Microbial formation of hydroxylated aromatic compounds from 4-chloro- and 4-nitrobenzoate

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Microbial formation of hydroxylated aromatic compounds from 4-chloro- and 4-nitrobenzoate

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
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STELLINGEN

1 Het is opmerkelijk dat in geen van de vele overzichtsartikelen over het metabolisme van gehalogeneerde aromaten door microorganismen aandacht geschonken wordt aan mogelijke opnamesystemen van deze verbindingen.
Dit proefschrift

2 De bewering van Zeyer dat bij de aerobe afbraak van nitroaromaten waarbij ammonium vrijkomt, een amine een intermediair is, is in zijn algemeenheid onjuist.
Zeyer J. (1988). Das Gas und Wasserfach Wasser und Abwasser 129:79-81.

3 Gezien het feit dat de omzetting van 4-fluorbenzoaat naar 4-hydroxybenzoaat redoxneutraal is, is het merkwaardig dat NADH wordt toegevoegd bij de bepaling van de aktiviteit van het dehalogenase.
Oltmanns R.H., Müller R., Otto M.K. and Lingens F. (1989) Applied Environmental Microbiology 55:2499-2504.

4 Sommige reincultures blijken toch Rijncultures te zijn.
Dit proefschrift

5 De overeenkomst tussen de geldende snelheidslimiet voor automobilisten en de gestelde tijdsduur voor een promotieonderzoek is, dat slechts een enkeling zich eraan houdt.

6 De vangkracht van kunstvliegen die bij het vliegvissen worden gebruikt, neemt evenredig af met pogingen een natuurgetrouwe imitatie van insekten te maken.

7 De groeiende kloof tussen het aantal veiligheidsvoorzieningen van auto's uit verschillende prijsklassen komt de verkeersveiligheid niet ten goede.

8 De geluidsproduktie van de huidige generatie deltavliegers maakt een verplichting tot het aanvragen van een hinderwetvergunning wenselijk.

9 Het oprichten van uitzendbureaus voor biowetenschappers zou de doorstroming vanuit de universiteit naar het bedrijfsleven kunnen vereenvoudigen.

10 De aanhoudende stroom van tegenstrijdige berichtgeving inzake de gevolgen van het broeikaseffect doet afbreuk aan het imago van de natuurwetenschappen.

Stellingen behorende bij het proefschrift "Microbial formation of hydroxylated aromatic compounds from 4-chloro- and 4-nitrobenzoate". Peter Groenewegen, Wageningen, 15 januari 1993.

VOORWOORD

Dit proefschrift is mede tot stand gekomen door de hulp en inzet van een groot aantal mensen. Ik wil er hier een aantal noemen en bedanken.
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CONTENTS

CHAPTER 1

INTRODUCTION

General

Increasing amounts of hydroxylated aromatic compounds are applied in pharmaceutical, agrochemical and petrochemical industries. Some compounds are used as precursors for the synthesis of antibiotics and other pharmaceuticals. In addition, hydroxylated aromatic compounds are used as building blocks for the synthesis of polymers, flavors and dyes. The major feedstocks for these compounds are obtained from both crude oil and coal (Franck and Stadelhofer 1988).

Also in nature, aromatic compounds are formed extensively and used as building blocks in the synthesis of lignin, alkaloids, certain hormones, etc. Consequently, several hydroxylated aromatic compounds may be obtained from natural sources. However, for reasons of availability and economics, they are mostly prepared chemically from fossil fuels. These chemical routes either depend on direct hydroxylation reactions or more commonly are achieved by indirect syntheses. Biological catalysts might also be used in the production of these compounds since a variety of enzymatic conversions has been characterized yielding hydroxylated aromatic compounds.

Chemical production of hydroxy-aromatics

Chemical synthesis of hydroxylated aromatic compounds by a direct hydroxylation reaction is rather difficult in preparative organic chemistry. A major problem is that after a hydroxyl-group is introduced on the aromatic nucleus the hydroxy-aromatic formed is easily further hydroxylated (Olah et al. 1981). Under the performed hydroxylation conditions, phenols are further oxidized to catechols and quinones. Hydroxylation of optically pure substrates to the corresponding products is even more difficult, since under the hydroxylation conditions racemic mixtures of the product are formed.

Most synthesis methods for the production of hydroxy-aromatics rely on indirect chemical syntheses. In such syntheses an aromatic compound generated or isolated, is modified to the desired hydroxy-aromatic. For instance, phenol may be obtained from benzene *via* sulfonation, chlorination or from cymene (Franck and Stadelhofer, 1988). Such processes are however often laborious.

Therefore, biotechnological methods for the production of hydroxylated aromatic compounds are of interest as an alternative for (in)direct chemical syntheses.

Biological production of hydroxy-aromatics by direct hydroxylation

In microorganisms various biochemical mechanisms have evolved which lead to the formation of aromatic compounds. The biosynthesis of these compounds can be achieved

by different routes: direct hydroxylation either by oxidases or by oxygenases, addition or modification of side-chains, and by replacement of substituents by hydroxyl groups (Fig. 1).

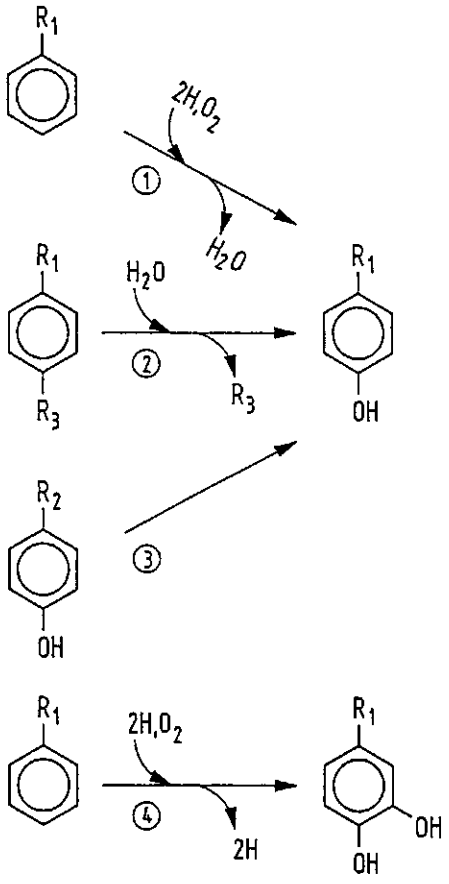


Fig. 1. Mechanisms involved in the bioformation of hydroxylated aromatic compounds
(1) Involvement of a monooxygenase
(2) Replacement of a substituent by a hydroxyl-group
(3) Modification of a sidechain of a hydroxylated aromatic compound
(4) Involvement of a dioxygenase

Several of such reactions have been discussed by van den Tweel (1988), and by Sikkema and de Bont (1991). In view of their potential regio- and enantioselectivity, biological oxidations might be attractive in commercial processes. However, in such processes other aspects, such as catalyst stability, efficiency, recycling of the catalyst,

product recovery etc., are also important. Consequently, for most products, chemical synthesis is applied. Presently, the commercial application of biological hydroxylation is restricted to the production of sterols and some specialty chemicals, which are produced on a very limited scale (Sikkema and de Bont 1991, Ribbons et al. 1990). Biological oxidation of aromatic compounds involves either oxidases or oxygenases. Oxidases catalyze the formation of hydrogen peroxide or hydroxyl radicals. As a result of this type of biological oxidation of the substrate the reaction is rather unspecific. In contrast, oxygenases are highly regiospecific, but the number of substrates that are oxidized by these enzymes is limited. Oxygenases can be divided into two groups: dioxygenases which incorporate both atoms of molecular oxygen into the substrate and monooxygenases which incorporate one oxygen atom into the substrate, whereas the other atom is reduced to water.

Biological production of hydroxy-aromatics by substitions

Compared with oxygenases and oxidases, replacement of a substituent of an aromatic compound by a hydroxyl-group is of special interest for the production of hydroxylated aromatic compounds since in these reactions no molecular oxygen is involved. Formation of hydroxylated aromatic compounds can thus be achieved under anaerobic conditions, preventing further oxidative degradation of the product.

In the degradative pathways of some halogenated aromatic compounds, hydrolytic dehalogenases have been detected. In these reactions a regioselective hydroxylation of an aromatic compound is achieved by replacement of the halogen by a hydroxyl group derived from water (van den Tweel 1986). Other cheap substituted aromatics may also be used as starting material to obtain hydroxylated aromatic compounds by biological substitution reactions. Nitro-aromatic compounds might be of interest in this respect, since they are easily obtained.

Chemical production of halogenated- and nitro-aromatic compounds

Halogenated aromatic compounds have been produced in large quantities during the last decades. These compounds are used in a variety of industrial and agricultural applications and are primarily used as solvents, lubricants, insulators, herbicides and as intermediates in chemical synthesis. The annual world production of chlorinated benzenes, for example, is estimated at 8×10^5 metric tons (Merians and Zander 1982).

Nitro-aromatic compounds are also important feedstocks for the synthesis of a wide rang of industrial chemicals, including explosives, pesticides and dyes. It has been estimated that as much as 10% of the sales by chemical industry are based on processes which, at some step, involve nitro-aromatic compounds. In particular, dinitrotoluenes are used extensively in the production of polyurethane and explosives. In 1982, the United

States alone produced approximately 325 million kg of dinitrotoluenes. Nitrobenzene is widely used in the manufacture of aniline, in the refining of lubricating oils and in the production of soaps and shoe polishes (Merck and Co. 1983).

