OXIDATION OF *N*-ALKYL- AND *NN*-DIALKYLHYDROXYLAMINES BY PARTIALLY PURIFIED PREPARATIONS OF TRIMETHYLAMINE MONO-OXYGENASE FROM *PSEUDOMONAS AMINOVORANS*

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Received 5 May 1975

1. Introduction

Partially purified preparations of trimethylamine mono-oxygenase from *Pseudomonas aminovorans* catalyse the oxidation of trimethylamine to trimethylamine *N*-oxide (equation [1]),

$$(CH_3)_3N + NADPH + H^+ + O_2 \longrightarrow (CH_3)_3NO + NADP^+ + H_2O \quad (1)$$

and of dimethylamine to formaldehyde and another product which was not methylamine [1]. In the present paper we present evidence suggesting that the unknown oxidation product is *N*-methylhydroxylamine. We also show that *NN*-dimethylhydroxylamine is a substrate for the enzyme, and gives the same oxidation products as dimethylamine. The reaction product *N*-methylhydroxylamine is itself slowly oxidized by preparation in the presence of NADPH and oxygen to an unidentified product which may possibly be formamide. The corresponding *N*-ethyl- and *NN*-diethylhydroxylamines are also oxidized by the enzyme. We suggest the following reaction sequence to explain our observations:

$$(CH_3)_2NH \xrightarrow[O_2]{NADPH} (CH_3)_2NOH \xrightarrow[O_2]{NADPH} HCHO + CH_3NHOH \xrightarrow[O_2]{NADPH} ? \; HCONH_2 \quad (2)$$

2. Materials and methods

2.1. *Materials*

Amine hydrochlorides were obtained from BDH Chemicals Ltd, Poole, England. NADPH was from Sigma London Chemical Co., Kingston on Thames England. *N*-Methylhydroxylamine hydrochloride and *NN*-diethylhydroxylamine were from Ralph N. Emanuel Ltd, Wembley, England. 2,4-Dichloro-6-phenylphenoxyethylamine and its *NN*-diethyl derivative were gifts from Eli Lilly and Co., Indianapolis, USA. Trimethylsulphonium chloride was from K and K Chemicals, Plainview, New York USA. *NN*-Dimethylhydroxylamine hydrochloride was synthesized from *NN*-dimethylcyclohexylamine (K and K Chemicals) by pyrolysis of the *N*-oxide [2] as described by Rogers [3]. *N*-Ethylhydroxylamine was synthesized by reduction of nitroethane as described in [4] for *N*-phenylhydroxylamine and isolated as the oxalate [5]. Purity of the two synthetic materials was checked by thin-layer chromatography on silica gel G in acetone methanol (60:40) [6], and quantitatively confirmed by two methods of analysis [7,8].

2.2. *Enzyme purification and assay*

Trimethylamine mono-oxygenase was partially purified from trimethylamine grown *Pseudomonas aminovorans* and assayed spectrophotometrically at pH 8.0 as previously described [1].

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Table 1

V_{max} and apparent K_m values for the oxidation of amines and substituted hydroxylamines by partially purified trimethylamine mono-oxygenase

Substrate	V_{max} (μmol/min/mg protein)	Apparent K_M (mM)	Substrate	V_{max} (μmol/min/mg protein)	Apparent K_M (mM)
Trimethylamine	0.34	0.009	Triethylamine	0.12	0.082
Dimethylamine	0.17	0.51	Diethylamine	0.59	0.72
NN-Dimethylhydroxylamine	0.20	0.077	*NN*-Diethylhydroxylamine	0.18	0.23
N-Methylhydroxylamine	0.11	19.2	*N*-Ethylhydroxylamine	0.70	2.30

Assay cuvettes contained in total volume of 3 ml: enzyme preparation (170 μg of protein), 67 mM phosphate buffer pH 8.0, 0.167 mM NADPH, dissolved oxyge in equilibrium with air (0.24 mM), substrate and water. The blank cuvette contained buffer. The reciprocal rate of NADPH oxidation was plotted against the reciprocal of substrate concentration. Temperature: 25°C.

2.3. *Chemical determinations*

Oxygen was measured polarographically [1], and NADPH spectrophotometrically at 340 nm. *N*-Methylhydroxylamine was measured by the method of Kadlubar et al. [7] except that the ethanol deproteinizing step was omitted. Under these conditions phosphate buffer interfered with the colorimetric reaction, and was replaced by glycine-NaOH buffer when *N*-methylhydroxylamine was to be estimated. Other estimation methods were: formaldehyde [9] and methylamine [10].

