Novel degradative pathway of 4-nitrobenzoate in *Comamonas acidovorans* NBA-10

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A *Comamonas acidovorans* strain, designated NBA-10, was isolated on 4-nitrobenzoate as sole carbon and energy source. When grown on 4-nitrobenzoate, it was simultaneously adapted to 4-nitrosobenzoate and 4-hydroxylaminobenzoate but not to 4-hydroxybenzoate or 4-aminobenzoate. In cell extracts with NADPH present, 4-nitrobenzoate was degraded to 4-hydroxylaminobenzoate and 3,4-dihydroxybenzoate. Partial purification of the 4-nitrobenzoate reductase revealed that 4-nitrobenzoate is degraded via 4-nitrosobenzoate to 4-hydroxylaminobenzoate. The substrate specificity of the enzyme was narrow and NADPH was 15 times more effective as a cofactor than NADH. The results provide evidence for a novel pathway for aerobic degradation of 4-nitrobenzoate, since neither 4-hydroxybenzoate nor 4-aminobenzoate were involved in the degradative pathway.

Introduction

Aromatic nitro-compounds, e.g. nitrophenols, nitrotoluenes and nitrobenzoates, are used in the manufacture of pesticides, dyes, explosives and industrial solvents. These compounds enter industrial waste streams and in several instances accumulate in the environment. The biological conversion of these compounds is consequently of great interest. Furthermore, microbes degrading nitro-aromatics may contain enzymes yielding hydroxylated aromatics. Such enzymes are of interest because hydroxylated aromatics are chemically difficult to prepare. Microbial methods for the preparation of hydroxylated aromatics have been considered previously in the biotransformation of halogenated aromatic compounds (Groenewegen *et al.*, 1992).

In general two different systems have been described for the removal of the nitro-group from nitro-aromatic compounds by micro-organisms. In the first pathway, the nitro substituent is directly removed as nitrite, as demonstrated for the metabolism of *o*- and *p*-nitrophenol (Zeyer & Kearney, 1986; Spain *et al.*, 1979), and in the metabolism of 2,6-dinitrophenol (Bruhn *et al.*, 1987).

In the second pathway, the nitro-group is reduced by a nitroreductase to an amine via nitroso and hydroxylamino intermediates (Schackmann & Müller, 1991; Kinouchi & Onishi, 1983; Liu *et al.*, 1984; McCormick *et al.*, 1976). Such reduction of the nitro-aromatic compound to the corresponding amino-aromatic compound has been demonstrated in various organisms which are able to use the nitro-aromatic compound as an electron acceptor. The amino intermediate is a metabolic end-product in these organisms (Rafii *et al.*, 1991; Schackmann & Müller, 1991).

In addition, the involvement of nitroreductases in the complete degradative pathways of nitro-aromatic compounds has also been reported (Durham, 1956; Germanier & Wuhrmann, 1963; Haller & Finn, 1978). In the aerobic metabolism of 2- and 4-nitrobenzoate, reduction of the nitro-group via nitroso- and hydroxylaminobenzoate was demonstrated (Cain, 1966*a, b*; Cartwright & Cain 1959*a, b*). The amino intermediate transiently accumulated during growth on the nitro-aromatic compound but no evidence was provided that the amino-aromatic compound was an intermediate in the degradative pathway of 2-nitrobenzoate and 4-nitrobenzoate (4-NBA) (Cain 1966*a*; Ke *et al.*, 1958; Cain & Cartwright, 1960). In *Nocardia erythropolis*, degradation of 4-NBA after reduction was suggested to proceed via 3,4-dihydroxybenzoate (3,4-diHBA) with some 4-nitrocatechol formed, probably from a side reaction. Based on

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Abbreviations: 4-ABA, 4-aminobenzoate; 4-HBA, 4-hydroxybenzoate; 4-NBA, 4-nitrobenzoate; 3,4-diHBA, 3,4-dihydroxybenzoate; 4-HABA, 4-hydroxylaminobenzoate; 4-NOBA, 4-nitrosobenzoate.

oxygen consumption rates of whole cells, an intermediary role for 4-hydroxybenzoate (4-HBA) in *Nocardia erythropolis* was also suggested (Cartwright & Cain, 1959a,b).