The uptake of (substituted) aromatic compounds by microorganisms

The enzymology and genetics of the degradation of halogenated aromatic compounds is studied extensively as summarized recently (Engesser and Fischer 1991, van der Meer 1992), but the uptake of these compounds by microorganisms is often ignored or considered to proceed by passive diffusion. Nevertheless, uptake is the first and consequently a very important step in microbial metabolism of these compounds. Uptake of substituted aromatics and excretion of hydroxylated aromatics by whole cells is also very important when organisms are used to produce hydroxylated aromatics

The uptake of substrates such as halo-aromatics depends on the hydrophobicity of the compound. In principle the cell membrane, consisting of a phospholipid bilayer, is impermeable for hydrophilic substances. Therefore, specific uptake systems have evolved in microorganisms for the transport of such compounds into the cell. In contrast lipophilic compounds enter the cell by passive diffusion.

Halogenated aromatic compounds are generally more apolar than the unsubstituted analogue. Therefore, it is reasonable to assume that halogenated aromatic compounds enter the cell by passive diffusion if the non-substituted compound permeates freely in the organism.

Uptake of benzoate and mandelate has been studied in detail in different bacteria (Cook and Fewson 1972, Geisler et al. 1988, Thayer and Wheelis 1982, Poolman et al. 1987, Meagher et al. 1982). Conclusive evidence for carrier-mediated transport of benzoate by *Pseudomonas putida* was obtained by Thayer and Wheelis (1976, 1982). Benzoate uptake was mediated by an inducible saturable transport system that could be eliminated by a mutation in a gene designated benP. A mutant strain that was blocked in the subsequent metabolism of benzoate, accumulated benzoate 150-fold against a concentration gradient. The mechanism of benzoate uptake is poorly defined, although some evidence was presented for proton-motive force (Δp)-driven rather than ATP-driven uptake. Uptake of mandelate by *Pseudomonas putida* has also been studied. According to Hegeman (1966), mandelate enters the cell by passive diffusion. Uptake was found both in induced and in uninduced cells but no internal accumulation was observed. In contrast, a carrier-mediated mechanism for mandelate uptake was proposed by Higgins and Mandelstam (1972). Induced cells accumulated mandelate against a concentration gradient and uptake was blocked by the protonophore 2,4-dichlorophenol. The kinetics of uptake of benzoate and 4-hydroxybenzoate (4-HBA) have been studied in *Rhodopseudomonas palustris* (Merkel et al. 1989, Harwood and Gibson 1986). These

aromatic acids are immediately converted into their Coenzyme A (CoA) derivatives by an inducible benzoyl-CoA ligase; no evidence for an active transport system was obtained.

In reports discussing the degradative pathways of other substituted aromatic compounds no data have been published on the uptake mechanism of these compounds, although for some compounds specific uptake mechanisms have been suggested. Specific transport systems were suggested on the basis of the difference in substrate specificity between whole cells and cell-free extracts of an *Alcaligenes* sp. metabolizing sulfonated aromatics (Thurnheer et al. 1990). Energy-dependent transport systems for halo-aromatic compounds have been implied in some systems (Shimao et al. 1989, van den Tweel et al. 1986, 1987a) but no evidence for carrier-mediated transport has been presented. The uptake of 2,4-dichlorophenoxyacetic acid was studied to some extent in a strain of *Pseudomonas fluorescens* that is unable to degrade this compound. Accumulation of 2,4-dichlorophenoxyacetic acid proceeded only up to the equilibrium level, and a facilitated diffusion type of uptake mechanism was suggested mainly based on the inhibition of uptake by azide and fluorodinitrobenzene (Wedemeyer 1966). However, in the coryneform bacterium NTB-1 an energy-dependent uptake system for 4-chlorobenzoate was found. Under anaerobic, conditions no 4-chlorobenzoate accumulated unless an alternative electron acceptor was present (Groenewegen et al. 1990).

Halogenated aromatic compounds diffusing freely into the cell may pose a serious threat to the organism since cells are not able to control the internal concentration of such compounds. Such lipophilic compounds may be especially toxic since they are likely to partition preferentially in the lipid membranes and will thus impair several membrane functions (Sikkema et al. 1992).

Bacterial degradation of halogenated aromatic compounds

Halogenated aromatic compounds are often considered as man-made chemicals which are not generally found in the environment. It should be noted, however, that a number of halogenated organic compounds are also produced biologically (Siuda and DeBernardis 1973, Neidleman and Geigert 1986). In the last decades many microorganisms have been isolated capable of degrading halogenated aromatic compounds to CO_2 and halide, which is not surprising since many halogenated aromatic compounds are produced in nature (de Jong et al. 1992). These organisms use pathways similar to those for the degradation of non-halogenated aromatic compounds. Under aerobic conditions, halogenated aromatic compounds are generally oxidized by mono- and dioxygenases into dihydroxylated derivatives with halide release occurring after ring cleavage has taken place. Hydrolytic displacement of a halide from an aromatic structure has been observed in specific cases. Under anaerobic conditions, reductive dehalogenation occurs yielding nonsubstituted aromatic compounds.

In recent years, bacteria not utilizing halo-aromatics for growth but rather as an electron acceptor have been described.

Halogenated aromatic compounds utilized as carbon and energy source

In the degradation of halogenated aromatic compounds by bacteria various mechanisms have been demonstrated: (1) Oxygenolytic cleavage of halogen-carbon bonds, (2) displacement of halogen by hydroxyl group, and (3) displacement of halogen by hydrogen (Table 1). Bioconversion of halogenated aromatic compounds into (di)hydroxylated aromatic compounds can only be achieved by oxygenolytic or hydrolytic displacement of the halogen-group.

Mechanism of dehalogenation	Typical example	Strain	References
(1) Oxygenolytic	2-halobenzoates	*Pseudomonas putida*	Fetzner et al. 1992 Engesser and Schulte 1989
	4-chlorophenylacetate	*Pseudomonas* sp.	Klages et al. 1981
	pentachlorophenol	*Arthrobacter* sp.	Schenk et al. 1990
(2) Hydrolytic	3-chlorobenzoate	*Pseudomonas* sp.	Johnston et al. 1972
	4-chlorobenzoate	*Pseudomonas* sp.	Klages and Lingens 1980
		Arthrobacter sp.	Marks et al. 1984a
		strain NTB-1	van den Tweel et al. 1986
(3) Reductive	3-chlorobenzoate	strain DCB-1	Dolfing 1990
	2,4-dichlorobenzoate	strain NTB-1	van den Tweel et al. 1987a

Table 1. Mechanisms involved in the degradation of halogenated aromatic compounds

Oxygenolytic removal of halogen

Dioxygenases, enzymes that incorporate molecular oxygen into their substrates, play an important role in the aerobic catabolism of aromatic compounds (Smith 1990). They introduce two hydroxyl groups on the very stable benzene nucleus. The intermediary dihydrodiol formed is further degraded to a catechol. Similarly, halogen substituted catechols appear to be crucial intermediates in the degradation of many halo-aromatic compounds which are metabolized by broad substrate specific enzymes, pyrocatechase

and cycloisomerases. The involvement of dioxygenases in the degradation of several halogenated aromatic compounds has been reviewed extensively (Reineke and Knackmuss 1988, Commandeur and Parsons 1990, Engesser and Fischer 1991, van der Meer 1991, 1992).

2-Fluoro- and 2-chlorobenzoate are frequently employed as model substrates for oxygenolytic substituent removal. Dioxygenase attack yields halohydroxy compounds which spontaneously re-aromatize to unsubstituted catechol, with concomitant release of halide as illustrated in the upper part of Figure 2 (Engesser et al. 1980, Vora et al. 1988, Higson and Focht 1990). Goldman et al. (1967) described such an oxygenolytic dehalogenation in the metabolism of 2-fluorobenzoate by a *Pseudomonas* sp. (Milne et al. 1968).

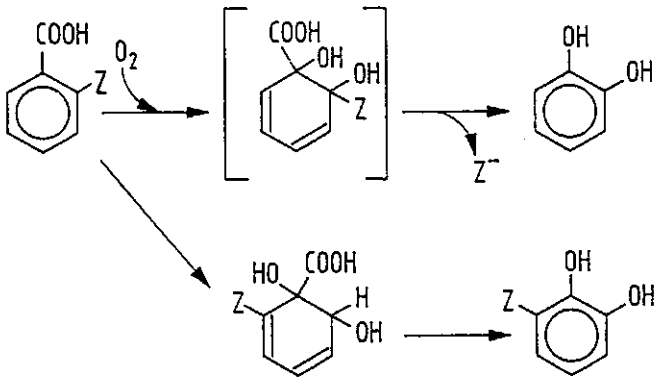


Fig. 2. Oxygenolytic degradation of halogenated aromatic compounds *via* halo-catechols (Z = halogen-group)

The 2-fluorobenzoate (2-FBA) may, however, be metabolized via two different pathways because nonselective dioxygenation by the benzoate 1,2-dioxygenase generated a mixture of 2- and 6-fluoro-1,2-dihydro-1,2-dihydroxybenzoate (Milne et al. 1968, Vora et al. 1988). In the first pathway 2-fluoro-1,2-dihydro-1,2-dihydroxybenzoate was defluorenated with the concomitant release of CO_2 yielding catechol, which was further degraded by catechol 1,2-dioxygenase. In the second pathway 6-fluoro-1,2-dihydro-1,2-dihydroxybenzoate was degraded via 3-fluorocatechol to 2-fluoro-*cis,cis*-muconate, which was not further metabolized (Fig. 2, lower part).

