NON-OXIDATIVE DEMETHYLATION OF TRIMETHYLAMINE *N*-OXIDE BY *PSEUDOMONAS AMINOVORANS*

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1. Introduction

Trimethylamine *N*-oxide is an important constituent of fish muscle and body fluids [1]. Most biochemical work, e.g. [2], has concentrated on its biological reduction to trimethylamine. This paper describes the partial purification of an enzyme from methylamine-grown *Pseudomonas aminovorans* catalysing the non-oxidative, non-hydrolytic cleavage of trimethylamine *N*-oxide (equation (1)):

$$(CH_3)_3NO \longrightarrow (CH_3)_2NH + HCHO \qquad (1)$$

An enzyme with somewhat different properties catalysing the same reaction has recently been described in *Bacillus* PM6 [3]. The enzyme from *P. aminovorans* has a pH optimum of 6.0 and a K_m for trimethylamine *N*-oxide of 2 mM. The equilibrium constant for reaction (1) was 6.06 mM at 25°. Triethylamine *N*-oxide was not a substrate, but inhibited the enzyme (K_i 8.1 mM). Other inhibitors included cyanide and mercurials. Pyridine, SKF 525-A and methylamines were not inhibitors. The enzyme may play an important role in growth on methylamines.

2. Materials and methods

2.1. *Growth and maintenance of the organism*

Pseudomonas aminovorans NCIB 9039 was grown as described previously [4].

2.2. *Assay for enzyme activity*

Trimethylamine *N*-oxide demethylase activity was measured in the following system: 100 μmoles of sodium hydrogen maleate buffer pH 6.0, 20 μmoles of trimethylamine *N*-oxide hydrochloride, enzyme and water to a final volume of 1.3 ml. The reaction was started by addition of substrate and terminated after 20 min incubation at 25° by addition of 0.2 ml of 2.5 M $HClO_4$. After centrifuging, formaldehyde in the supernatant was estimated [5].

2.3. *Chemical estimations*

These were performed by the following methods: dimethylamine [6], methylamine [7], total aliphatic aldehydes [8], protein [9], and trimethylamine *N*-oxide [10].

When enzymically-formed formaldehyde was present in the same solution as trimethylamine *N*-oxide, it was first eliminated by oxidation to formate as follows. The perchloric acid was neutralised by addition of 0.1 of a volume of 2.5 M NaOH. Then 0.05 ml of 100 volume H_2O_2 was added and the samples incubated at 50° for 10 min. The trimethylamine *N*-oxide was then estimated by the above method.

2.4. *Purification of the enzyme*

The enzyme was unstable when purified more than about 5-fold, and higher purifications were not obtained. The following method gave a reasonably stable preparation.

Crude ultrasonic extracts, after centrifuging at 25,000 *g* for 20 min, were made 50% saturated by addition of solid ammonium sulphate. The precipitated protein was removed by centrifuging at 25,000 *g* for 20 min at 0° and redissolved in 10 mM phosphate pH 7.4 and dialysed at 4° against the same buffer for 4 hr. It was then applied to a column (30 cm × 2 cm) of DEAE-cellulose (Whatman DE32) and eluted at 4°

with a linear gradient of 10–500 mM sodium potassium phosphate pH 7.5 (gradient volume, 400 ml). Activity was eluted between 0.28 and 0.3 M phosphate. Purification was 4.8-fold.

3. Results

3.1. *Formation of formaldehyde from various amine substrates by extracts*

The data in table 1 show that: (a) methylamine and formaldehyde were formed from dimethylamine in the presence of NADH and oxygen; (b) there was an oxygen – and NADH – independent formation of formaldehyde from trimethylamine *N*-oxide, and in the presence of oxygen and NADH, methylamine was also found; (c) trimethylamine cannot be an intermediate in the conversion of trimethylamine *N*-oxide to formaldehyde and methylamine. The formation of methylamine and formaldehyde under these conditions is due to the mixed function secondary amine oxidase present in these cells [4] (equation (2)):

$$(CH_3)_2NH_2^+ + NAD(P)H + H^+ + O_2 \longrightarrow$$

$$CH_3NH_3^+ + NAD(P)^+ + H_2O + HCHO \qquad (2)$$

It can thus be concluded that there is present in the preparation an additional enzyme system which catalyses the formation of formaldehyde from trimethylamine *N*-oxide in the absence of NADH and O_2 (equation (1)), and it seems probable that the other product of the reaction is dimethylamine.

