The Inducible Amine Dehydrogenase in *Pseudomonas putida* NP and its Role in the Metabolism of Benzylamine

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Pseudomonas putida NP utilizes benzylamine and other primary amines as the sole source of carbon, nitrogen and energy. Extracts of organisms grown on benzylamine and other amines contained an inducible amine dehydrogenase [amine:(acceptor) oxidoreductase (deaminating)]. The enzyme required either phenazine methosulphate, 2,6-dichlorophenolindophenol, ferricyanide or cytochrome *c* for activity; oxygen, FAD, FMN, NAD$^+$ and NADP$^+$ were not utilized. The substrate specificity of the amine dehydrogenase was independent of the amine utilized for growth; when cell-free extracts of organisms grown on benzylamine, *n*-propylamine or *n*-butylamine were subjected to polyacrylamide gel electrophoresis, a single band of enzymic activity was detected in an equivalent position in each gel. This indicated that an enzyme of broad specificity was involved in the deamination of these substrates. The amine dehydrogenase was heat labile; 97 % of the initial activity was lost after incubation at 65 °C for 3 min. By isolating intermediates and demonstrating the requisite enzyme activities, it was shown that benzylamine was deaminated to benzaldehyde and further metabolized through benzoate and via the *meta* (or α-keto acid) pathway.

INTRODUCTION

Microbial enzymes which oxidatively cleave alkyl-nitrogen bonds of primary mono- and diamines fall into two categories: oxidases and dehydrogenases. Amine oxidases transfer electrons from the prosthetic group directly to oxygen to form H_2O_2, while an alternative electron acceptor such as phenazine methosulphate (PMS) or cytochrome *c* functions as the immediate electron acceptor for amine dehydrogenases. There are numerous accounts of amine oxidases in bacteria (Bachrach, 1973; Kapeller-Adler, 1970; Large, 1971), but few primary monoamine dehydrogenases have been reported (Blevins & Perry, 1972; Cerniglia & Perry, 1975; Dahl, Mehta & Hoare, 1972; Eady & Large, 1968; Mehta, 1977). Eady & Large (1968) purified and characterized an amine dehydrogenase that was induced in *Pseudomonas* AM1 during growth on methylamine and this enzyme oxidized methylamine to formaldehyde in the presence of either PMS, cytochrome *c*, ferricyanide, brilliant cresyl blue or 2,6-dichlorophenolindophenol (DCPIP). The enzyme had a high affinity for primary mono- and diamines, but little or no activity for isoamines. Recently, Cerniglia & Perry (1975) isolated a strain of *Mycobacterium convolutum* by soil enrichment on *n*-propylamine; this strain also metabolized isopropylamine. Although it was shown that these amines were metabolized via divergent pathways, their initial oxidative deamination was catalysed by a non-specific amine dehydrogenase.

The ability of pseudomonads to utilize amines is particularly useful for the delineation of the fluorescent group of *Pseudomonas*: *P. putida* can be distinguished from *P. aeruginosa* and in most cases from *P. fluorescens* by the capacity to assimilate benzylamine (Stanier,

Palleroni & Doudoroff, 1966). Although the amine specificities of various *Pseudomonas* species were reported by Stanier *et al.* (1966) and Ballard *et al.* (1970), data for the characterization and metabolic studies of the enzymes responsible are lacking. This investigation was initiated to examine the degradation of amines by *P. putida* and to determine the nature of the enzyme responsible for the initial oxidative cleavage of primary amines.

METHODS

Organism. Pseudomonas putida NP was isolated by enrichment culture with naphthalene as a sole source of carbon and energy, and was obtained from Dr David T. Gibson, The University of Texas, Austin, U.S.A. This organism conforms to biotype A as it cannot grow on L-tryptophan, D-galactose or nicotinic acid. Stock cultures were maintained on nutrient agar slants and stored at 4 °C.