The above mechanism is only suitable for the purpose of obtaining catechols with or without a halogen substituent. Hydroxylated aromatics from halo-aromatics not retaining

their halogen atom and with no displacement of the side chain have only been obtained in special cases. In *Pseudomonas* sp. strain CBS3 the degradation of 4-chlorophenylacetate was also suggested to proceed via unstable *cis*-dihydrodiols yielding 3,4-dihydroxyphenylacetate (Klages et al. 1981, Markus et al. 1984) (Fig. 3).

An analogous scheme was proposed for dehalogenation of 2-halobenzoate by the 2-halobenzoate-1,2-dioxygenase purified from *Pseudomonas cepacia* 2CBS (Fetzner et al. 1992). The dioxygenase was NADH and Fe^{2+} dependent and exhibited a very broad substrate specificity.

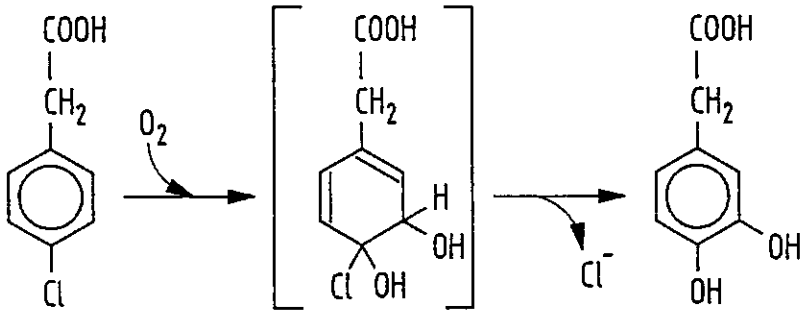


Fig. 3. Involvement of a dioxygenase in the degradation of 4-chlorophenylacetate.

Hydrolytic displacement of a halogen

The evidence implicating replacement of chlorine from the aromatic ring by a hydroxyl group were presented by Faulkner and Woodcock (1961). An *Aspergillus niger* strain converted 2-chloro- into 2-hydroxyphenoxyacetate.

Johnston et al. (1972) presented some data on the degradation of 3-chlorobenzoate *via* 3-hydroxybenzoate. Resting cells of a *Pseudomonas* sp. grown on 3-chlorobenzoate oxidized 3-hydroxybenzoate and 2,5-dihydroxybenzoate without delay. During growth on 3-chlorobenzoate, 3-hydroxybenzoate and 2,5-dihydroxybenzoate were excreted into the culture medium. The involvement of a hydrolytic dehalogenase was also suggested for the degradation of 2-bromobenzoate. Oxygen-depleted cells of a *Pseudomonas aeruginosa* strain accumulated salicylate from 2-bromobenzoate (Higson and Focht 1990).

The degradation of polychlorinated phenols, e.g. pentachlorophenol and tetrachlorophenol, may also involve hydrolytic dehalogenases (Häggblom et al. 1989, Apajalahti and Salkinoja-Salonen 1986). Apajalahti and Salkinoja-Salonen (1987a,b) showed that the degradation of pentachlorophenol by *Rhodococcus chlorophenolicus*

involved an initial substitution of chlorine by hydroxyl in the conversion of pentachlorophenol to 2,3,5,6-tetrachlorohydroquinone, and a second substitution step yielding 3,5,6-trichloro-1,2,4-trihydroxybenzene. Apajalahti and Salkinoja-Salonen demonstrated with extracts of *Rhodoccus phenolicus* in the presence of either H_2O^{18} and $^{18}O_2$ that in the conversion of 2,3,4,5-tetrachlorophenol to 2,3,5-trichlorohydroquinone the hydroxyl group was derived from water rather than from molecular oxygen. In contrast, the pentachlorophenol dehalogenases in cell extracts of *Rhodococcus phenolicus* and also in cell extracts of *Arthrobacter* and *Flavobacterium* sp. were only active in the presence of NADPH and in the presence of at least traces of oxygen (Apajalahti and Salkinoja-Salonen 1987b, Schenk et al. 1989, Xun and Orser 1991). Furthermore, Schenk et al. (1990) demonstrated with a control labeling experiments with H_2O^{18} and $^{18}O_2$, that unlabeled tetrachlorohydroquinone (instead of pentachlorophenol) was labeled after incubation with the enzyme. Therefore, distinction between an oxygenolytic or a hydrolytic dehalogenation mechanism of pentachlorophenol using labeling experiment is not possible. The need for oxygen and NADPH suggests that the first step in the degradation of pentachlorophenol by pentachlorophenol dehalogenase from *Arthrobacter* sp. represents an oxygenolytic mechanism (Schenk et al. 1990).

Most reports on hydrolytic dehalogenation involve the substitution of the halogen by a hydroxyl group at the *para* position of benzoate. Chapman (1975) isolated a *Micrococcus* sp. able to grow on 4-chlorobenzoate. Degradation of 4-CBA proceeded via 4-hydroxybenzoate and protocatechuate. Studies on the 4-chlorobenzoate dehalogenation system in cell-free extracts of an *Arthrobacter* sp. indicated that maximum activity occurred at low oxygen concentrations (Marks et al. 1984a). This observation did not favour dehalogenation catalyzed by an oxygenase. Conclusive evidence that 4-CBA is converted to 4-HBA by a hydrolytic dehalogenase was obtained by two groups of researchers studying different bacteria. Labeling experiments using ^{18}O were performed to determine the source of the hydroxyl group substituted onto the aromatic ring, during the dehalogenation of 4-chlorobenzoate. Marks et al. (1984b) for an *Arthrobacter* sp. and Müller et al. (1984) for *Pseudomonas* sp. CBS3 showed that the oxygen atom incorporated in the hydroxyl-group of 4-HBA is derived from water rather than from molecular oxygen. Another line of evidence consistent with the mechanism of hydrolytic dehalogenation is the observation that in cell extracts of an *Arthrobacter* sp. 4-CBA was converted to 4-HBA under anaerobic conditions (Shimao et al. 1989). The same degradative pathway has also been reported for other 4-CBA degrading bacteria: an *Arthrobacter* sp. (Ruisinger et al. 1976), a *Nocardia* sp. (Klages and Lingens 1979), a *Pseudomonas* sp. (Klages and Lingens 1980), an *Arthrobacter globiformis* (Zaitsev and Karasevich 1981a,b), an *Acinetobacter* sp. (Adriaens et al. 1989, 1991) and a coryneform bacterium (van den Tweel et al. 1986, 1987a,b). Most bacteria which are capable of 4-CBA dehalogenation also dehalogenated

4-bromobenzoate and 4-iodobenzoate but not 4-fluorobenzoate. In contrast to this general rule *Aureobacterium* sp. RH025 dehalogenated 4-fluorobenzoate but not 4-CBA (Oltmanns et al. 1989). Recently, in *Acinetobacter* sp. 4CB1 it was found that both 4-CBA and 3,4-diCBA were dehalogenated by a hydrolytic dehalogenase (Adriaens et al. 1991).

Despite the conclusive reports by Marks et al. (1984b) and Müller et al. (1984) on the origin of the hydroxyl group replacing the halogen it was not possible to detect the hydrolytic dehalogenases in cell extracts of other 4-CBA degrading strains (Zaitsev et al. 1981, van den Tweel et al. 1987a, Adriaens et al. 1989). Purification of any 4-CBA dehalogenase observed in crude extracts resulted in considerable losses of activity (Marks et al. 1984a, Müller et al. 1988). Consequently, the characterization of the 4-CBA dehalogenase and elucidation of the mechanism involved, has not been so far reported.

In view of the (presumably) hydrolytic nature of the 4-CBA dehalogenation an unexpected observation was reported by van den Tweel et al. (1987a). In the absence of oxygen whole cells of the coryneform bacterium NTB-1 did not form 4-HBA. Only under low and controlled oxygen concentrations an almost quantitative conversion of 4-CBA to 4-HBA was demonstrated.

In a different approach to unravel the mechanism of the 4-CBA dehalogenase, molecular-genetic work was undertaken by three groups of researchers. Savard et al (1986) cloned the genes specifying the dehalogenation of 4-CBA from a Sau3A-generated gene bank of *Pseudomonas* sp. strain CBS3 (Savard et al. 1986). The gene bank was examined in a *Pseudomonas putida* strain capable of metabolizing 4-hydroxybenzoate. One clone designated B389 was able to grow on mineral medium containing 4-CBA as the sole carbon and energy source. The hybrid plasmid (pPSA843) was purified from *Pseudomonas putida* B389 and further characterized. pPSA843 did not confer on a *Pseudomonas putida* mutant unable to grow on 4-HBA the ability to use 4-HBA. Hybridization analysis of pPSA843 was carried out with a plasmid of 108 kbp detected in *Pseudomonas* sp. strain CBS3 and total DNA of this strain. No homologous sequences were detected between pPSA843 and the plasmid, whereas pPSA843 did hybridize with a 9-kbp fragment of the total DNA, demonstrating the genes specifying 4-CBA dehalogenation are located on the chromosome.

