchell & Jollow (1969). In preliminary reports (Eady & Large, 1969; Jarman, Eady & Large, 1970) of some of the work described in the present paper it was shown that the enzyme system contains a carbon monoxide-sensitive haemoprotein.

* Present address: Agricultural Research Council Nitrogen Fixation Unit, Chemical Laboratory, University of Sussex, Falmer, Brighton BN1 9QJ, U.K.

MATERIALS AND METHODS

Maintenance and growth of the organism. Stock cultures of *Ps. aminovorans* N.C.I.B. 9039 were maintained on slopes made by solidifying methylamine growth medium with 1% (w/v) agar-agar. The cultures were grown at 30°C, stored at 4°C and subcultured monthly. Initially cells were grown in the liquid medium of Large, Eady & Murden (1969), which is referred to as salts medium A. Later it was found that better growth is obtained with salts medium B, which has the following composition (mg/l): K_2HPO_4 , 1360; $NaH_2PO_4 \cdot 12H_2O$, 5370; $(NH_4)_2SO_4$, 500; disodium EDTA (adjusted to pH 6.8), 202; $MgSO_4 \cdot 7H_2O$, 290; $CaCl_2 \cdot 2H_2O$, 66; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 186; $FeSO_4 \cdot 7H_2O$, 7; $ZnSO_4 \cdot 7H_2O$, 11; $MnSO_4 \cdot 4H_2O$, 1.5; $CuSO_4 \cdot 5H_2O$, 0.4; $Co(NO_3)_2 \cdot 6H_2O$, 0.25; $Na_2B_4O_7 \cdot 10H_2O$, 0.18. Various carbon sources were added to both media. The following concentrations (g/l) were used: methylamine hydrochloride, 5; dimethylamine hydrochloride, 1; trimethylamine hydrochloride, 2; trimethylamine *N*-oxide hydrochloride, 2; sodium succinate hexahydrate, 2.

Bacteria from slopes were transferred to 10 ml of methylamine medium and incubated at 30°C until growth occurred (usually 3 days) before being used to inoculate 2 litres of medium in a wide-necked 4-litre conical flask. Cultures were grown at 30°C on a Gyrotory Shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The cells were harvested at 4°C by centrifugation and washed twice with 0.9% (w/v) KCl. Large batches of cells were obtained by inoculating 14 litres of medium in a 20-litre aspirator with 4 litres of fully grown culture and aerating them vigorously for 24 h at 30°C. The cells were harvested in a Sharples Super centrifuge and stored in liquid N₂.

When cells grown on carbon sources other than methylamine were required, they were taken through at least five subcultures on the new substrate before being used experimentally.

Measurement of oxygen. Oxygen uptake was measured at 30°C with a Beckman model 777 Oxygen Analyser in a 3.3 ml thermostatically controlled reaction vessel. This was calibrated by measuring the extent of oxygen uptake for the chemical reoxidation of various amounts of spectrophotometrically standardized NADH by phenazine methosulphate by a method similar to that described by Robinson & Cooper (1970).

Partial purification of the mixed-function secondary-amine oxidase system. Step 1. Preparation of crude extract. Methylamine-grown cells (20 g wet wt.) were suspended in 50 ml of 50 mM-phosphate buffer, pH 7.5. The suspension was cooled to 2°C and subjected in two batches to the full output of a 100 W Dawe Soniprobe ultrasonic disintegrator (Dawe Instruments Ltd., London W.3., U.K.) for a total exposure time of 2 min/batch, the temperature being kept below 10°C. The suspension was centrifuged for 20 min at 30000g at 2°C. The centrifuged pellet was resuspended in 50 ml of phosphate buffer and treated as before. This process was repeated until most cells were broken (usually three exposures). The combined supernatants were centrifuged for 40 min at 30000g at 2°C and the small pellet formed was discarded. The supernatant at this stage is referred to as the crude extract. All subsequent operations were performed at 4°C with as little delay as possible.

Step 2. First ammonium sulphate fractionation. The crude extract was made 30% saturated with (NH₄)₂SO₄ by the slow addition of solid (NH₄)₂SO₄ without adjustment of the pH and allowed to equilibrate for 30 min. The precipitate formed was centrifuged and discarded. The supernatant was then made 55% saturated by the addition of further (NH₄)₂SO₄. The resulting precipitate, after equilibration as before, was sedimented and redissolved in 50 mM-phosphate buffer, pH 6.8, containing 5% (v/v) of ethanol to stabilize the activity.