In this paper, evidence is presented for a new metabolic pathway in the degradation of 4-NBA involving neither 4-HBA nor 4-aminobenzoate (4-ABA) as intermediates.

Methods

Media and culture conditions. Enrichments of 4-NBA-utilizing bacteria from various soil and water samples were done at 30 °C in 100 ml serum flasks containing 10 ml mineral salts medium with 1 mM-4-NBA. After incubation for 1 week, plates of yeast/glucose agar were streaked with material from the enrichment cultures. Colonies were streaked to purity on the same medium and the ability to grow on 4-NBA was examined by growing the pure cultures in liquid medium in the presence and absence of 1 mM-4-NBA. The pure culture exhibiting the highest growth rate on 4-NBA was designated NBA-10. It was classified by the National Collections of Industrial and Marine Bacteria (NCIMB, Aberdeen, UK) as a strain of *Comamonas acidovorans*. Strain NBA-10 was routinely grown in a chemostat ($D = 0.1 \text{ h}^{-1}$) under carbon-limited conditions at pH 7.0 and at 30 °C. The mineral salts medium in the medium reservoir contained 20 mM-4-NBA. Strain NBA-10 was also grown on 4-HBA, 3,4-diHBA or succinate in the chemostat.

Experiments with resting cells. Cells grown in a chemostat were harvested by centrifugation at 16000 g (10 min at 4 °C), washed in potassium phosphate buffer (pH 7.0, 50 mM) and resuspended in the same buffer. Endogenous oxygen uptake by suspensions (3 ml) of washed cells was measured for at least 3 min at 30 °C using a YSI model 53 monitor equipped with a YSI model 5331 polarographic oxygen probe (Yellow Springs Instruments, USA). Subsequently, 0.1 ml of a substrate solution (30 mM) was added and the oxygen uptake was recorded for at least another 5 min.

Experiments to determine the aerobic degradation rate of 4-NBA and 4-nitrosobenzoate (4-NOBA) by washed cell suspension (10 ml) were done in 100 ml serum flasks in a shaking water-bath (30 °C, 1 Hz). Degradation of 4-NBA and 4-NOBA was also examined by incubating 100 ml serum flasks under stationary (oxygen-limited) conditions at 30 °C. Before starting anaerobic experiments, incubation flasks were flushed for at least 15 min with N_2 gas (oxygen-free). The degradation of 4-NBA or 4-NOBA by whole cells was started by adding substrate with a gas-tight syringe from a N_2 -flushed concentrated stock solution. Samples were periodically withdrawn from the incubation mixture (with a gas-tight syringe) and immediately centrifuged (10000 g); the supernatant was then analysed by HPLC.

Experiments with cell-free extracts. Cell-extracts were prepared by disrupting washed cell suspensions by ultrasonic disintegration (Branson Sonic Power) for $8 \times 15 \text{ s}$ with a power input of 10 W at 0 °C. The resulting homogenate was centrifuged at 30000 g for 20 min at 4 °C. The supernatant was used as crude cell-free extract and contained 10–15 mg protein ml^{-1} .

Experiments to determine the degradation of 4-NBA and 4-hydroxylaminobenzoate (4-HABA) by cell-free extracts were done in 5 ml tubes with gas-tight rubber septa. Anaerobic experiments in these tubes were done as described above for the serum flasks. The reaction was stopped by addition of 6 M-HCl to give a final concentration of 0.6 M. The precipitated protein was removed by centrifugation and samples of the supernatant were analysed by HPLC.

4-NBA reductase assay. 4-NBA consumption by cell-free extracts or partially purified enzyme was measured spectrophotometrically by following at 340 nm the rate of oxidation of NADPH in the presence of 4-NBA. In some experiments, 4-NBA reductase activity was determined by using HPLC to monitor the degradation of 4-NBA.