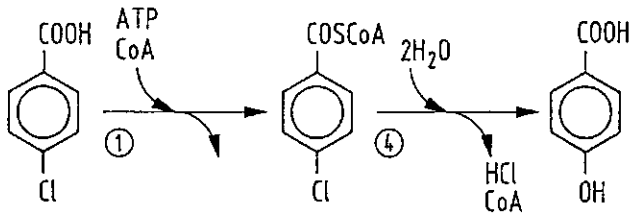
Interestingly also the 2,4,5-trichlorophenoxyacetic acid degradative gene that encodes for the enzyme metabolizing the intermediate, 5-chloro-1,2,4-trihydroxybenzene, is located on the chromosome (Sangodkar et al. 1988). In contrast other genes specifying degradation of halogenated aromatic compounds, e.g. 3-chlorobenzoate and 1,2-dichlorobenzene are located on plasmid rather than on the chromosome (Chatterjee and Chakrabarty 1981, Don and Pemberton 1981, van der Meer et al. 1991). However, recently, several plasmids isolated from strains capable of degrading 4-chlorobiphenyl (4CB) were characterized (Layton et al. 1992). *Alcaligenes* sp strain A5 (containing

pSS50) upon repeated subculturing lost the ability to mineralize 4-chlorobiphenyl to CO2, but retained the ability to produce the metabolite 4-CBA. The loss in ability to mineralize 4-CB was associated with a change in plasmid size from 50Mdal to 35Mdal. Furthermore, another 4-CB degrading strain ALP83 containing a plasmid (pSS70) similar to pSS50 with an additional 13 kb was studied and evidence was presented that the 4-CBA dehalogenase activity was correlated with a 10 kb fragment carried on plasmid pSS70 (Layton et al. 1992).

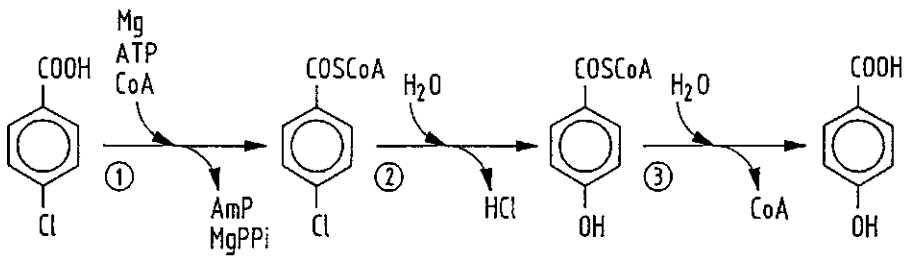
Elsner et al. (1991a) showed that the 4-CBA dehalogenase from *Pseudomonas* sp. CBS3 consists of three components. Apart from two stable proteins with molecular weights of about 86,000 and 92,000, also a third component appeared to be necessary for 4-CBA dehalogenation. This component was highly unstable and had a molecular weight of about 3,000. The involvement of this unstable component might explain why the attempts to purify the 4-CBA-dehalogenase, so far, failed. After subcloning the genes encoding for both proteins from pPSA843 separately into *Escherichia coli* both proteins were expressed constitutively in *Escherichia coli*. The activity of 4-CBA dehalogenase was only detectable in a mixture of the cell extracts of both clones. These two protein components were partly purified from *Escherichia coli* and were identical to the components purified earlier from *Pseudomonas* sp. CBS3 (Elsner et al. 1991b). The nature of the low molecular weight component was elucidated, and revealed a mixture of ATP, Coenzyme A and Mg^{2+}, since addition of these compounds restored activity in dialyzed extracts from *Pseudomonas* sp. CBS3 and *Escherichia coli* clones (Löffler et al. 1991a, Elsner et al. 1991b). Subsequent to these findings the mechanism of the dehalogenation reaction was partly elucidated. It was demonstrated that the Coenzyme A ester of 4-CBA is an intermediate in the dehalogenation of 4-CBA by 4-CBA dehalogenase of Pseudomonas sp. CBS3 (Löffler and Müller 1991b). Furthermore, after synthesis of 4-CBA:CoA with Component I (4-CBA:CoA ligase), the 4-CBA dehalogenase component II converted the 4-chlorobenzoyl CoA ester to 4-HBA and CoA in the absence of cofactors. Since it had been demonstrated previously that the chlorine was substituted by a hydroxyl derived from water, this second enzyme involved in the degradation of 4-CBA was identified as a hydrolase (Löffler and Müller 1991b).

From these data it can be concluded that before conversion of 4-CBA to 4-HBA, 4-CBA first has to be activated to the CoA derivative by a ligase as reported also for other aromatic compounds (Schennen et al. 1985, Harwood et al. 1986, Ziegler et al. 1989). The involvement of 4-CBA:CoA in the degradative pathway of 4-CBA was also demonstrated in another 4-CBA degrading bacterium. Addition of ATP, Coenzyme A to cell-free extracts of the coryneform bacterium NTB-1 also resulted in a pronounced stimulation of the 4-CBA dehalogenase activity (Groenewegen et al. 1992a). The conversion of this 4-chlorobenzoyl CoA ester (chemically synthesized) to 4-

hydroxybenzoate and CoA was shown in cell-free extracts of the coryneform bacterium NTB-1 (Groenewegen et al. 1992a).



Löffler and Müller 1991b



Scholten et al. 1991

Fig. 4 Proposed mechanisms for 4-chlorobenzoate dehalogenation catalyzed by 4-CBA-dehalogenase from *Pseudomonas* sp. CBS3
(1) 4-CBA:CoA ligase
(2) 4-CBA:CoA dehalogenase
(3) 4-HBA:CoA thioesterase
(4) 4-CBA:CoA hydratase]

Another mechanism for the dehalogenation of 4-CBA was proposed by Scholten et al. (1991). After subcloning pPSA843 from *Pseudomonas* sp. CBS3, three ORF's (open reading frames) were detected encoding for a 30 kD, 57 kD and a 19.4 kD polypeptide. All three polypeptide subclones were required for dehalogenase activity. The dehalogenase activity was separated into two components: component I; 80 kD revealed to be a dimer of the 57 and 30 kD polypeptides, and component II; a 65 kD (α 4 tetramer). Component I catalyzed the cleavage of ATP to AMP an PPi coupled with the formation of 4-HBA:CoA, Component II catalyzed the hydrolysis of 4-HBA:CoA to 4-HBA and CoA. From these results it was concluded that dehalogenase activity was the sum of a 4-CBA:CoA ligase, 4-CBA:CoA dehalogenase and 4-HBA:CoA thioesterase.

The mechanism of 4-CBA dehalogenation proposed by Scholten et al is different with

respect to function of components I an II of 4-CBA dehalogenase proposed by Löffler and Müller (1991b) (Fig. 4). Since both groups used the same DNA fragment from the same organism, the two components of the 4-CBA dehalogenase purified must be similar. Löffler and Müller (1991b) demonstrated the first component catalyzed the formation of 4-CBA:CoA from 4-CBA and CoA. In contrast Scholten et al. (1991) concluded from their results (not shown) the formation of 4-HBA:CoA. As a result of these different findings the second component must be either a 4-CBA:CoA hydrolase or a 4-HBA:CoA thioesterase. Although Scholten et al. (1991) reported component II hydrolyzed 4-HBA:CoA unfortunately no data were presented and no attention was given to the possibility (shown by Löffler and Müller 1991b) that component II in fact hydrolyses 4-CBA:CoA.

Reductive displacement of halogen by a hydrogen group

Anaerobic reductive dehalogenation involves the replacement of a halogen by a hydrogen. Many halo-aromatic compounds such as 3-chlorobenzoate, 2,4,5-trichlorophenoxyacetic acid, chlorophenols and 1,2,4-trichlorobenzene are reductively dehalogenated by bacteria under anaerobic conditions as an initial step in their degradation (Reineke and Knackmuss 1988). Partial metabolism was observed in many cases, with only one chlorine ion being removed and lesser-chlorinated metabolites being accumulated (Engesser and Fischer 1991, Fathepure et al. 1988).

However, the involvement of reductive dechlorination in the complete degradation of halo-aromatics by pure cultures has been described in only a few cases. Polyhalophenols are metabolized by both hydrolytic and reductive processes. A cell-free extract of a *Rhodococcus* sp. (Häggblom et al. 1989) was found to catalyze formation of 1,2,4-trihydroxybenzene from 2,3,5,6-tetrachlorohydroquinone, following a reductive mechanism. This same mechanism seems to operate in the degradation of polychlorophenols by a *Flavobacterium* sp. isolated with pentachlorophenol as selection substrate (Steiert et al. 1987). In addition, in the degradation of 2,4-dichlorobenzoate by the coryneform bacterium NTB-1 a reductive dechlorination 2,4-dichlorobenzoate to 4-chlorobenzoate was demonstrated (van den Tweel et al. 1987a).