3.2. *Properties of the partially purified enzyme*

3.2.1. pH optimum and effect of pH on stability

The enzyme had a pH optimum of 6.0 (fig. 1, curve A) and sodium hydrogen maleate pH 6.0 was selected as the buffer for the standard assay. The fall-off in activity below pH 5 or above pH 7.5 is not due to inactivation of the enzyme, since exposure of the enzyme for 30 min at 0° to buffers of pH values in the region 4 to 9 did not result in loss of activity (fig. 1, curve B) when the enzyme was brought back to pH 6.0 and tested in the standard assay.

3.2.2. Substrate specificity and effect of substrate concentration on activity

By varying the concentration of trimethylamine *N*-oxide in the standard assay, its K_m was found to be 2 mM from a double reciprocal plot. Triethylamine *N*-oxide when tested in the assay did not give any detectable amount of acetaldehyde. Benzyldimethylamine *N*-oxide was a substrate, the maximum rate being about 50% of that with trimethylamine *N*-oxide, but the K_m for this substrate was very high (about 0.14 M).

Table 1

Formation of formaldehyde and methylamine from various amines by partially-purified preparations from methylamine-grown *P. aminovorans*.

Tube no.	Substrate	Other reactants	NADH consumed (μmoles)	HCHO formed (μmoles)	Methylamine formed (μmoles)
1	Trimethylamine oxide	Complete	4.37	13.2	2.3
2	Trimethylamine oxide	Complete, but enzyme boiled	0	0	0
3	Trimethylamine oxide	No NADH	–	9.9	0
4	Trimethylamine oxide	N_2 gas phase	0.87	13.6	1.37
5	Trimethylamine	Complete	2.09	0	0
6	Dimethylamine	Complete	2.80	7.2	2.23
7	Methylamine	Complete	0	0	–

The enzyme preparation was a crude extract which had been centrifuged at 150,000 *g* for 90 min and the supernatant was treated with ammonium sulphate. The 35–50% saturation fraction was dialysed before use. The complete system contained: enzyme (2.1 mg protein), 40 μmoles of substrate, 20 μmoles of NADH, 200 μmoles of phosphate buffer pH 7.5 and water to 5 ml. The tubes were incubated for 60 min at 25° (tube 4 under nitrogen) and the absorbance of each mixture was read at 340 nm before stopping the reaction with 0.5 ml of 2.5 M $HClO_4$.

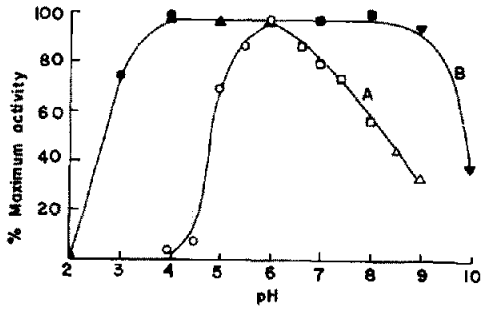

Fig. 1. Effect of pH on the activity and stability of trimethylamine *N*-oxide demethylase. Curve A shows the effect of pH on the activity of the partially-purified enzyme (see section 2.4) at 25°. Buffers: ○, succinate; □, phosphate and △, Tris-HCl. Curve B shows the effect of incubating partially-purified enzyme at the pH values shown, for 30 min at 0°, followed by adjustment of the pH to 6.0 with excess maleate buffer and assaying under standard conditions (see section 2.2). Buffers: ●, glycine-HCl; ▲, acetate; ■, phosphate and ▲, glycine-NaOH.