Partial purification of 4-NBA-reductase. Protein was precipitated with saturated ammonium sulphate solution, centrifuged at 10000 g (15 min at 4 °C), dissolved in 10 ml Tris/HCl (50 mM, pH 8.0), dialysed against the same buffer and applied to a DEAE-Sephacrose CL-6B column (Pharmacia). The enzyme was eluted with a linear gradient of 0–0.5 M-NaCl in Tris/HCl (50 mM, pH 8.0). Fractions of 10 ml were collected and active fractions were pooled, concentrated with ammonium sulphate and dialysed as above. This concentrated fraction was applied onto a FPLC Mono Q column (Pharmacia), and the enzyme was eluted with a linear gradient of NaCl (0–0.5 M).

Chemicals. 4-HABA was synthesized chemically (Bauer & Rosenthal, 1944) and was free of 4-ABA as determined by HPLC analysis. 4-NOBA was synthesized by the method described by Cartwright & Cain (1959a) and was free of 4-NBA and 4-ABA.

All other chemicals were of the highest purity commercially available.

Analytical methods. The synthesized 4-HABA was also analysed with a Finnegan Q 70 mass spectrometer. The DCI (direct current introduction) probe was heated to 400 °C. The source temperature was 150 °C and ionization took place at 70 eV. The [quadruple] mass filters were kept at 70 °C. The measurements were with 100 ng 4-HABA and 3,4-diHBA dissolved in ethyl acetate dried with Na_2SO_4 . Since DCI-MS resulted in a disturbed picture, the standards and extracted HPLC samples were scanned by CID (collision induced decomposition) MS with DCI introduction.

The purity of the chemically synthesized 4-NOBA and 4-HABA were measured in DMSO- d_6 solution on a Bruker AC 200E instrument.

Concentrations of 4-NBA, 4-NOBA, 4-HABA and 3,4-diHBA were routinely determined by reversed phase HPLC on a C-18 column (200 $\times$ 3 mm, Chrompack). For determinations of 4-NBA and 4-NOBA, acetonitrile/water/acetic acid (40:59:1, by vol.) and for 4-HABA and 3,4-diHBA, acetonitrile/0.005 M-sulphuric acid (10:90, v/v) were used as mobile phases. The concentration of NADP was determined by HPLC using a C-18 column with 50 mM-phosphate buffer (pH 7.0) as mobile phase.

Protein contents of whole cells and cell extracts were determined by the Lowry method using crystalline bovine serum albumin as a standard.

Ammonium was determined by following the oxidation of NADH in the presence of 2-oxoglutarate and L-glutamate dehydrogenase using a test kit from Sigma (kit number 170-A).

Results

Isolation and characterization of the 4-NBA-degrading organisms

Several 4-NBA-utilizing strains were isolated from aerobic enrichment cultures with 4-NBA as sole carbon and energy source. One organism, designated strain NAB-10, was a motile rod and transmission electron microscopic photographs showed it possessed one to six polar flagella. It was further characterized by the National Collections of Industrial and Marine Bacteria Limited as Gram-negative and oxidase-positive. The

Fig. 1

Fig. 1. Growth of *C. acidovorans* NBA-10 at various concentrations of 4-NBA. The initial concentrations of 4-NBA (mM) were 0.5 (●), 1.0 (■), 2.0 (▼), 4.0 (◆) and 5.0 (▲).

Fig. 2

Fig. 2. Degradation of 4-NBA (●) and formation of 4-HABA (▲) and 3,4-diHBA (■) by resting cells of *C. acidovorans* NBA-10 grown on 4-NBA. The cell suspensions (5.6 mg protein in 10 ml) were incubated in the presence of phenanthroline (0.1 mM) under oxygen-limited conditions.

organism was obligately aerobic, did not grow above 41 °C and lacked diffusible fluorescent pigment. The API test revealed reduction of nitrate, assimilation of mannitol, gluconate, adipate, malate and phenylacetate. Other biochemical tests revealed no acid production from glucose, alkalization on tartrate and acetamide, restricted hydrolysis of gelatine and sensitivity to polymyxin B. Furthermore, NBA-10 utilized *p*-hydroxybenzoate, *m*-hydroxybenzoate and Simmon's citrate. On the basis of these taxonomic and biochemical characteristics strain NBA-10 was identified as a *Comamonas acidovorans* strain according to Willems *et al.* (1991).