Step 3. Chromatography on DEAE-cellulose. The redissolved 30–55%-saturated-(NH₄)₂SO₄ precipitate was dialysed against 4 litres of 33 mM-phosphate buffer, pH 6.8, containing 5% (v/v) of ethanol for 4 h. It was then loaded on to a column (2.5 cm × 40 cm) of microgranular DEAE-cellulose (Whatman DE 32) equilibrated with the same buffer. The column was then washed with the same buffer with an LKB type 4912A ReCyChrom peristaltic pump (LKB Produkter A.B., Stockholm-Bromma 1, Sweden), and approx. 4 ml fractions were collected in a Gilson Mini Escargot (model MDTC)

fraction collector (Anachem Ltd., Luton, U.K.). The eluate was monitored for u.v.-absorbing material at 265 nm in a Gilson 265 IF ultraviolet-absorption meter with a Rustrak model (88)146 recorder [Gulton Industries (Britain) Ltd., Sheffield 1, U.K.]. The enzyme activity did not usually bind significantly to the column and was eluted as a brown-red band. Occasionally some activity did bind to the column under these conditions, in which case a linear gradient of KCl (0–400 mM-Cl⁻, total volume 600 ml) was used to elute the activity.

Step 4. Second ammonium sulphate fractionation. Enzyme-containing fractions from the previous step were combined and made 30% saturated with (NH₄)₂SO₄ as in step 1. After 1 h of equilibration the precipitate formed was centrifuged off and discarded. Solid (NH₄)₂SO₄ was then added to 45% saturation, and after 1 h of equilibration the precipitate formed was centrifuged off and redissolved in 50 mM-phosphate buffer, pH 6.8, containing 5% (v/v) of ethanol. This procedure is summarized in Table 1.

Enzyme assays. (a) Spectrophotometric assay of the secondary-amine oxidase system. The activity of the secondary-amine oxidase system was measured as the rate of the substrate-dependent oxidation of NADH or NADPH determined by the decrease in extinction at 340 nm. The assay mixture contained in quartz cuvettes (1 cm light-path) 200 μmol of phosphate buffer, pH 6.8, 0.5 μmol of NAD(P)H, 3 μmol of dimethylamine hydrochloride and enzyme preparation in a total volume of 3 ml, at 28°C. The reaction was started by the addition of substrate. One unit of enzyme is defined as the amount required to oxidize 1 μmol of NAD(P)H/min under these conditions.

(b) Formaldehyde formation by the secondary-amine oxidase system. The secondary-amine oxidase system could also be assayed by measuring the formation of formaldehyde from dimethylamine hydrochloride. The assay mixture, set up in test tubes, was as in section (a) except that 1 μmol of NAD(P)H was used. The incubation was started at 28°C by addition of dimethylamine hydrochloride and stopped after 15 min by the addition of 0.3 ml of 10% (w/v) trichloroacetic acid. The protein precipitate was centrifuged off and the formaldehyde formed was determined.

(c) Trimethylamine *N*-oxide demethylase. The activity of trimethylamine *N*-oxide demethylase was measured by incubating the enzyme preparation at 28°C with 100 μmol of sodium hydrogen maleate buffer, pH 6.0, and 10 μmol of trimethylamine *N*-oxide hydrochloride in a total volume of 1.35 ml. The reaction was started by addition of substrate and after 20 min stopped by addition of 0.15 ml of 2.5M-HClO₄. After centrifuging the mixture, formaldehyde in the supernatant was determined.

(d) Dye reduction assays. The enzyme-dependent reduction of dyes by NAD(P)H was measured at 28°C in glass cuvettes containing 200 μmol of phosphate buffer, pH 6.8, 0.5 μmol of NAD(P)H, electron acceptor, enzyme and water to 3 ml. The following electron acceptors were used in the assay mixture: 0.1 ml of 2% (w/v) horse heart cytochrome *c* (ϵ_{550} 21 × 10³), 0.05 ml of 0.1% (w/v) 2,6-dichlorophenol-indophenol (ϵ_{500} 21.5 × 10³) or 0.1 ml of 30 mM-potassium ferricyanide (ϵ_{420} 1.02 × 10³). The reaction was started by addition of NAD(P)H and the rate of reduction of dye was measured

Table 1. Purification of the secondary amine oxidase and NADPH-2,6-dichlorophenol-indophenol reductase activity from *Ps. aminovorans*

Experimental details are given in the text.											
Step. no.	Fraction	Volume (ml)	Protein concn. (mg/ml)	Secondary-amine oxidase activity				NADPH-2,6-dichlorophenol-indophenol reductase activity			
				Total activity (units)	Sp. activity (units/mg of protein)	Purification factor	Yield (%)	Total activity (units)	Sp. activity (units/mg of protein)	Purification factor	Yield (%)
1	Crude extract	160	11.6	126	0.068	1	100	1900	1.025	1	100
2	Fraction precipitated by 30-55%-satd. (NH ₄) ₂ SO ₄ and then dialysed	23	49.6	121	0.106	1.56	96	1700	1.50	1.46	90
3	DEAE-cellulose effluent (combined fractions)	31.5	11.4	77	0.215	3.2	61	1700	4.75	4.6	90
4	Fraction precipitated by 30-45%-satd. (NH ₄) ₂ SO ₄	4.6	30.2	49	0.353	5.2	39	546	3.95	3.85	29

Experimental details are given in the text.

spectrophotometrically at the wavelengths indicated, by using the molar extinction coefficients shown. When 2,6-dichlorophenol-indophenol was used as electron acceptor, 3 μmol of KCN was also added to the assay mixture. With 2,6-dichlorophenol-indophenol and ferricyanide the rate was corrected for non-enzymic reduction. One unit is defined as the amount of enzyme required to reduce 1 μmol of electron acceptor/min under the above conditions.