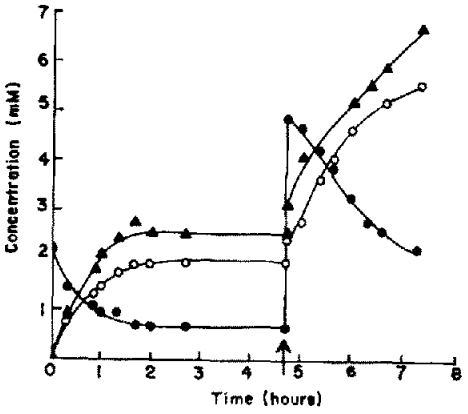

Fig. 2. Equilibrium constant of the trimethylamine *N*-oxide demethylase-catalysed reaction. The initial concentration of trimethylamine *N*-oxide hydrochloride was 2.5 mM in 33 mM maleate buffer pH 6.0, and 3.2 mg of enzyme preparation (section 2.4) were present. Samples were removed at intervals for analysis (see section 2.3). Concentrations: ●, trimethylamine *N*-oxide; ▲, dimethylamine and ○, formaldehyde. At the point indicated by the arrow, further trimethylamine *N*-oxide was added. Incubation temperature was 25°.

3.2.3. Effect of inhibitors

Chelating agents were not inhibitory at 2 mM, nor were carbon monoxide and -SH compounds which are potent inhibitors of the secondary amine oxidase reaction [4]. Pyridine (10 mM) and SKF 525-A (0.1 mM) did not inhibit, nor did methylamine, dimethylamine or trimethylamine. Cyanide and mercurials were the most effective inhibitors, being active at 1 μM. The former showed competitive inhibition, with a K_i of 40 μM. Triethylamine *N*-oxide was an inhibitor, showing mixed inhibition with a K_i of 8.1 mM.

3.3. *Equilibrium constant, stoichiometry and identity of the reaction products*

The equilibrium constant was determined by analyzing samples removed at intervals from the reaction mixture as shown in fig. 2. At equilibrium, when the millimolar concentration of each product was 1.94, that of trimethylamine *N*-oxide was 0.62. This gives an equilibrium constant at 25° of 6.06 mM. To ensure that the enzyme was still active in the equilibrium system, further trimethylamine *N*-oxide was added and the reaction resumed. Despite the anomalously high dimethylamine values, the stoichiometry of the reaction conforms to equation (1). Since the amine product reacts with 1-fluoro-2,4-dinitrobenzene, it must be a primary or a secondary amine. The 2,4-dinitrophenyl derivative formed in the Dubin [6] reaction had an absorption maximum in dioxan-HCl of 370 nm. The derivative of dimethylamine under these conditions had an absorption maximum at 372 nm, while that of methylamine had an absorption maximum at 353 nm. The R_F of the amine formed was identical with the R_F of authentic dimethylamine on thin layer chromatography in three different solvent systems [11, 12].

4. Discussion

The evidence presented shows that in *P. aminovorans*, trimethylamine *N*-oxide is metabolized via a non-oxidative cleavage to dimethylamine and formaldehyde. This reaction can proceed nonenzymically under certain conditions [13], but in the present conditions no formaldehyde was ever detected in the boiled enzyme controls. These observations, and those of [3] are the first to demonstrate this reaction in bacteria, although it has been suggested that it occurs in fish pyloric caeca [14].

The partially purified preparation from *P. aminovorans* differs in many respects from the more highly

purified enzyme from *Bacillus* PM6 [3]. Thus the former has a different pH optimum (6.0 compared with 7.5), is not inhibited by trimethylamine or proadifen hydrochloride (SKF 525-A), nor is it stimulated by glutathione or ferrous ions. It also differs from the dialkylarylamide *N*-oxide dealkylase of pig liver microsomes [15]; although the overall stoichiometry is similar. The microsomal enzyme is inhibited by SKF 525-A, carbon monoxide and pyridine, none of which affect the *P. aminovorans* enzyme. The enzyme is present in cells grown on methylamine, dimethylamine, trimethylamine and trimethylamine *N*-oxide and is induced before growth begins when succinate-grown *P. aminovorans* is transferred to an amine growth medium [16], suggesting an important role for the enzyme during growth on amines.

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