The following compounds served as sole carbon and energy source: 4-NBA, 3,4-diHBA, 4-HBA, acetate and succinate. No growth occurred with 4-ABA, or 3-nitro- and 2-nitrobenzoate. The growth of NBA-10 on 4-NBA was dependent on the concentration of 4-NBA; a lag phase of 10 h was observed at an initial concentration of 4 mM-4-NBA and no growth at all was observed at concentrations above 5 mM (Fig. 1). The doubling time of strain NBA-10 on 2 mM-4-NBA was 2 h as calculated from the growth curve.

Degradation of various nitro-aromatics under resting cell conditions

Cells grown on 4-NBA oxidized 4-NBA, 4-NOBA, 4-HABA and 3,4-diHBA but no enhanced oxygen uptake was recorded with either 4-HBA or 4-ABA. Cells grown on 3,4-diHBA or 4-HBA oxidized neither 4-NBA nor 4-NOBA nor 4-HABA (Table 1).

Table 1. Rates of oxygen uptake by washed cell suspensions of *C. acidovorans* NBA-10 grown on various carbon sources

Rates of oxygen uptake are expressed as nmol O₂ consumed min⁻¹ (mg protein)⁻¹ after correction for the endogenous oxygen uptake rate.

Substrate tested	Oxygen uptake			
	Growth substrate:			
	4-NBA	4-HBA	3,4-diHBA	Succinate
4-Nitrobenzoate	160	<5	<5	<5
4-Nitrosobenzoate	150	<5	<5	<5
4-Hydroxylaminobenzoate	180	<5	<5	<5
4-Hydroxybenzoate	<5	230	10	5
3,4-Dihydroxybenzoate	380	180	280	20
4-Aminobenzoate	<5	<5	<5	<5
4-Nitrophenol	<5	20	10	10
Succinate	20	15	20	400

4-NBA metabolism by washed cells was also examined by measuring the disappearance of the substrate by means of HPLC. Cells grown on 4-NBA in a chemostat readily degraded 4-NBA aerobically [220 nmol 4-NBA min⁻¹ (mg protein)⁻¹] and a stoichiometric amount of ammonium was detected as degradation product. Under strict anaerobic conditions, no 4-NBA was degraded. Incubating whole cells with 4-NBA under oxygen-limited conditions did result in the accumulation of traces of other degradation products apart from ammonium. A more pronounced accumulation of these products (detected by HPLC) was observed when under oxygen-limited conditions phenanthroline (0.1 mM) was added as an inhibitor of, presumably, the 3,4-diHBA dioxygenase (Fig. 2). One of these compounds accumulated was

Fig. 3. Absorption spectrum of chemically synthesized 4-hydroxylaminobenzoate.

identified as 3,4-diHBA on the basis of HPLC retention time and absorption spectrum.

Because the other compound did not match the characteristics of a range of possible intermediates tested, e.g. 4-HBA or 4-ABA, additional efforts for identification were necessary. 4-NOBA and 4-HABA were synthesized and their identity and purity were checked by H-NMR. NMR spectra recorded in DMSO- d_6 revealed the proton shifts for 4-NOBA to be δ (p.p.m.) 8.01 d(2H) and 8.24 d(2H), and for 4-HABA δ (p.p.m.) 6.82 d(2H) and 7.74 d(2H). The retention time of the chemically synthesized 4-HABA, as well as its absorbance spectrum (Fig. 3) determined by stop-flow scanning HPLC, were identical to these characteristics of the unknown compound found in the incubation mixture. The identity of the so-far unknown intermediate was additionally confirmed by extracting the compound via preparative HPLC followed by GC/MS analysis. However, procedures using diazomethane to methylate 4-NOBA and 4-HABA prior to GC/MS analysis resulted in degradation products. Therefore, direct current mass spectrometric analysis was used. For both the synthesized 4-HABA and for the extracted compound a parent ion m/e of 153 was found. In addition four fragment ions were detected at m/e 136 ($C_7H_6NO_2$), 108 (C_6H_6NO), 120 (C_7H_6NO) and 90 (C_6H_6N).

Incubation of 4-NBA-grown cells aerobically with 4-NOBA resulted in a rapid degradation of 4-NOBA. In the presence of phenanthroline under oxygen-limited condition a transient accumulation of 4-HABA was found (Fig. 4).