Chemical determinations. (a) Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard.

(b) Formaldehyde. This was determined by the acetylacetone method of Nash (1953). A standard calibration was prepared with a solution of formaldehyde previously standardized gravimetrically with dimedone (Frisell & Mackenzie, 1958).

(c) Aliphatic aldehydes. These were determined by the anthranilaldehyde method of Albrecht, Scher & Vogel (1962).

(d) Methylamine. This was determined by the 1-fluoro-2,4-dinitrobenzene method of Dubin (1960) after microdiffusion at 40°C for 12 h in a Conway (1962) unit. The enzymic method of Large *et al.* (1969) was also used.

(e) Dimethylamine. This was determined by the 1-fluoro-2,4-dinitrobenzene method of Dubin (1960).

(f) Thiols. These were determined by the 5,5'-dithiobis-(2-nitrobenzoic acid) method of Ellman (1959).

(g) Iron. Acid-labile iron was measured by adding to 1 ml of sample [that had been previously passed down a column of Chelex 100 chelating resin (Na^+ form) to remove any unbound iron] 0.5 ml of 1M-HCl and then incubating the mixture for 45 min at room temperature. The sample was then deproteinized by addition of 1 ml of 1.23M-trichloroacetic acid and centrifuged. The iron content of the supernatant was then determined with 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) disulphonate in the form of the Boehringer test combination TC-FE for the determination of serum iron [Boehringer Corporation (London) Ltd., London W.5, U.K.]. Extinctions were read at 535 nm against a reagent blank, with standards of 1 μg of iron. Non-acid-labile iron was determined by the same method after digestion of the denatured protein precipitate from the above procedure as described below.

(h) Flavin. Total flavin, FMN and FAD were determined fluorometrically by the method of Burch (1957), with a spectrophotometrically standardized solution of FMN for calibration.

Thin-layer chromatography of aldehyde 2,4-dinitrophenylhydrazones. This method was adapted from the paper-chromatographic method of Meigh (1952). Enzyme incubation mixtures [see (b) under 'Enzyme assays' above] were treated with 1 ml of 1 mM-2,4-dinitrophenylhydrazine in 2M-HCl, and after 10 min 0.5 ml of chloroform was added. Samples of the chloroform extract were applied to freshly prepared plates of MN-cellulose powder 300G (Macherey, Nagel and Co.; obtained from Camlab Ltd., Cambridge, U.K.) made by suspending the powder in methanol instead of water. After 2 h of equilibration, the plates were developed in heptane saturated with methanol. After drying, the spots were intensified by spraying with aq. 10% (w/v) NaOH.

2,4-Dinitrophenylhydrazones of authentic aldehydes gave the following R_F values: formaldehyde, 0.29; acetaldehyde, 0.51; propionaldehyde, 0.68; butyraldehyde, 0.80. These R_F values are considerably more satisfactory than those obtained with paper (Meigh, 1952).

Spectrophotometry. Absolute u.v. and visible spectra were recorded on a Unicam SP.1800 recording spectrophotometer against a water blank. Difference spectra were either recorded directly or calculated from absolute spectra. Spectrophotometric enzyme assays were performed in a Unicam SP.800 recording spectrophotometer fitted with an SP.820 constant-wavelength scan unit. Fluorescence measurements were made on a Farrand Mark I spectrofluorimeter.

Digestion of denatured protein for iron determination. The precipitate from the acid-labile iron determination was digested in a micro-Kjeldahl flask with 0.1 ml of conc. H_2SO_4 until charring occurred. The flask was then cooled, 0.1 ml of conc. HNO_3 was added and the mixture heated until fumes of sulphuric acid appeared. Addition of HNO_3 dropwise was continued until no further charring occurred. After cooling the flask, 2 drops of 100-volume H_2O_2 were added and the flask was heated until fumes were evolved. This procedure was repeated until the flask contents were colourless. After cooling the flask, 1 ml of water was added and the flask reheated until it fumed. After cooling the flask, 0.6 ml of water, 0.5 ml of 1M-potassium metabisulphite, 0.8 ml of 5M-sodium acetate and 1 ml of iron reagent were added to the mixture. Recovery experiments indicated that 2 μg of Fe could be recovered in 97% yield from 10 mg of bovine serum albumin.

Special chemicals. Ethylmethylamine and methyl-octylamine were Eastman products (Kodak Ltd., Kirkby, Liverpool, U.K.), *n*-butylmethylamine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., methyl-*n*-propylamine and ethyl-*n*-propylamine were from K & K Laboratories, Plainview, N.Y., U.S.A., CO was from Matheson Gas Products, East Rutherford, N.J., U.S.A., *N*-methylglutamate and Chelex 100 (Na^+ form) from Calbiochem Ltd., London W.1, U.K., and 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 obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., Sigma (London) Chemical Co. Ltd., London S.W.6., U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Buffers. Sodium-potassium phosphate and sodium hydrogen maleate buffers were prepared as described by Dawson, Elliott, Elliott & Jones (1969).