Media and growth conditions. Pseudomonas putida NP was cultured on the mineral salts medium of Leadbetter & Foster (1958) supplemented with 10 mM-sodium benzoate or sodium succinate as carbon source. Amines were neutralized with HCl, filter sterilized, and added to give a final concentration of 5 mM. Liquid cultures were inoculated with cells pre-adapted to growth on the appropriate substrate (the amine served as both carbon and nitrogen source) and incubated at 28 °C on a reciprocating shaker at 200 rev. min^{-1}.

Preparation of cell extracts. Cells were grown to late-exponential phase, harvested by centrifuging at 10000g at 4 °C and washed twice with cold 50 mM-sodium phosphate buffer, pH 7·0. Washed cell pastes were resuspended in buffer and disrupted by two passages through a French press at 35 MPa. Cell debris was removed by centrifuging at 37000g for 30 min at 4 °C. Crude extracts were prepared by centrifuging the supernatant at 102000g for 60 min in a Beckman L2–65 ultracentrifuge.

Enzyme assays. Amine dehydrogenase activity was determined spectrophotometrically at 24 °C by measuring the PMS-coupled reduction of DCPIP by the method of Eady & Large (1968).

The following enzymes were assayed as described by Hegeman (1966) and Feist & Hegeman (1969): benzaldehyde dehydrogenase (EC 1.2.1.28; benzaldehyde:NAD$^+$ oxidoreductase), benzyl alcohol dehydrogenase, catechol 1,2-oxygenase [EC 1.13.11.1; catechol:oxygen 1,2-oxidoreductase (decyclizing)] and catechol 2,3-oxygenase [EC 1.13.11.2; catechol:oxygen 2,3-oxidoreductase (decyclizing)].

Protein was determined by the method of Lowry *et al.* (1951).

Polyacrylamide gel electrophoresis. Disc gel electrophoresis was performed as described by Davis (1964) using 7% (w/v) acrylamide gels. The gel and reservoir buffers were prepared according to Laemmli (1970) except that sodium dodecyl sulphate was omitted. Crude extracts were mixed with bromothymol blue in Tris/HCl buffer, pH 7·3, containing 50% (w/v) glycerol. Electrophoresis was conducted at a constant current of 2 mA per gel at 4 °C until the tracking dye had migrated through the gel. Amine dehydrogenase activity in gels was located by coupling enzymic activity to the reduction of tetrazolium chloride (Cerniglia & Perry, 1975).

Other techniques. Manometric techniques were those described by Umbreit, Burris & Stauffer (1964). Manometric vessels contained (in a total volume of 2·4 ml) whole cells (5 mg dry wt) or cell extract (5·4 mg protein) in 200 μmol phosphate buffer pH 7·0, 20 μmol substrate and, in the centre well, 0·2 ml 20% (w/v) KOH. For amine dehydrogenase assays, 3 μmol KCN and 1 μmol PMS were also added. The incubation temperature was 30 °C. All values were corrected for endogenous respiration. The 2,4-dinitrophenylhydrazones were determined as described previously (Blevins & Perry, 1972).

RESULTS

Specificity of P. putida for growth on various amines

Amines used as the sole source of carbon, nitrogen and energy by *P. putida* were limited to C_3, C_4, C_5 and C_7 primary aliphatic monoamines and the substituted primary amines, benzylamine, ethanolamine and histamine. *n*-Butylamine, benzylamine and *n*-propylamine were the most readily utilized growth substrates with generation times of 60, 75 and 130 min, respectively. No growth occurred on methylamine, isopropylamine, 1,2-diaminopropane, *sec*-butylamine, *tert*-pentylamine, octylamine, decylamine, tryptamine, tyramine and phenylethylamine.