3. Results

3.1. *Oxidation of NN-dialkyl- and N-alkyl-hydroxylamine derivatives by the enzyme preparation*

In addition to the tertiary and secondary amines previously reported [1] to be oxidized by the enzyme preparation, table 1 shows that *NN*-dialkylhydroxylamines are good substrates, having similar V_{max} values to the corresponding secondary amines and lower apparent K_M values. Thus if an *NN*-dialkylhydroxylamine (*N*-hydroxy amine) were the first product of secondary amine oxidation sequence [2]), it would not be detected, since it would be rapidly oxidized further. The corresponding *N*-monoalkylhydroxylamines are poorer substrates, and would be expected to accumulate if they were formed by oxidation of secondary amines or *NN*-dialkylhydroxylamines. The pH optimum for the oxidation of *NN*-dimethylhydroxylamine was 8.5, the same as for the oxidation of dimethylamine [1].

3.2. *Products and stoichiometry of the enzymic oxidation of dimethylamine and NN-dimethylhydroxylamine*

Formaldehyde was an oxidation product of both dimethylamine and *NN*-dimethylhydroxylamine (table 2); the stoichiometry for the oxidation was in accordance with sequence [2] above. No methylamine was detected as a product of *NN*-dimethylhydroxylamane oxidation. These observations support, although they do not prove, the concept that dimethylhydroxylamine is the first product of dimethylamine oxidation. Under the conditions of table 2, *N*-methylhydroxylamine ought to accumulate if it is a reaction product. Thin-layer chromatography [6] of reaction mixtures containing enzyme, NADPH and either dimethylamine or *NN*-dimethylhydroxylamine showed in both cases a product with reducing properties when sprayed with aqueous 1% (w/v) $KMnO_4$ which had an identical R_f value to that of authentic *N*-methylhydroxylamine. Fig.1 demonstrates that the formation of *N*-methylhydroxylamine and formaldehyde from dimethylamine showed close stoichiometry in the first 10 min of the reaction.

3.3. *Product of the enzymic oxidation of N-methylhydroxylamine*

The further oxidation of *N*-methylhydroxylamine by the enzyme did not give rise to formaldehyde,

Table 2

Stoichiometry of NADPH and O_2 consumption and formaldehyde formation with dimethylamine and *NN*-dimethylhydroxylamine as substrates for trimethylamine mono-oxygenase

Substrate	O_2 consumed (μmol)	NADPH consumed (μmol)	HCHO formed (μmol)
Dimethylamine	0.439	0.396	0.170
NN-Dimethylhydroxylamine	0.223	0.227	0.162

Assay cuvettes were as table 1 except that 33 mM sodium pyrophosphate buffer pH 8.5 was used, 0.3 mM NADPH and 0.20 μmol of substrate were present in the reaction mixture with 300 μg of enzyme protein in a total volume of 3.3 ml. The temperature was 25°C. The reaction was followed to completion on a Unicam SP1800 spectrophotometer at 340 nm or in a Beckman model 777 oxygen electrode, and formaldehyde formed was measured at the end of the reaction after deproteinization with 0.3 ml 2.5 M $HClO_4$ (see section 2.3).

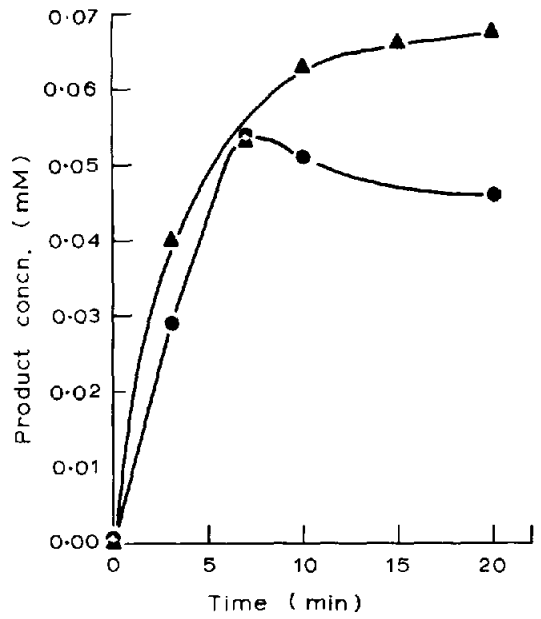

Fig.1. Formation of formaldehyde and *N*-methylhydroxylamine from dimethylamine by the trimethylamine monooxygenase preparation. The reaction mixture contained 0.21 mM NADPH, 4.2 mM dimethylamine hydrochloride, enzyme (17 mg of protein), 40 mM glycine-NaOH buffer pH 8.0, and water in a total vol of 12 ml. The incubation was at 25°C and duplicate samples (0.5 ml) were removed at intervals for formaldehyde (•) and *N*-methylhydroxylamine (▲) estimation (see section 2.3).