Gas mixtures. These were prepared as described by Umbreit, Burris & Stauffer (1964).

RESULTS

It has previously been shown that whole cells of *Ps. aminovorans* grown on dimethylamine will readily oxidize methyl amines (Eady & Large, 1969). This ability is shared by cells grown on primary and tertiary methyl amines and trimethylamine *N*-oxide (T. R. Jarman & P. J. Large, unpublished work). Extracts of cells grown on

Table 2. *Formation of formaldehyde from dimethylamine by ammonium sulphate-treated crude extracts of Ps. aminovorans grown on dimethylamine*

The complete system contained: 100 μ mol of phosphate buffer, pH 6.5, 1 μ mol of dimethylamine hydrochloride, 0.5 μ mol of NADP⁺, 5 μ mol of sodium DL-isocitrate, crude extract precipitated with 100%-saturated $(NH_4)_2SO_4$ and dialysed (3.5 mg of protein), and water to 1.1 ml. After 30 min at 30°C, the reaction was terminated by adding 0.1 ml of 10% (w/v) trichloroacetic acid, and formaldehyde in the supernatant was determined as described in the Materials and Methods section.

Component omitted from incubation mixture	Formaldehyde formed (nmol)
No omission	432
No omission, extract boiled	60
Dimethylamine	72
NADP ⁺	24
Isocitrate	36
NADP ⁺ and isocitrate replaced by NADPH (1 μ mol)	264
No omission, N_2 gas phase	120
No omission, but dimethylamine replaced by 10 μ mol of methylamine	48

methylamine, dimethylamine, trimethylamine or trimethylamine *N*-oxide (but not succinate) catalyse the oxygen- and NADPH-dependent formation of formaldehyde from dimethylamine (Table 2). To demonstrate the nicotinamide nucleotide requirement in this system, precipitation of crude extracts with 100%-saturated ammonium sulphate was necessary. Methylamine would not substitute for dimethylamine.

Purification of the enzyme system. Preparations of the enzyme purified from methylamine-grown cells as indicated in Table 1 still contained large amounts of trimethylamine *N*-oxide demethylase activity (Myers & Zatman, 1971), which is present in *Ps. aminovorans* (P. J. Large, unpublished work). The preparation also had 'diaphorase' [NAD(P)H-dye reductase] activity with 2,6-dichlorophenol-indophenol, cytochrome *c* or potassium ferricyanide as electron acceptor, and to a considerable extent this activity co-purified with the dimethylamine-dependent oxidation of NADPH (Table 1).

Attempts to improve the purification by addition of further steps resulted in the loss of most of the activity.

Properties of the partially purified enzyme

Stabilization of the enzyme. The partially purified enzyme was unstable, but could be stabilized considerably by the addition either of dimethylamine or of organic solvents, of which 5% (v/v) ethanol

Fig. 1. Stability of the partially purified mixed-function secondary-amine oxidase system. The preparation (3.4 mg of protein/ml) was incubated at 0°C in 20 mM-phosphate buffer, pH 7.5, containing the following: ○, no addition; △, 5% (v/v) ethanol; ▲, 10% (v/v) ethanol; □, 5 mM-dimethylamine hydrochloride. Samples (0.1 ml) were removed at intervals and assayed by using method (a) of the Materials and Methods section.

Fig. 2. Optimum pH of the partially purified mixed-function secondary-amine oxidase system. The spectrophotometric assay method (a) in the Materials and Methods section was used, except that the pH of the reaction mixture was varied as indicated. ○, Sodium-potassium phosphate buffer; △, tris-HCl buffer.

was most effective (Fig. 1). Accordingly, ethanol was present at this concentration in all buffers, columns etc. used during the purification.

Optimum pH. The enzyme had a broad optimum at about pH 6.8 (Fig. 2).

Sedimentation behaviour. On centrifuging at 150 000g for 5.5 h in 50 mM-phosphate pH 6.8 containing 5% (v/v) ethanol, 35% of the enzyme activity was found in the pellet, and the remaining activity in the lower quarter of the tube. The upper part of the supernatant was devoid of activity.

Substrate specificity. Table 3 shows K_m values and relative reaction rates for active substrates of the enzyme, which were all secondary methyl or ethyl amines. The Table also lists compounds that were not substrates. Primary amines (methylamine, ethylamine and *n*-propylamine) were inhibitors of dimethylamine oxidation. These compounds also showed slight activity in the assay system, but this is probably because of secondary-amine impurities. Dimethylamine, which was a good substrate for the enzyme, showed substrate inhibition at concentrations greater than 1 mM. NADH and NADPH were both active as electron donors, and both had the same V_{max} , but the K_m for NADH (6.5 μ M) was lower than that for NADPH, which was 13.2 μ M. To avoid complications because of NADH oxidase activity, NADPH was used as electron donor during the enzyme purification, but in experiments with the purified enzyme preparation NADH was used except where indicated.