Halogenated aromatic compounds utilized as electron acceptor

Many reductive dehalogenation reactions have been described in the degradation of halo-aromatics by mixed cultures (Engesser and Fischer, 1991). However, despite many examples of the involvement of reductive dehalogenation little is known about bacteria catalyzing this process and about the exact mechanism of reductive dechlorination. The crucial question is whether the microorganisms can derive energy from the dehalogenation reaction.

The best studied model system is the dehalogenation of 3-halo-substituted benzoates (Shelton and Tiedje 1984). Dolfing and Tiedje (1987) described the reductive dehalogenation of 3-chlorobenzoate by a defined methanogenic bacterial consortium. The key organisms from this consortium were characterized. Apart from the dechlorinating organism *Desulfomonile tiedjei* DCB-1, a benzoate degrader (BZ-1) and a lithotrophic methanogen (*Methanospirillum* strain PM-1) were isolated from enrichment on 3-chlorobenzoate. *Desulfomonile tiedjei* DCB-1, a strict anaerobe, dechlorinated meta-chlorobenzoates replacing the chlorine on 3-CBA by hydrogen yielding benzoate and chlorine. (Dolfing and Tiedje 1987, Deweerd et al. 1991). This strain apparently conserves energy from this reaction since dechlorination supported formation of a proton-motive force which in turn supports ATP synthesis via a proton-driven ATPase (Dolfing and Tiedje 1987, Mohn and Tiedje 1990, Dolfing 1990 and Mohn and Tiedje 1991). As terminal electronacceptor for this form of respiration protons transferred to chlorobenzoate were derived from water and not from the initial electron donor acetate. Whole cells of strain DCB-1 converted 2,5-dichlorobenzoate into 2-chlorobenzoate in buffer containing D_2O and replacement of the position 5 proton with deuterium was demonstrated (Griffith et al. 1992). Similarly Nies and Vogel (1991) showed that protons from water were the source of hydrogen atoms added to the aromatic ring in the reductive dechlorination of 2,3,4,5,6-pentachlorobiphenyl to 2,3,4,6-tetrachlorobiphenyl.

Bacterial degradation of nitro-aromatic compounds

Aerobic metabolism of homocyclic nitro-aromatics seemingly follows the general degradation pattern of homocyclic aromatics. The nitro compounds are converted into hydroxylated aromatics, either in an initial oxygenation reaction also yielding nitrite, or in a sequence of reactions yielding ammonium rather than nitrite (Zeyer 1988).

Oxygenolytic degradation of nitro-aromatic compounds

Direct removal of the nitro group from the aromatic nucleus with the liberation of nitrite was demonstrated in the degradation of various nitro aromatic compounds, and enzymes catalyzing this reaction have been identified. Simpson and Evans (1953) suggested an oxidative removal of the nitro group of 2-nitrophenol by a *Pseudomonas* spp.. The 2-nitrophenol oxygenase from *Pseudomonas putida* was NADPH-dependent and catalyzed the conversion of 2-nitrophenol to catechol and nitrite. Subsequently, catechol is converted by a catechol 1,2-dioxygenase to *cis,cis*-muconate (Zeyer et al. 1986). Zeyer and Kocher (1988) reported the purification of 2-nitrophenol oxygenase and demonstrated that the enzyme exhibited a broad substrate specificity. Spain et al. (1979) demonstrated that 4-nitrophenol is converted to hydroquinone in cell-free extract of a *Moraxella* sp. Although 1,4-benzoquinone was not detected, this compound was proposed

as intermediate and a possible role of 1,2,4 benzenetriol was ruled out (Spain and Gibson 1991). In contrast Raymond and Alexander (1971) suggested that a *Flavobacterium* sp. converted 4-nitrophenol to 4-nitrocatechol as a first step in the degradation. Also the degradation of 2,6-dinitrophenol yielded nitrite as an end-product (Bruhn et al. 1987).

Recently, also the oxidation of apolar nitro-aromatic compounds was demonstrated. Nitrobenzene (Haigler and Spain 1991), 1,3-dinitrobenzene (Dickel and Knackmuss 1991) and 2,4-dinitrotoluene (Spanggord et al. 1991) were all degraded *via* oxygenolytic reactions. A general scheme for oxygenolytic degradation of nitro-aromatic compounds is given in Figure 5.

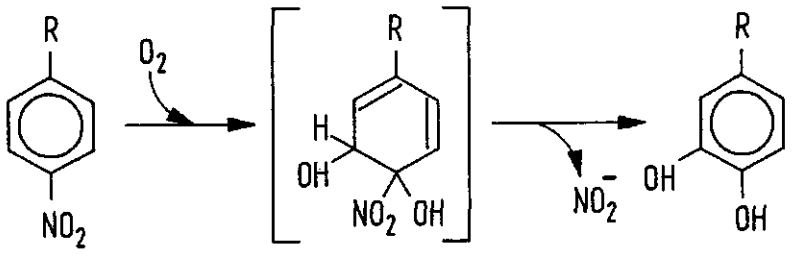


Fig. 5 General scheme for oxygenolytic degradation of nitro-aromatic compounds

Reductive degradation of nitro-aromatic compounds

The involvement of nitroreductases in the complete degradative pathways of nitro-aromatic compounds has been reported (Durham 1958; Ke et al. 1959; Germanier and Wuhrman 1963; Cartwright and Cain 1959a,b; Cain 1966; Zeyer and Kearney 1984). In the aerobic metabolism of 4-nitrobenzoate, reduction of the nitro group *via* 4-nitrosobenzoate to 4-hydroxylaminobenzoate was demonstrated. Although *Nocardia erythropolis* produced 4-aminobenzoate from 4-nitrobenzoate, no evidence was provided that the amino-aromatic compound is a direct intermediate in the degradative pathway of 4-nitrobenzoate (Cartwright and Cain 1959a,b, Cain and Cartwright 1960). After reduction of 4-nitrobenzoate further degradation was suggested to proceed via 3,4-diHBA (Fig. 6).

A *Flavobacterium* sp. studied by Ke et al (1959) when grown on 2-nitrobenzoate was adapted to 2-hydroxylaminobenzoate but not to 2-aminobenzoate. *Nocardia opaca* (Cain 1966) when growing on 2-aminobenzoate transiently accumulated 2-aminobenzoate under oxygen-limited conditions, but not under fully aerobic conditions. The organism was only adapted to 2-aminobenzoate during growth on 2-nitrobenzoate under conditions allowing

accumulation of 2-aminobenzoate. It was consequently concluded that the amino compound was not an intermediate of the pathway of 2-nitrobenzoate metabolism in the organism (Cain 1966). Furthermore, also in the reductive degradation of 3-nitrophenol it was not possible to show an involvement of 3-aminophenol (Zeyer and Kearney 1984).

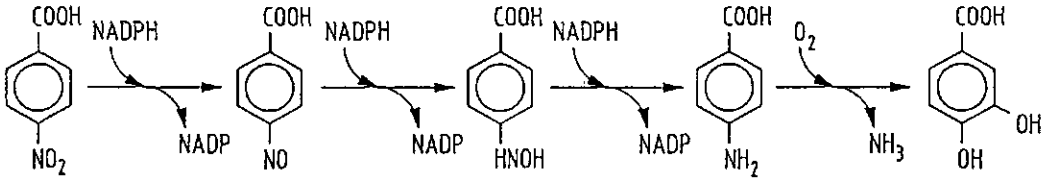


Fig. 6 Degradative pathway of 4-nitrobenzoate by reductive displacement of the nitro-group

In chapter 4 and 5 the degradation of 4-nitrobenzoate by *Comamonas acidovorans* NBA-10 is described. In this strain a novel degradative pathway of 4-nitrobenzoate was detected. 4-Nitrosobenzoate and 4-hydroxylaminobenzoate (4-HABA) were identified as intermediates. Furthermore, evidence was obtained that 4-HABA, also in the absence of oxygen, was further degraded via 3,4-dihydroxybenzoate (3,4-diHBA). The degradative pathway for 4-NBA consequently does not follow the general pattern of aerobic metabolism of homocyclic aromatic compounds since no involvement of molecular oxygen was implied in the formation of 3,4-diHBA (Groenewegen et al. 1992b,c).

Degradation of nitro-aromatic compounds has also been demonstrated in fungi. Under ligninolytic conditions, *Phanerochaete chrysosporium* mineralizes 2,4-dinitrotoluene. The multistep pathway involves the initial reduction to 2-amino-4-nitrotoluene, which was further oxidized to 4-nitro-1,2-benzoquinone (Valli et al. 1992).

Nitro-aromatic compounds utilized as electron acceptor

In various bacterial strains, lacking the capability to mineralize nitro-aromatics, reduction of nitro-aromatic compounds has been demonstrated. The nitroreductases involved were constitutively expressed in these organisms. Nitroreductases from these organism seemed to have a wide spectrum of substrates, in contrast to the reductases detected in organisms capable of utilizing nitro-aromatic compounds as sole carbon and energy source (Rafii et al. 1991, Kitamura et al. 1983). Schackman and Müller (1991) demonstrated that resting cells of *Pseudomonas* sp. CBS3 also reduced a range of nitroaromatic compounds to the corresponding amines. The conversion rates with 1-

chloro-2,4-dinitrobenzenes were higher than with the nitrobenzoates and nitrophenols, and dinitro-aromatics were better substrates than mononitro-aromatics. These results suggest that the rate of reduction increases with electron withdrawing substituents.