formate (sought by the method of Johnson et al. [11]) ammonia (tested for by the indophenol method [12]), or formaldoxime. The product of the reaction gave a hydroxamate when incubated with neutral hydroxylamine and ferric chloride, but has not yet been identified. A possible product is formamide (equation [3]):

$$CH_3NHOH + NADPH + H^+ + O_2 \longrightarrow HCONH_2 + NADP^+ + 2\,H_2O \quad (3)$$

but further experimental evidence will be needed for confirmation of this proposal.

3.4. *Inhibition of amine and substituted hydroxylamine oxidation by the enzyme*

The oxidation of substituted hydroxylamines was sensitive to the same inhibitors as dimethylamine and trimethylamine oxidation, although the percentage inhibition observed (at substrate concentrations approx. 10 times the apparent K_M) showed some variation. 2,4-Dichloro-6-phenylphenoxyethylamine (Lilly 53325) and 2,4-dichloro-6-phenylphenoxyethyldiethylamine (Lilly 18947) when tested at a concentration of 0.3 mM showed between 20 and 80% inhibition of the oxidation of each of the substrates listed in table 1. Tetramethylammonium chloride (5 mM) and trimethylsulphonium chloride (5 mM) caused between 60 and 90% inhibition of the oxidation of the same substrates.

4. Discussion

With the present state of purity of the preparation, it is not possible to decide whether all the activities are due to a single enzyme or to several similar enzymes, but the simplest explanation of our observations would be a single *N*-hydroxylating enzyme of broad specificity, catalysing the successive *N*-hydroxylation of dimethylamine to *NN*-dimethylhydroxylamine and of the latter to its *N*-oxide. This would decompose spontaneously to *N*-methylnitrone, which would be instantly hydrolysed [13] to formaldehyde and *N*-methylhydroxylamine (as in equations [4] to [7] below)

$$(CH_3)_2NH + NADPH + H^+ + O_2 \longrightarrow (CH_3)_2NOH + NADP^+ + H_2O \quad (4)$$

$$(CH_3)_2NOH + NADPH + H^+ + O_2 \longrightarrow (CH_3)_2N(\rightarrow O)OH + NADP^+ + H_2O \quad (5)$$

$$(CH_3)_2N(\rightarrow O)OH \longrightarrow CH_2{=}N(\rightarrow O)\text{-}CH_3 + H_2O \quad (6)$$

$$CH_2{=}N(\rightarrow O)\text{-}CH_3 + H_2O \longrightarrow HCHO + CH_3NHOH \quad (7)$$

This would make the trimethylamine mono-oxygenase very similar to the liver microsomal dimethylaniline mono-oxygenase (*N*-oxide forming) (EC 1.14.13.8) which oxidises secondary amines to *NN*-dialkylhydro-

xylamines [14]. Kadlubar et al. [7] have demonstrated the NADPH- and oxygen-dependent formation of nitrones and nitrone hydrolysis products from *NN*-disubstituted hydroxylamines by microsomal preparations, and also [15] by the purified microsomal mono-oxygenase (EC 1.14.13.8). Unlike the liver system [7], our bacterial preparation also catalyses the slow oxidation of *N*-alkylhydroxylamines to products which are possibly the corresponding acid amides.

The products of dimethylamine oxidation (equations [4] to [7]) with trimethylamine mono-oxygenase are different from those (formaldehyde and methylamine) formed from dimethylamine by the secondary-amine mono-oxygenase (EC 1.14.99.-) of this organism [16,17] which is present in the same cells. The presence of this second mechanism for dimethylamine oxidation suggests to us that the role of the trimethylamine mono-oxygenase is to oxidize trimethylamine only as far as its *N*-oxide (equation [1]), and that its activity with secondary amines and substituted hydroxylamines is non-physiological.

Acknowledgements

We thank Eli Lilly and Company, Indianapolis, USA for the gift of the inhibitors. The bacterial cells used in this work were obtained under grant B/RG/08971 from the Science Research Council, which is gratefully acknowledged.

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