Stoichiometry of the reaction and identity of the products. Table 4 shows that the stoichiometry of dimethylamine oxidation agrees with eqn. (1):

Formaldehyde is identified by its reaction with the specific Nash reagent (Nash, 1953) and by t.l.c. of its 2,4-dinitrophenylhydrazone (see below). The identity of methylamine as the other product was shown (a) by its reaction with the amine dehydrogenase of *Pseudomonas* AM1, which is specific for primary amines (Eady & Large, 1968; Large *et al.* 1969), and (b) by the fact that the spectrum of its *N*-2,4-dinitrophenyl derivative in dioxan-hydrochloric acid (Dubin, 1960) was identical with that of the derivative formed from authentic methylamine (λ_{max} , 353 nm) and different from that of dimethylamine (λ_{max} , 373 nm).

The nature of the aldehyde products with substrates other than dimethylamine was investigated. Total aldehyde formed was determined by the method of Albrecht *et al.* (1962) and formaldehyde separately determined by the Nash (1953) method. Table 5 shows the proportion of aldehyde product that was formaldehyde. T.l.c. of the 2,4-dinitrophenylhydrazones of the aldehydes confirmed that

Table 3. *Substrate specificity of the partially purified secondary-amine oxidase system*

Amine hydrochlorides were tested for activity in the standard spectrophotometric assay at concentrations of 0.6 mM and 66 mM. NADH was used as electron donor. The K_m values for active substrates were determined by Lineweaver-Burk plots. Relative velocities for the various active amines at saturating substrate concentration are expressed as a percentage of the rate with 1 mM-dimethylamine.

(a) Active compounds		
Substrate	Relative velocity	K_m (mM)
Dimethylamine	100	0.058
Ethylmethylamine	65	0.041
Diethylamine	40	0.345
Methyl- <i>n</i> -propylamine	87	0.630
Ethyl- <i>n</i> -propylamine	22	4.16
<i>n</i> -Butylmethylamine	54	5.40
<i>N</i> -Methylethanolamine	19	0.75
(b) Inactive compounds		
Methylamine*	Di-isobutylamine	Sarcosine
Ethylamine*	Di- <i>n</i> -butylamine	<i>N</i> -Methylglutamate*
<i>n</i> -Propylamine*	Diethanolamine	Noradrenaline
Ethanolamine*	Methyl- <i>n</i> -octylamine	Trimethylamine*
Aniline	<i>N</i> -Methylaniline*	Triethylamine
1,2-Diaminopropane	Benzylmethylamine	<i>NN</i> -Dimethylaniline
1,3-Diaminopropane	Putrescine	Tetramethylammonium
Di-isopropylamine	Spermidine	chloride
Di- <i>n</i> -propylamine	Piperidine	Tetraethylammonium
		chloride

* These compounds gave a slow substrate-dependent oxidation of NADH that was judged to be caused by contaminating amines.

Table 4. *Stoichiometry of dimethylamine oxidation by the partially purified secondary-amine oxidase system*

The complete system contained in a final volume of 3.3 ml: 200 μ mol of phosphate buffer, pH 6.8, 1.5 μ mol of NADPH and 370 μ g of partially purified enzyme (or boiled enzyme). The extinction at 340 nm was measured and the reaction started by addition of 1 μ mol of dimethylamine hydrochloride. Oxygen uptake was followed to completion with a Beckman oxygen analyser. After measurement of the final extinction at 340 nm, the mixture was deproteinized by the addition of 0.1 ml of 2.5M-HClO₄. Formaldehyde was determined in the supernatant, and amines after micro-distillation at 40°C overnight in a Conway unit. Methylamine was also measured enzymically (denoted by *). For further details see the Materials and Methods section.

	Complete system	Boiled enzyme
Reactants disappearing (μ mol):		
NADPH	1.20	0.29
Oxygen	1.05	0.02
Products formed (μ mol):		
Methylamine	1.10 (1.02*)	0
Formaldehyde	1.04	0.03
Residual dimethylamine (μ mol)	0	0.99

formaldehyde was the sole product of dimethylamine oxidation, as was acetaldehyde of diethylamine oxidation. Ethylmethylamine gave rise to both these aldehydes, methyl-*n*-propylamine produced formaldehyde and propionaldehyde, *n*-butylmethylamine gave formaldehyde and butyraldehyde, and ethyl-*n*-propylamine gave acetaldehyde and propionaldehyde. Only formaldehyde

was detected as the product of *N*-methylethanolamine oxidation.

Inhibitors. The enzyme was inhibited by cyanide, by bivalent cations and by reagents attacking thiol groups. After 15 min preincubation at 0°C with the enzyme, 2mM-Fe²⁺, Zn²⁺ or Mn²⁺ all produced total inhibition. Potassium cyanide (1mM) produced 70% inhibition under these conditions, and

Table 5. *Formation of aldehydes from secondary amines by the partially purified secondary-amine oxidase system*

Reaction mixtures in stoppered tubes contained: 200 μ mol of phosphate buffer, pH 6.8, 2 μ mol of NADH, substrate at the final concentration shown below, enzyme preparation and water to a final volume of 3 ml. After 30 min at 25°C, the reaction was stopped by adding 0.3 ml of 10% (w/v) trichloroacetic acid. Precipitated protein was centrifuged off and the tubes were cooled to 0°C before removal of the stopper. Products were determined as described in the Materials and Methods section.