Acceptor specificity of amine dehydrogenase

In manometric experiments with cell-free extracts of benzylamine-grown organisms, no oxygen uptake occurred with benzylamine as substrate unless PMS was added. Enzymic

Table 1. *Specific activities of amine dehydrogenase in P. putida grown on various amines*

Enzymic activity in crude extracts was measured by the standard spectrophotometric assay with the substrate added at 6·6 mM. Specific activities are expressed as μmol DCPIP reduced min^{-1} (mg protein)$^{-1}$.

	Growth substrate		
Test substrate	Benzylamine	n-Propylamine	n-Butylamine
Benzylamine	0·21	0·12	0·06
n-Butylamine	0·32	0·20	0·10
n-Propylamine	0·26	0·13	0·07
Methylamine	0·10	0·04	0·03
1,3-Diaminopropane	0·05	0·02	0·02
Ethanolamine	0·03	0·01	0·01

activity could be measured spectrophotometrically by replacing oxygen with DCPIP. Other electron acceptors included ferricyanide and cytochrome *c*. FAD, FMN, NAD$^+$ and NADP$^+$ could not serve as the initial electron acceptor (Durham & Perry, 1977).

Induction of amine dehydrogenase activity

Amine dehydrogenase activity was not detected in cell extracts of benzoate- or succinate-grown cells by the standard assay procedure or by gel electrophoresis. Induction of the enzyme by benzylamine was studied by growing the organism for several subcultures on succinate and then using a washed suspension of organisms harvested in mid-exponential growth to inoculate mineral salts medium containing benzylamine. Growth was followed spectrophotometrically at 660 nm; samples were removed periodically, cell-free extracts were prepared and assayed for amine dehydrogenase activity. Initially no enzymic activity was present (Fig. 1), but appreciable levels of amine dehydrogenase activity were detected before growth occurred (after a 5 h lag phase).

Specificity of amine dehydrogenase

The specific activities of amine dehydrogenase in crude extracts of *P. putida* NP grown on benzylamine, *n*-propylamine and *n*-butylamine are presented in Table 1. The substrate specificity of the enzyme was independent of the growth substrate. Highest activities were induced by benzylamine and *n*-butylamine was the most rapidly oxidized substrate. There was no activity with the iso-substituted amines, isopropylamine, 1,2-diaminopropane or isobutylamine.

Crude extracts obtained from organisms grown on benzylamine, *n*-propylamine or *n*-butylamine were subjected to electrophoresis and stained for amine dehydrogenase activity. Regardless of the assay substrate, one band of enzymic activity was detected and was located in an equivalent position in each gel.

Heat stability

Figure 2 shows the stability of the enzyme after incubation at 55, 60 and 65 °C. The enzyme was stable at 55 °C for 10 min after which there was a decline in activity. At 60 and 65 °C the amine dehydrogenase activity declined rapidly, 97% inactivation occurring after incubation for 12 and 3 min, respectively.

Metabolism of benzylamine

Pseudomonas putida was tested to determine whether proposed intermediates of benzylamine biodegradation would support growth. Benzyl alcohol, benzoate and catechol were
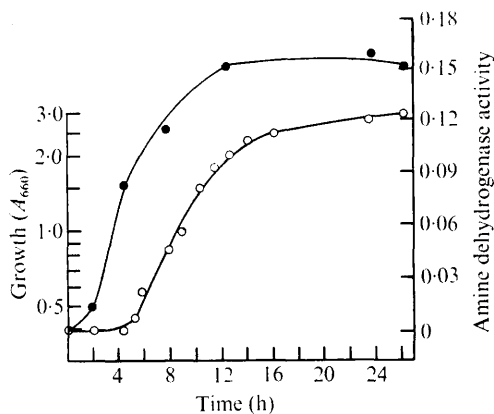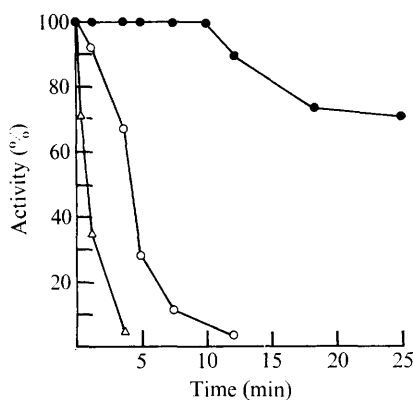
Fig. 1   Fig. 2