Degradation of 4-NBA in cell extracts

From the experiments with whole cells, it was assumed that the first step in the degradation of 4-NBA is catalysed by a reductase via 4-NOBA to 4-HABA. Activity of the 4-NBA reductase was measured both by spectrophotometrically recording NADPH oxidation

and by HPLC measuring the disappearance of 4-NBA in the presence of NADPH. Cell-extracts of strain NBA-10 did indeed contain a NAD(P)H-dependent 4-NBA reductase activity, which was present when cell were grown on 4-NBA but not when grown on other substrates. The specific activity of the 4-NBA reductase measured as NADPH oxidation was $1060 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ and $550 \text{ nmol 4-NBA min}^{-1} (\text{mg protein})^{-1}$ as measured directly by the HPLC method.

Under anaerobic conditions, an accumulation of 4-HABA and 3,4-diHBA was observed (Fig. 5). Under both aerobic and oxygen-limited conditions the same 4-NBA reductase activity was measured, although only traces ($<0.01 \text{ mM}$) of 4-HABA and 3,4-diHBA accumulated.

Partial purification and characterization of 4-NBA reductase

Purification of the 4-NBA-reductase was assessed by monitoring NADPH oxidation in the presence of 4-NBA. The enzyme purification scheme (Table 2) consisted of three steps and resulted in a 18.4-fold purification. After Mono Q chromatography, only 3.2% of the 4-NBA reductase activity from the cell-extract was recovered.

Gel filtration, hydroxyapatite chromatography, and Cibacron blue affinity chromatography were tested as subsequent purification steps. However, no increase in the specific activity of 4-NBA reductase was obtained by any of these methods. After gel electrophoresis of the purified 4-NBA reductase ($20 \mu\text{g}$) only one major band was detected, and consequently no other efforts were undertaken to further purify the enzyme.

The partially purified enzyme was characterized. The optimal temperature of the enzyme reaction was at 30°C . Only 20% of the maximal activity was retained at 40°C . Enzymic activity was completely absent at 45°C . Heating the enzyme for 10 min at 40°C resulted in complete loss of activity.

Incubation of the partially purified enzyme with a range of possible substrates revealed a rather narrow substrate specificity. Only 4-nitrophenyl chloride, 4-nitrophenyl acetate and 4-nitrophenylalanine were degraded, albeit at very low rates of 0.5, 7.0 and 0.5% respectively of the specific activity found with 4-NBA. No activity was found with 4-halobenzoates, 4-hydroxy-, 4-hydroxylamino- and 4-aminobenzoate or with benzoate.

The degradation rate of 4-NBA with NADPH was 15 times higher than with NADH. The difference in specific activities of the 4-NBA reductase with either NADPH or NADH did not change during the purification procedure. Adding FAD or FMN or metal ions to

Fig. 4

Fig. 4. Degradation of 4-NOBA (▼) to 4-HABA (▲) by resting cells of *C. acidovorans* NBA-10 grown on 4-NBA. The cell suspensions (0.8 mg protein in 10 ml) were incubated in the presence of phenanthroline (0.1 mM) under oxygen-limited conditions.

Fig. 5

Fig. 5. Anaerobic degradation of 4-NBA (●) and formation of 4-HABA (▲) and 3,4-diHBA (■) in cell extracts. The incubations contained 2.1 mg protein in 1 ml.

Table 2. Partial purification of 4-NBA reductase from *C. acidovorans* NBA-10

1 Unit (U) is 1 nmol 4-NBA degraded.

Purification step	Vol. (ml)	Total act. (U)	Protein (mg ml ⁻¹)	Sp. act. (U min ⁻¹ mg ⁻¹)	Yield (%)	Purification (-fold)
Cell-free extract	41	580	22.4	630	100	1
(NH ₄) ₂ SO ₄ (35–55%)	10	312	49.6	630	54	1
DEAE	30	182	1.1	5600	31	8.9
Mono Q (FPLC)	5	18	0.3	11 600	3.2	18.4

the incubation mixture did not enhance the specific activity.