Anaerobic bacteria commonly associated with the gastrointestinal tract also reduce nitro polycyclic aromatic to the corresponding amines (Rafii et al. 1991, Kitamura et al. 1983). Kinouchi and Onishi (1983) have purified four reductases from *Bacteroides fragilis* that convert 1-nitropyrene to 1-aminopyrene. The ability of bacteria isolated from the human intestinal flora to reduce these nitroaromatic compounds decreases the mutagenicity of nitro polycyclic aromatic compounds.

Recently, Oren et al. (1991) demonstrated that the anaerobes *Haloanaerobicum praevalens* and *Sporohalobacter marismortui* reduced a wide variety of nitosubstituted aromatics. Compounds reduced to the corresponding amines included nitrobenzene, nitrophenols and nitroanilines.

Outline of this thesis

The biotechnological production of hydroxylated aromatic compounds is of interest since chemical syntheses of these compounds is rather difficult. Formation of hydroxylated aromatic compounds might be achieved by replacement of a substituent on the aromatic ring by a hydroxyl group.

Two different groups of substituted aromatic compounds have been studied halogenated and nitro-aromatic compounds, as starting material for bioformation of hydroxylated aromatic compounds. Both these classes of compounds are readily obtained by chemical methods. Organisms capable of degrading these substituted aromatic compounds were isolated and enzymes involved in the conversion of these compounds to hydroxy-aromatics were studied.

In this thesis the research concentrated on the characterization of the enzymes catalyzing the replacement the substituent by a hydroxyl group. Furthermore, the potential of the enzymatic conversion was studied in more detail and conditions for the formation of the hydroxy-aromatic compound were optimized. The investigation of the degradative pathways of two model substrates yielding hydroxylated aromatic compounds resulted in the discovery of several novel enzymes.

In the first part of this thesis the conversion of 4-chlorobenzoate to 4-hydroxybenzoate catalyzed by the coryneform bacterium NTB-1 is described. In chapter 2 the involvement of an energy-dependent uptake system for 4-chlorobenzoate in this bacterium is described. Conditions for the formation of 4-hydroxybenzoate from 4-chlorobenzoate by resting cells were optimized. Some attention was given to the enzymatic mechanism of the 4-chlorobenzoate dehalogenation, and a novel mechanism of dehalogenation of this halogenated aromatic compound is proposed. Evidence is provided for the involvement

of 4-chlorobenzoyl CoA, which is formed in the presence of ATP, as intermediate in the conversion of 4-chlorobenzoate to 4-hydroxybenzoate (chapter 3)

In the second part of this thesis a novel degradative pathway in *Comamonas acidovorans* of 4-nitrobenzoate is proposed. In chapter 4 the initial steps of 4-nitrobenzoate degradation are described and evidence is provided for an incomplete reduction of the nitro-substituent yielding 4-hydroxylaminobenzoate. The 4-nitrobenzoate reductase was partly purified and some properties are given. Furthermore, in this *Comamonas* sp. a new type of anaerobic enzymatic conversion of 4-hydroxylaminobenzoate yielding 3,4-dihydroxybenzoate was detected (chapter 5). The enzyme involved was purified and initially characterized.

Finally in chapter 6 some concluding remarks dealing with the production of hydroxylated aromatic compounds from halogenated and nitro-aromatic compounds are presented, and some applied aspects concerning these biocatalysts are discussed.

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CHAPTER 2

Energy-dependent uptake of 4-chlorobenzoate in the coryneform bacterium NTB-1

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ABSTRACT

The uptake of 4-chlorobenzoate (4-CBA) in intact cells of the coryneform bacterium NTB-1 was investigated. Uptake and metabolism of 4-CBA were observed in cells grown in 4-CBA and not in glucose-grown cells. Under aerobic conditions, uptake of 4-CBA occurred with a high apparent affinity (apparent K_t 1.7 μM) and a maximal velocity (V_{max}) of 5.1 nmol min^{-1} mg of $protein^{-1}$. At pH values below 7, the rate of 4-CBA uptake was strongly greatly by nigericin, an ionophore which dissipates the pH gradient across the membrane (Δ pH), while at higher pH values inhibition was only observed with valinomycin, an ionophore which collapses the electrical potential across the membrane (Δ ψ). Under anaerobic conditions, no uptake of 4-CBA was observed unless an alternative electron acceptor was present. With nitrate as terminal electron acceptor, 4-CBA was rapidly accumulated by the cells to a steady state level at which uptake of 4-CBA was balanced by excretion of 4-hydroxybenzoate. The mechanism of energy coupling to 4-CBA transport under anaerobic conditions was further examined by the imposition of an artificial Δ ψ, Δ pH or both. Uptake of 4-CBA was shown to be coupled to the proton-motive force, suggesting a proton symport mechanism. Competition studies with various substrate analogs revealed a very narrow specificity of the 4-CBA uptake system. This is the first report of carrier-mediated transport of halogenated aromatic compounds in bacteria.

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INTRODUCTION

The coryneform bacterium NTB-1 contains a hydrolytic dehalogenase that catalyzes the initial step in the degradation of 4-chlorobenzoate (4-CBA), and various other halobenzoates, with forming of 4-hydroxybenzoate (4-HBA) and halide ions (van den Tweel et al. 1986, 1987). In this dehalogenation reaction water is used instead of oxygen as the hydroxyl donor (Marks et al. 1984; Müller et al. 1984). Under aerobic conditions, 4-HBA is rapidly oxidized to 3,4-dihydroxybenzoate by an NADPH-dependent 4-hydroxybenzoate hydroxylase, and further degradation proceeds via ring-cleavage (van den Tweel et al. 1987). The finding that no dehalogenation of 4-CBA takes place under anaerobic conditions is unexpected (van den Tweel et al. 1986); cell-free extracts of NTB-1 readily dehalogenate 4-CBA in the absence of molecular oxygen (Groenewegen et al. 1992). When the cells are supplied with an alternative terminal electron acceptor, such as nitrate or ferricyanide, 4-CBA is dehalogenated and 4-HBA accumulates in the suspending medium, since further hydroxylation can not take place. The lack of dehalogenase activity in intact cells under anaerobic conditions, and in the absence of alternative electron acceptors, suggests that an energy-dependent transport system is involved in the uptake of 4-CBA by NTB-1 cells.

Energy-dependent transport systems for halo-aromatic compounds have been implied in some systems (Shimao et al. 1989; van den Tweel et al. 1986, 1987), but so far no solid evidence for carrier-mediated transport has been supplied. Uptake of these compounds as the first step in the degradative pathway is often ignored or considered to proceed by passive diffusion. The uptake of 2,4-dichlorophenoxy acetic acid in a strain of *Pseudomonas fluorescens* which is unable to degrade this compound was studied to some extent (Whedemeyer 1966). Accumulation of 2,4-dichlorophenoxy acetic acid proceeded only up to the equilibration level, and a facilitated diffusion type of uptake mechanism was suggested mainly on the basis of the inhibition of uptake by azide and fluorodinitrobenzene (Whedemeyer 1966). The uptake of nonhalogenated aromatic compounds, such as aromatic carboxylic acids, has been studied in more detail (Cook and Fewson 1972, Harwood and Gibson 1986, Hegeman 1966, Higgins and Mandelstam 1972, Meagher et al. 1972, Merkel et al. 1989, Ornston and Parke 1976, Thayer and Wheelis 1976, 1982). Conclusive evidence for the carrier-mediated transport of benzoate by *Pseudomonas putida* was obtained by Thayer and Wheelis (1976, 1982). Benzoate uptake is mediated by an inducible saturable transport system that can be eliminated by a mutation in a gene designated benP. A strain that was blocked in the subsequent metabolism of benzoate, accumulated benzoate 150-fold against a concentration gradient. The mechanism of benzoate uptake is not sufficiently defined, although ample evidence has been presented

for proton motive force-(Δ p)-driven rather than ATP-driven uptake. The uptake of mandelate by P. putida has also been studied (Hegeman 1966; Higgins and Mandelstam 1972). According to Hegeman (1966), mandelate enters the cell by passive diffusion. Uptake occurred in both induced and uninduced cells but no internal accumulation was observed. In contrast, a carrier-mediated mechanism for mandelate uptake was proposed by Higgins and Mandelstam (1972). They suggested that induced cells accumulate mandelate against a concentration gradient and uptake is blocked by the protonophore 2,4-dichlorophenol. The kinetics of benzoate (Hegeman 1966) and 4-hydroxybenzoate (4-HBA) (Merkel et al. 1989) uptake in *Rhodopseudomonas palustris* grown photosynthetically on these compounds have been studied. These aromatic acids are immediately converted into their Coenzyme A derivatives by an inducible benzoyl-CoA ligase; no evidence of an active transport system was obtained. The kinetic properties of the benzoate- and 4-HBA-COA-ligases correspond to the kinetics of benzoate (Geissler et al. 1988, Harwood and Gibson 1986) and 4-HBA (Merkel et al. 1989) uptake by intact cells respectively, which confirms their role in catalyzing the first degradation steps.