Fig. 1. Induction of amine dehydrogenase during adaptation of succinate-grown *P. putida* to growth on benzylamine. Washed suspensions of succinate-grown organisms were used to inoculate mineral salts medium containing benzylamine. Growth (○) was measured spectrophotometrically and, at intervals, samples were removed for determination of amine dehydrogenase activity (●) [expressed as μmol DCPIP reduced min^{-1} (mg protein)$^{-1}$].

Fig. 2. Temperature stability of the amine dehydrogenase from *P. putidia* NP. Samples of enzyme (2·5 mg protein ml^{-1}) were heated in a water bath at 55 °C (●), 60 °C (○) or 65 °C (△) and assayed at intervals for enzymic activity at 24 °C.

utilized, whereas benzaldehyde was not. These four substrates were oxidized by non-proliferating cell suspensions of benzylamine-grown organisms; however, the Q_{O_2} value for benzyl alcohol of 54 μl O_2 h^{-1} (mg dry wt)$^{-1}$ was less than half the Q_{O_2} values for benzylamine, benzaldehyde, benzoate and catechol, which were 119, 160, 135 and 130 μl O_2 h^{-1} (mg dry wt)$^{-1}$, respectively.

To confirm that benzyl alcohol and benzaldehyde were intermediates in benzylamine bio-degradation, the presence of the requisite enzymes was investigated. Benzaldehyde dehydro-genase was induced by growth on benzylamine [specific activity 0·42 μmol benzoate min^{-1} (mg protein)$^{-1}$], but not by growth on benzoate. However, benzyl alcohol dehydrogenase activity was not detected in soluble or particulate fractions of cell extracts after growth of *P. putida* on benzylamine. Benzaldehyde was isolated, as its 2,4-dinitrophenylhydrazone, from the culture medium of benzylamine-grown organisms, and from the reaction mixture resulting from manometric assays of amine dehydrogenase activity in extracts of these organisms. *Pseudomonas putida* grown on benzylamine or benzoate showed the characteristic yellow colour of 2-hydroxymuconic semialdehyde when incubated with catechol in the cleavage test (Stanier *et al.*, 1966). The specific activity of catechol 2,3-oxygenase in extracts of benzylamine- and benzoate-grown organisms was 0·73 and 0·55 μmol product min^{-1} (mg protein)$^{-1}$, respectively. There was insignificant catechol 1,2-oxygenase activity ($< 0·01$) in extracts of *P. putida* grown on benzylamine.

DISCUSSION

Little is known about the microbial metabolism of primary monoamines with the exception of methylamine (Anthony, 1975; Quayle, 1972; Ribbons, Harrison & Wadzinski, 1970) and propylamines (Blevins & Perry, 1972; Cerniglia & Perry, 1975). The ability of pseudomonads to utilize amines is well documented (Ballard *et al.*, 1970; Stanier *et al.*, 1966); however, the pathways of carbon assimilation and the enzyme(s) which catalyse the initial oxidative step in amine metabolism in *P. putida* have not been investigated. The observation that cell-free extracts of benzylamine-grown *P. putida* do not consume oxygen except on addition of PMS, suggested that the enzyme responsible for the deamination was not an

oxidase but an amine dehydrogenase [amine:(acceptor) oxidoreductase (deaminating)]. The ability of DCPIP, PMS, ferricyanide and cytochrome *c* to function as an electron acceptor was similar to the acceptor specificities of previously reported amine dehydrogenases (Eady & Large, 1968; Tabor & Kellogg, 1970).