Substrate	Final concn. (mM)	Formaldehyde formed (μ mol)	Total aldehyde formed (μ mol)	Formaldehyde as % of total aldehyde
Dimethylamine	1	1.000	1.02	98
Ethylmethylamine	1	0.103	0.66	16
Diethylamine	3	0.013	0.305	4
Methyl- <i>n</i> -propylamine	5	0.127	0.78	16
Ethyl- <i>n</i> -propylamine	10	0	0.311	0
<i>n</i> -Butylmethylamine	20	0.442	0.78	56
<i>N</i> -Methylethanolamine	1	0.210	0.195	100

mercurials (sodium salts of mersalyl and *p*-chloro-mercuribenzoate) were effective in the micromolar concentration range. In contrast, *N*-ethylmaleimide and iodoacetamide were only effective above 1 mM, even with 15 min preincubation. Chelating agents (EDTA, 8-hydroxyquinoline, 2,2'-bipyridyl and diethyldithiocarbamate) and proadifen hydrochloride (SKF 525-A) were not inhibitory at concentrations of 1 mM. Cysteine and glutathione at 3 mM did not inhibit the enzyme, but other thiol compounds, notably 2-mercaptoethanol, dithiothreitol and 2-mercaptoethylamine were very effective inhibitors and preincubation was not necessary. 2-Mercaptoethylamine was especially potent and seemed to act by a mechanism different from that of the other inhibitors, since it showed competitive inhibition with a K_i of 0.9 μ M, whereas 2-mercaptoethanol showed non-competitive inhibition with a K_i of 0.16 mM. The method of Dixon (1953) was used in these kinetic analyses. Methylamine, ethylamine and ethanolamine (all 3 mM) produced 20–30% inhibition. The K_i for ethanolamine was 2.3 mM.

Carbon monoxide was a potent inhibitor. When the gas was bubbled through the enzyme solution for 15 s, 88% loss of activity was observed when tested in the standard spectrophotometric assay. This loss of activity could be completely reversed by bubbling nitrogen through the carbon monoxide-treated enzyme for 3 min in the dark. Illumination of the enzyme without passing nitrogen through the solution did not reactivate the enzyme. The effect of carbon monoxide concentration was investigated by determining the amount of formaldehyde formed in sealed Thunberg tubes under an atmosphere of air containing increasing carbon monoxide concentrations. Carbon monoxide (5%,

v/v) caused 92.5% inhibition of formaldehyde formation, 10% caused 95.9% inhibition and 26% caused 98% inhibition. This effect occurred because of positive inhibition: it was not due merely to dilution of oxygen since addition of 26% (v/v) nitrogen to air caused only a 4% inhibition of formaldehyde formation.

Spectral properties. The absorption spectrum of the partially purified preparation is shown in Fig. 3(b). There was an absorption maximum at 412 nm and shoulders at 427 and 550 nm. Reduction with dithionite bleached the 412 nm peak and enhanced the shoulder at 426 nm so that it became a peak, and new peaks appeared at 550 and 580–588 nm in the visible region (Fig. 4b). Essentially similar results were obtained with NADH (Fig. 3b). The difference spectrum (Fig. 3a) of reduced-minus-oxidized enzyme had peaks at 430, 549 and 578 nm and troughs at 408 and 460–500 nm. The spectral changes produced by addition of 0.25 mM-NADH were reversed by addition of 1.25 mM-dimethylamine hydrochloride.

The effect of dithionite reduction on enzyme that had been bubbled with carbon monoxide is shown in Fig. 4(b). There was a pronounced enhancement and sharpening of the Soret band and a shift to 422 nm, and the peaks in the visible spectrum became shoulders. The difference spectrum (dithionite-reduced enzyme as reference) (Fig. 4a) had a peak at 420 nm and a trough at 440 nm (cf. Peterson, 1970).

Content of iron and flavin in the preparation. The absorption spectrum of the enzyme (Fig. 3) suggests that it contains a haem derivative. The effect of reduction suggests also that a component of the preparation absorbing in the 450–470 nm region of the spectrum is being bleached. Such a component

Fig. 3. Effect of reduction on the spectrum of the partially purified mixed-function secondary-amine oxidase. To 0.4 ml of enzyme preparation (25 mg of protein/ml) in a 1 cm-light-path micro-cuvette was added 0.5 μ mol of NADH or 1 mg of solid $\text{Na}_2\text{S}_2\text{O}_4$. (a) Difference spectra: ----, NADH-reduced minus untreated enzyme; —, dithionite-reduced minus untreated enzyme. (b) Absolute spectra (water in reference cuvette): ----, untreated enzyme; — NADH-reduced enzyme. (For dithionite-reduced absolute spectrum, see Fig. 4.)