The polar nature of aromatic acids, such as benzoate, is such that they are often used as rapidly diffusible acid (Kihara and Macnab 1981) for measuring of the pH gradient across membranes (Δ pH). Assuming that these compounds enter the cell by passive diffusion, uptake depends on the concentration gradient and Δ pH only. Nevertheless, as indicated above, bacteria have developed highly specific mechanisms for the scavenging of (halo)aromatic acids from the external medium. In this report, evidence that the uptake of 4-CBA by the coryneform bacterium NTB-1 is mediated by an inducible transport system is presented. Some of the characteristics of the 4-CBA carrier are described.

METHODS

Media and culture conditions

The coryneform strain NTB-1 (NCIB 12617) was grown routinely in a chemostat (dilution rate, 0.04 h^{-1}) under carbon-limited conditions. Glucose or 4-CBA was added as the sole carbon source. The cells were grown aerobically at pH 7.0 on a mineral salts medium at 30°C as described previously (van den Tweel et al. 1986). In some experiments, cells capable of using nitrate as a terminal electron acceptor were used. For this purpose, cells were grown at low controlled oxygen concentrations with 60 mM KNO_3 added to the medium.

Assay of ^{14}C-labeled 4-CBA uptake by intact cells

Cells grown in a chemostat were harvested by centrifugation (10 min at 16,000 x g),

washed twice in 50 mM potassium phosphate (pH 7.0) and resuspended in the same buffer. A sample of 2-4 μl of the concentrated cell suspension (20 mg of protein ml^{-1}) was added to 200 μl potassium phosphate buffer at 30°C, and uptake was initiated by the addition of ^{14}C-labeled 4-CBA. Transport was terminated by dilution into 2 ml ice-cold 100 mM LiCl followed by filtration through 0.45 μm - pore size cellulose nitrate membrane filter. The filters were washed once with 2 ml of 100 mM LiCl and dried for 25 min at 120°C. The dried filters were transferred to scintillation counter (Tri-Carb-460CD, Packard Instruments Co. Rockville, Md.). In some experiments, cells were preincubated with ionophores 15 min prior to the addition of ^{14}C-labeled 4-CBA. Nigericin and valinomycin were used at a concentration of 0.5 and 5.0 μM, respectively.

Uptake experiments under anaerobic conditions were performed essentially as described above. A sample (20-40 μl) of the concentrated cell suspension was diluted to 2 ml of 50 mM potassium phosphate, pH 7.0. This solution was magnetically stirred and flushed with oxygen-free nitrogen gas to ensure anaerobic conditions. Uptake was initiated by the addition of 20 μM ^{14}C-labeled 4-CBA. At the times indicated in the figures, 50 μl samples were removed with a Hamilton syringe (Bonaduz, Switzerland) and treated as described above.

Artificially imposed proton motive force-driven uptake of ^{14}C-labeled 4-CBA

For artificially imposed proton motive force-driven uptake of ^{14}C-labeled 4-CBA, cells were incubated for 1 h at 4°C in a solution containing 50 mM potassium phosphate (pH 7.0) and 100 mM potassium acetate in the presence of 5.0 μM valinomycin. Loaded cells were washed once in 100 mM potassium acetate and resuspended in the same buffer. The concentrated suspension was incubated in a sealed bottle, and flushed with oxygen-free nitrogen gas. A gas-tight Hamilton syringe was filled anaerobically with the cell suspension (approximately 50 mg of protein ml^{-1}), and uptake of 4-CBA was initiated by diluting 20 μl of the cell suspension to 2 ml of a solution which contained, in addition to the indicated concentration of ^{14}C-labeled 4-CBA, 50 mM sodium phosphate (pH 7.0)- and 100 mM sodium 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (Δ p, interior negative and alkaline), 50 mM sodium phosphate (pH 7.0)- and sodium acetate ($\Delta\ \psi$, interior negative), 50 mM potassium phosphate (pH 7.0)- 100 mM potassium HEPES, (Δ pH, interior alkaline), or 50 mM potassium phosphate (pH 7.0)- and potassium acetate (no gradient) (Driessen et al. 1987). The suspension was magnetically stirred, and flushed with oxygen-free nitrogen gas. At times indicated in the figures, 50-μl samples were removed and assayed for 4-CBA uptake.

Materials

Ring-U-^{14}C-labeled 4-CBA (4.03 GBq $mmol^{-1}$) was obtained from the Radiochemical Centre, Amersham (Buckinghamshire, United Kingdom). The stock ^{14}C-labeled 4-CBA (3.1mM) was diluted in the experiments as indicated in the legends to the figures. The

halogenated aromatic compounds were obtained from Janssen Chimica (Beerse, Belgium). All other chemicals were of the highest purity commercially available.

RESULTS

Uptake of 4-chlorobenzoate under aerobic conditions

The uptake of ^{14}C-labeled 4-CBA under aerobic conditions by a washed cell suspension of NTB-1 cells grown in the presence of 4-CBA is shown in Fig. 1. Under aerobic conditions, uptake and respiration of 4-CBA occurred at the same time. Uptake was linear for several minutes and ceased when most of the label was removed from the external medium (data not shown). A low rate of 4-CBA uptake was observed with glucose-grown cells, either in the absence (Fig. 1) or presence of glucose (data not shown).

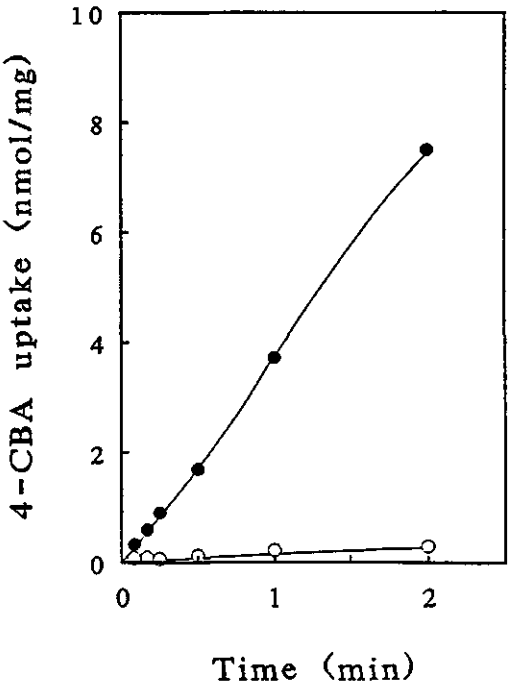

Fig. 1. Uptake of ^{14}C-labeled 4-CBA (final concentration, 78 μM) by NTB-1 cells grown in the presence of 4-CBA (●) or glucose (○)

Previously, it was shown that the hydrolytic dehalogenase is only present in cells induced with 4-CBA (van den Tweel et al. 1987). These results suggest that in NTB-1 cells uptake of 4-CBA is mediated by an inducible transport system.

Uptake of 4-chlorobenzoate under anaerobic conditions

The accumulation of 4-CBA under aerobic conditions may be the result of passive diffusion in conjunction with the conversion of this halo-aromatic compound to a less lipophilic derivative. The degradation of 4-CBA involves a dehalogenation step yielding 4-HBA, which is further oxidized *via* protocatechuate. As shown previously, 4-CBA is not metabolized under anaerobic conditions (van den Tweel et al. 1986). When 4-CBA grown cells were incubated anaerobically, no 4-CBA entered the cell (Fig. 2). Restoring aerobic conditions caused a rapid uptake of the labeled substrate. These results demonstrate that 4-CBA is not accumulated under anaerobic conditions.

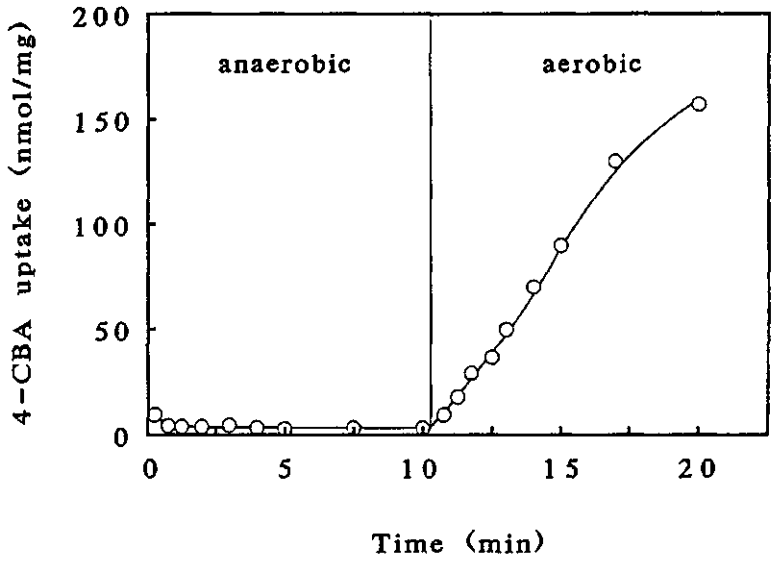

Fig. 2. Uptake of ^{14}C-labeled 4-CBA (final concentration, 78 μM) by NTB-1 in the absence and presence of oxygen

Cells grown on 4-CBA in the presence of nitrate at low oxygen tension express a nitrate reductase system (Groenewegen et al. 1992). These cells are able to use nitrate as the terminal electron-acceptor under anaerobic conditions. Addition of nitrate to the

cells, during anaerobic transport experiments, resulted in an immediate uptake of 4-CBA (Fig. 3). Unlike the uptake observed under aerobic conditions, the uptake under anaerobic conditions with nitrate as electron acceptor, a steady state was reached corresponding to a 30-fold accumulation of the label. Under these conditions, 4-HBA is excreted in the suspending medium (Groenewegen et al. 1992). Therefore, the steady state most likely represents an equilibrium between uptake of 4-CBA and excretion of 4-HBA. The introduction of molecular oxygen in the system instantly resulted in a further uptake of label (Fig. 3). These results show that under anaerobic conditions, uptake of 4-CBA is impeded, because of the lack of a suitable electron acceptor. With nitrate as the terminal electron acceptor, the accumulation of 4-CBA against a concentration gradient is evident, although a passive diffusion mechanism cannot definitely be ruled out.