The absence of amine dehydrogenase activity in organisms grown on succinate or benzoate suggested that the enzyme was inducible. The enzymic specificity of the amine dehydrogenase was independent of the growth substrate. This, together with the equivalent temperature sensitivity of the amine dehydrogenase obtained from benzylamine-, *n*-butyl-amine- and *n*-propylamine-grown organisms (unpublished data), suggested that the same enzyme was induced by growth on these amines. Polyacrylamide gel electrophoresis of cell extracts yielded one band of enzymic activity, confirming that one enzyme of broad specificity was involved in the deamination of primary amines in *P. putida* NP. Similar results have been reported by Cerniglia & Perry (1975) for *Mycobacterium convolutum*.

The substrate specificity of the enzyme was different from that of a methylamine dehydrogenase present in *Pseudomonas* AM1 (Eady & Large, 1968), and the rate of oxidation of amines by the enzyme from *P. putida* NP was considerably higher. Neither of these enzymes acts on iso- or tertiary-substituted amines in contrast to an amine dehydrogenase from *Mycobacterium convolutum* which oxidized isopropylamine (Cerniglia & Perry, 1975).

The substrate specificity (Table 1) and temperature sensitivity (Fig. 2) of the primary amine dehydrogenase in *P. putida* NP suggest that this enzyme is different from those previously described in *Pseudomonas* AM1 (Eady & Large, 1968), *Methylomonas methylovora* (Mehta, 1977), *Mycobacterium convolutum* (Cerniglia & Perry, 1975), *Serratia marcescens* (Tabor & Kellogg, 1970) and *Achromobacter* sp. (Satake & Fujita, 1953).

Pseudomonas putida NP utilized as growth substrates all of the putative intermediates of benzylamine metabolism except benzaldehyde. According to the theory of sequential induction (Stanier, 1947), the results suggested that benzaldehyde, benzyl alcohol, benzoate and catechol may be intermediates in benzylamine degradation. Benzaldehyde dehydrogenase was induced in benzylamine-grown organisms, but benzyl alcohol dehydrogenase was not detected in cell extracts. These data, together with the isolation of benzaldehyde from cell extracts, indicated that benzylamine was deaminated directly to benzaldehyde and subsequently oxidized to benzoate by an inducible benzaldehyde dehydrogenase. The oxidation of benzyl alcohol in whole organisms, not evident in extracts, may be due to a non-specific alcohol dehydrogenase that was not active under our assay conditions.

The conversion of benzoate to catechol proceeded via a benzoate oxidase system, which was measured manometrically in whole organisms grown on benzylamine. In many microorganisms benzoate is converted to catechol through 1,2-dihydro-1,2-dihydroxybenzoic acid via a peroxidation mechanism (Reiner, 1971). Whether a *cis*-dihydrodiol dehydrogenase was present in cell extracts of benzylamine-grown cells was not determined. However, *P. putida* NP has been previously reported to possess a *cis*-naphthalene dihydrodiol dehydrogenase following growth on naphthalene (Patel & Gibson, 1976).

The oxidative cleavage of catechol in pseudomonads occurs by the *β*-ketoadipic acid pathway (Stanier & Ornston, 1973) or by the *α*-keto acid pathway (Dagley *et al.*, 1964; Evans, 1963). The cleavage test (Stanier *et al.*, 1966) to determine which catabolic route was used suggested that 2-hydroxymuconic semialdehyde was formed by both benzoate- and benzylamine-grown organisms. Catechol 2,3-oxygenase activity was detected in cell-free extracts indicating that benzylamine and benzoate were metabolized by the *meta* (or *α*-keto acid) pathway. *Pseudomonas putida* NP appeared to be similar to *P. putida* mt-2 (previously designated *P. arvilla*, Murray *et al.*, 1972) in that it metabolized benzoate by the *meta* pathway, rather than the *ortho* pathway, which is induced in most benzoate-grown strains of *P. putida*.

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