Incubation of 4-NBA reductase [11 600 nmol 4-NBA min⁻¹ (mg protein)⁻¹] with NADPH and 4-NBA revealed a stoichiometric conversion of 4-NBA to 4-HABA. The amount of NADP formed was approximately twice the amount of 4-NBA degraded or 4-HABA formed (results not shown). No 4-NOBA was detected during this incubation. Under both anaerobic and aerobic conditions, the same rate of 4-NBA reduction was found. When 4-NOBA was offered as alternative substrate, no conversion was found unless NADPH was added. However, in the absence of the 4-NBA reductase but in the presence of NADPH, 4-NOBA was chemically unstable. Apart from 4-HABA, some unidentified degradation products were detected also.

Discussion

Bacteria growing on 4-NBA as sole carbon and energy source were easily isolated from soil. In the isolate *Comamonas acidovorans* NBA-10 the initial attack in the

degradation of 4-NBA is on the nitro-group as reported for the 4-NBA-degrading bacterium *Pseudomonas fluorescens* (Durham, 1958; Cartwright & Cain, 1959*b*) and for *Nocardia erythropolis* (Cartwright & Cain, 1959*a, b*). However, from the growth characteristics and simultaneous adaptation experiments it appeared that 4-NOBA and 4-HABA but not 4-ABA or 4-HBA are intermediates in the degradation of 4-NBA by strain NBA-10. This was supported by demonstrating that when grown on 4-NBA under oxygen-limited conditions cells excreted 4-HABA and 3,4-diHBA. Furthermore, after partial purification of the 4-NBA reductase it was shown that with 2 mols NADPH present 1 mol of 4-NBA was converted stoichiometrically to 4-HABA. These results clearly demonstrate that the reduction of 4-NBA by strain NBA-10 does not involve the complete reduction of 4-NBA to 4-ABA. Such a pathway, without the involvement of an amino derivative, was suggested initially by Ke *et al.* (1959) on the basis of simultaneous adaptation experiments in the degradation of 2-nitrobenzoate by a *Flavobacterium*. The reduction of the nitro-group of 4-NBA by strain NBA-10 to an hydroxylamino-group is the first example of an incomplete reduction

Fig. 6. Proposed degradative pathway of 4-NBA by *C. acidovorans* NBA-10.

involved in the degradative pathways of nitro-aromatic compounds.

From the arguments given below we concluded that no similar 4-NBA reductases from bacterial sources other than *C. acidovorans* strain NBA-10 have been purified. Three methyl-4-nitrobenzoate reductases with a broad substrate specificity purified from *Escherichia coli* exhibited 4-NBA reductase activity also, although their activities were rather low. The characteristics of these reductases (Kitamura *et al.*, 1983) do not match the properties of 4-NBA reductase from strain NBA-10, since after incubation with methyl-4-nitrobenzoate methyl-4-aminobenzoate was detected as well as methyl-4-hydroxylaminobenzoate; also the dialysed NAD(P)H-dependent *E. coli* reductases required addition of FMN for activity. From results obtained after purification of *p*-dinitrobenzene reductase from *Nocardia* V, it was also similarly assumed that the three subsequent steps involved in *p*-dinitrobenzene reduction to *p*-nitroaniline were catalysed by only one reductase (Villanueva, 1964). Furthermore, in *Bacteroides fragilis* four nitropyrene reductases were detected with different properties, one of them requiring NADH while the other reductases were NADPH linked. The purified nitropyrene reductase also seemed to catalyse all three steps of nitropyrene reduction (Kinouchi & Onishi, 1983).

Fractionation by anion-exchange chromatography of the 4-NBA reductase from *C. acidovorans* NBA-10 resulted in an 18·4-fold purification but considerable loss of activity occurred (Table 2). Other purification procedures tested gave even less satisfactory results. This loss of activity could not be prevented by adding antioxidants (dithiothreitol) or by excluding oxygen during purification.

The further degradation of 4-HABA in *C. acidovorans* NBA-10 is puzzling. Based on results from adaptation experiments (Table 1), from oxygen-limited incubations of whole cells (Fig. 2) as well as from results with crude cell-free extracts (Fig. 5), it would seem that 4-HABA is degraded to 3,4-diHBA (Fig. 6). However, attempts to characterize the enzyme involved in the conversion of 4-HABA into 3,4-diHBA have not been successful because activity was lost upon dialysing extracts.

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