could be flavin, and the fluorescence spectrum of the enzyme preparation showed excitation maxima at 250, 370 and 465 nm (uncorrected). The emission maximum caused by excitation at any of these wavelengths was 522 nm. Such behaviour is typical of flavins and certain flavoproteins. Acid-labile (non-haem) iron and non-acid-labile iron were both also present in the preparation. A typical analysis showed the presence of 0.40 nmol of FMN and 0.15 nmol of FAD/mg of protein, and 0.94 nmol of acid-extractable iron and 0.48 nmol of non-acid-extractable iron/mg of protein. This corresponds to ratios of 1.7:1 for acid-extractable iron to total flavin and 1.9:1 for acid-extractable to non-acid-extractable iron. Preliminary experiments indicated that acid-labile sulphide may also be present in the enzyme preparation. On reduction of the preparation with 2 mM-dithionite, the electron-paramagnetic-resonance spectrum at 77°K showed a signal centred around $g = 1.946$. This is characteristic of non-haem iron-sulphur proteins (Palmer & Sands, 1966; Hall & Evans, 1969). The signal was also produced on reduction of the preparation with 2 mM-NADPH.

Fig. 4. Effect of carbon monoxide on the spectrum of the partially purified mixed-function secondary-amine oxidase. The preparation (16 mg of protein/ml) was bubbled for 20 s with CO and then reduced by the addition of 1 mg of solid $\text{Na}_2\text{S}_2\text{O}_4$. (a) Difference spectrum of carbon monoxide-treated reduced enzyme minus reduced enzyme. (b) Absolute spectra (water in reference cuvette): —, dithionite-reduced enzyme; ----, carbon monoxide-treated dithionite-reduced enzyme.

DISCUSSION

Apart from their involvement in a large variety of hydroxylation and *O*- and *N*-demethylation reactions in liver microsomal preparations (Estabrook *et al.* 1968), haemoproteins of the cytochrome *P*-450 type (Omura & Sato, 1964a) have been shown to be involved in similar reactions in plants [19-hydroxylation of (–)-kaurene in *Echinocystis macrocarpa* (Murphy & West, 1969)] and bacteria [1-hydroxylation of *n*-octane in *Corynebacterium* 7E1C (Cardini & Jurtshuk, 1970); 5-hydroxylation of (+)-camphor in *Pseudomonas putida* (Katagiri, Ganguli & Gunsalus, 1968); and, possibly, hydroxylation of δ -carotene in *Staphylococcus aureus* (Hammond & White, 1970)]. Reports on *O*-demethylation reactions (Bernhardt, Staudinger & Ullrich, 1970) and ω -hydroxylation of fatty acids or 1-hydroxylation of *n*-hexane (McKenna & Coon, 1970; Peterson, 1970) suggest,

however, that not all such bacterial systems involve cytochrome-*P*-450-type haemoproteins. The present work reports a novel finding, that the *N*-demethylation of dimethylamine in *Ps. aminovorans* involves a carbon monoxide-sensitive haemoprotein with spectral properties resembling enzymically active cytochrome *P*-420 rather than cytochrome *P*-450.

Although cytochrome *P*-420 (Omura & Sato, 1964b) is commonly regarded as an inactive degradation derivative of cytochrome *P*-450, indications that a bacterial cytochrome-*P*-420-type haemoprotein may be active in the *n*-octane hydroxylation system of *Corynebacterium* 7EIC were obtained by Cardini & Jurtshuk (1970). When this organism was grown on acetate, although devoid of cytochrome *P*-450, it contained small quantities of cytochrome *P*-420 and also showed activity in the hydroxylation system when combined with the flavoprotein fraction from *n*-octane-grown cells. Additional evidence of the possible functionality of compounds the carbon monoxide derivative of which has an absorption maximum at 420 nm is suggested by observations that there is a peak at 420 nm in the photochemical action spectra for the reversal of carbon monoxide inhibition of cyclohexane hydroxylation in rat liver microsomal preparations (Diehl, Capalna & Ullrich, 1969) and of steroid 11 β -hydroxylation in adrenal-cortex mitochondria (Wilson & Harding, 1970). Appleby (1969) seems to regard the cytochrome-*P*-420-type and cytochrome-*P*-428-type haemoproteins of *Rhizobium japonicum* bacteroids as being functional rather than degraded entities, although no enzymic function has yet been found for them.

Of the *b*-type cytochromes found in bacteria, apart from haemoprotein *P*-450, only haemoprotein-558 from *Acetobacter suboxydans* and cytochrome *o* (Castor & Chance, 1959; Revsin & Brodie, 1969) react with carbon monoxide (Kamen & Horio, 1970), and these have spectral maxima in the 500–600 nm region that are different from the present preparation. The spectral properties of the haemoprotein obtained by us show resemblances in the Soret region to those of cytochrome *o*, which Peterson (1970) has shown to be present in *Pseudomonas oleovorans* when grown on *n*-hexane, but not when it is grown on glucose. There are differences between the two spectra in the 500–600 nm region, however. The close association of cytochrome *o* obtained by Peterson (1970) with the hexane 1-hydroxylation system of *Ps. oleovorans* has not been demonstrated, and the latter reaction is not inhibited by carbon monoxide (Peterson, Basu & Coon, 1966; McKenna & Coon, 1970).

The substrate specificity of the present enzyme is much more limited than the substrate specificity

reported for solubilized microsomal cytochrome *P*-450 preparations (Lu, Strobel & Coon, 1970) in that only secondary amines containing a methyl or an ethyl group seem to be active substrates, and reactivity decreases with increasing alkyl chain length (Table 3). Although microsomal preparations will demethylate or de-ethylate secondary amines, the substrate specificity of the purified mixed-function amine oxidase from microsomal preparations shows a different pattern from the present system (Ziegler *et al.* 1969). The *Ps. aminovorans* enzyme does not oxidize tertiary amines. Moreover, the purified microsomal enzyme contained only flavin, cytochrome *P*-450 being absent.

The observation that the enzyme system is stabilized by organic solvents is not new. Several mammalian enzymes are stabilized by organic solvents (George, McMahan, Bowler & Elliott, 1969), as well as bacterial dioxygenases (Nazaki, Kagamiyama & Hayaishi, 1963) and also *p*-anisate *O*-demethylase (Bernhardt *et al.* 1970) and benzyl alcohol dehydrogenase (Suhara, Takemori & Katagiri, 1969).

The stoichiometry of the reaction is in accordance with eqn. (1), which is typical of a monooxygenase (mixed-function oxidase). Of the products of the reaction, methylamine is oxidized well by whole cells, and formaldehyde scarcely at all. Extracts, however, contain a glutathione- and NAD-dependent formaldehyde dehydrogenase (Eady, 1970), but no cell-free methylamine-oxidizing system has yet been detected.

It seems possible that the sonic disruption used to prepare extracts in the present work has broken down and solubilized a much larger particulate enzyme complex similar to that observed in *Pseudomonas* MS by Kung & Wagner (1970), who prepared their extracts with a French pressure cell. Preliminary observations suggest that the mixed-function secondary-amine oxidase activity and trimethylamine *N*-oxide demethylase activity may be closely associated in a sedimentable particulate fraction in *Ps. aminovorans*. This association may extend to the enzymes oxidizing mono- and trimethylamine. Trimethylamine oxidation in *Ps. aminovorans* is mediated not by a dehydrogenase of the type found in bacterium 4B6 by Colby & Zatman (1971) but by an NADPH-dependent mixed-function oxidase (M. J. C. Crabbe & P. J. Large, unpublished work).

Although organic solvents are known to convert cytochrome *P*-450 into cytochrome *P*-420 (Ichikawa, Yamano & Fujishima, 1969), the characteristic spectral properties of the active cytochrome-*P*-420-type haemoprotein can be observed in crude extracts that have not been exposed to ethanol, and hence an ethanol-mediated

conversion is unlikely. The retention of activity supports this conclusion, since in the microsomal system conversion of cytochrome *P*-450 into cytochrome *P*-420 is accompanied by a loss of activity (Ichikawa *et al.* 1969).

Although acid-extractable and non-acid-extractable iron and flavin have been detected in the enzyme preparation, and reduction with NADPH or dithionite at 77°K gives a characteristic electron-paramagnetic-resonance signal at around $g = 1.946$, suggestive of a non-haem iron-sulphur component, there is as yet no direct evidence implicating these components in the overall process of dimethylamine-dependent electron transfer from NAD(P)H to oxygen. In the absence of this evidence, particularly in a preparation that has only a low degree of purity, we can only tentatively suggest that the system resembles the camphor-methylene hydroxylase system described by Katagiri *et al.* (1968); this involves three proteins, one of which, putidaredoxin, is a non-haem iron-sulphur protein. The electron-paramagnetic-resonance spectrum suggests that the non-haem iron component is of the iron-sulphur type rather than rubredoxin, which is a component of the *Ps. oleovorans* ω -hydroxylation system, and which does not show an electron-paramagnetic-resonance signal at $g = 1.94$ (Peterson & Coon, 1968). Spinach or *Clostridium pasteurianum* ferredoxin failed to stimulate formaldehyde formation by the unfractionated system (T. R. Jarman, unpublished work). This may not be significant, since the components are probably very tightly associated. Fractionation of the system with reconstitution of the presumed multiple protein components to give an active mixed-function oxidase has not so far proved possible. The NAD(P)H-dye reductase activity may well reflect interaction of the electron acceptors with the reduced enzyme system, as is observed in the *n*-octane-hydroxylation system (Cardini & Jurtshuk, 1970), and the co-purification of 'diaphorase' activity with mixed-function dimethylamine oxidase activity supports this hypothesis. But with a partially purified preparation of the present kind it is not possible to draw a definite conclusion, since the dye reductase activity may be an unrelated contaminant of the system.

The enzyme has been found in other amine-metabolizing micro-organisms. It is present in *Hyphomicrobium vulgare* NQ grown on trimethylamine (M. J. C. Crabbe & P. J. Large, unpublished work). Colby & Zatman (1971) have shown its presence in trimethylamine-grown bacterium 4B6, and it is also present in *Bacillus* PM6 (P. A. Myers, personal communication).

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