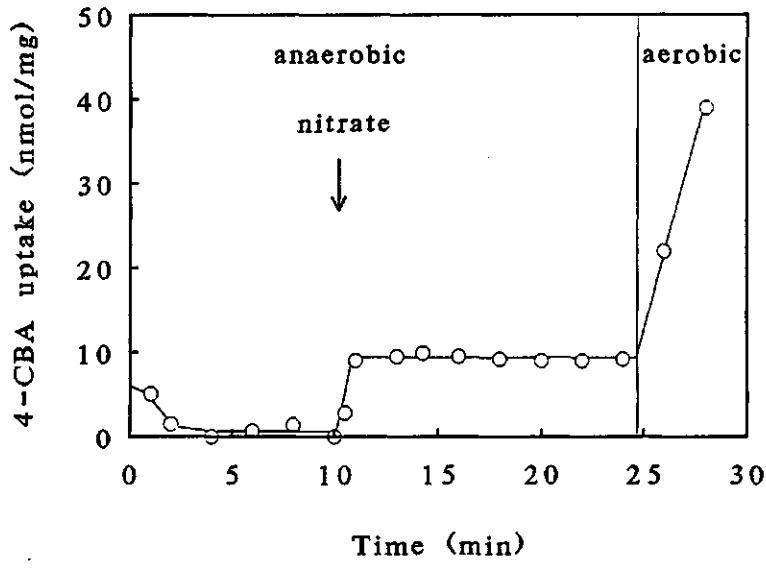

Fig. 3. Uptake of ^{14}C-labeled 4-CBA (final concentration, 78 μM) by NTB-1 cells of NTB-1 under anaerobic conditions in the absence and presence of nitrate. The addition of KNO_3 to a final concentration of 60 mM is indicated by the arrow. At t = 25 min, molecular oxygen was introduced in the uptake vessel

Artificially imposed protonmotive-force driven uptake of 4-CBA under anaerobic conditions

The inability of NTB-1 cells to accumulate 4-CBA under anaerobic conditions may be caused by a low Δ p. To test this hypothesis, the effect of an artificially imposed Δ p on 4-CBA transport under anaerobic conditions was studied. A Δ ψ, inside negative, can be created by diluting K^+-loaded cells into a K^+-free (i.e. Na^+) buffer in the presence of valinomycin. A Δ pH, inside alkaline, can be generated by diluting acetate-loaded cells into a solution containing a less permeable anion, such as HEPES. A Δ p, inside negative and alkaline, can be established by the simultaneous imposition of outwardly-directed diffusion gradients of K^+-ions and acetate. Transient accumulation of 4-CBA was observed with a Δ pH or a Δ ψ (Fig. 4). No accumulation of 4-CBA was observed in the absence of a gradient, while the highest level of accumulation was observed when both a Δ pH and Δ ψ were imposed simultaneously (Fig. 4). In contrast, the imposition of an artificial Δ p in glucose-grown cells failed to promote uptake of 4-CBA (data not shown). These results unequivocally demonstrate that uptake of 4-CBA is dependent on the Δ p and can only be explained if the uptake is carrier-mediated.

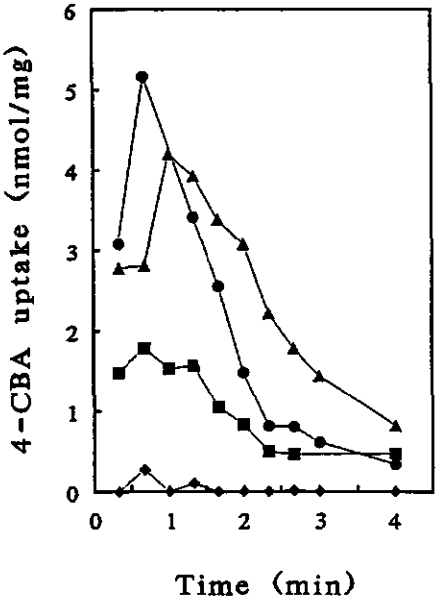

Fig. 4. Uptake of ^{14}C-labeled 4-CBA (final concentration of 12 μM) by NTB-1 cells under anaerobic conditions (pH 7.0), in the absence of a Δ p (♦) or with an artificially imposed Δ ψ (■), Δ pH (▲), and Δ p (●)

Effect of ionophores on 4-chlorobenzoate transport

To define the role of the Δ p in more detail, the effects of the ionophores nigericin and valinomycin on the initial rate of 4-CBA uptake under aerobic conditions were investigated. Nigericin mediates electroneutral H^+-K^+ exchange, thereby collapsing the Δ pH. At pH 5.0, the rate of 4-CBA uptake was dramatically reduced by this ionophore (Fig. 5). Nigericin was ineffective at more high pH values. An opposite effect was obtained with valinomycin, which dissipates the Δ ψ in the presence of K^+-ions. At pH 5.0, the rate of 4-CBA uptake was stimulated by valinomycin at least two-fold. With increasing pHs, the stimulating effect of valinomycin was gradually changed to an inhibitory effect (Fig. 5). The simultaneous addition of both ionophores completely prevented 4-CBA uptake at low pH, although at pH 7.0 and above, some residual uptake remained (Fig. 5). The data indicates that the uptake of 4-CBA at low pH is predominately coupled to the Δ pH. At high pHs, uptake is an electrogenic process, which suggests that transport occurs in symport with more than one proton.

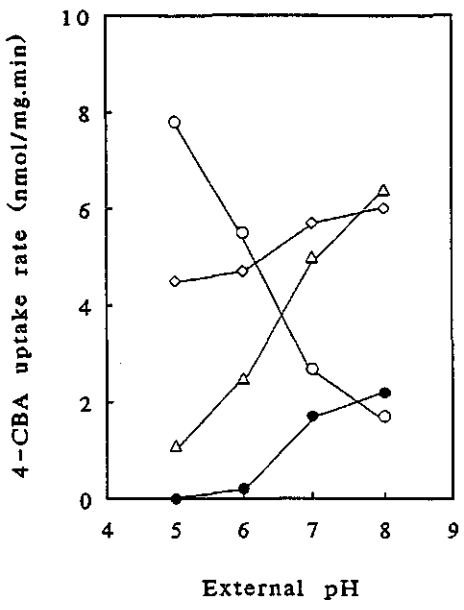

Fig. 5. Effect of ionophores on ^{14}C-labeled 4-CBA (5.9 μM) uptake by NTB-1 cells at different pH values. Cells were incubated under aerobic conditions, and the initial rate of uptake was determined in the absence (◇) or presence of 0.5 μM nigericin (△), 5 μM valinomycin (○), or both 0.5 μM nigericin and 5 μM valinomycin (●)

Kinetics and specificity of 4-CBA transport

The kinetic parameters of 4-CBA uptake were estimated from the initial rate of uptake under aerobic conditions at concentrations from 0.9 to 12 μM. At pH 7.0, uptake of 4-CBA displayed monophasic saturation kinetics (Fig. 6) with an apparent affinity constant (K_t) of 1.7 μM. The maximal velocity (V_{max}) was 5.1 nmol min^{-1} mg of cell $protein^{-1}$ but varied between different experiments. The rate of 4-CBA uptake decreased slowly upon prolonged storage of the NTB-1 cells at 4°C.

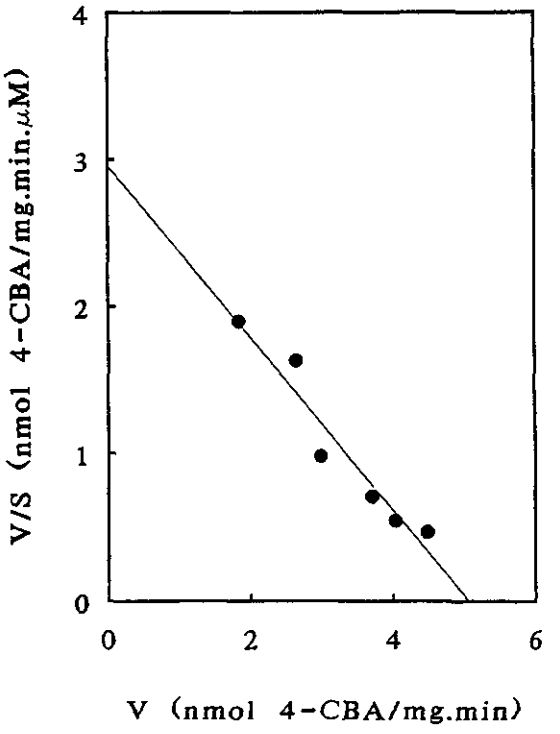

Fig. 6. Kinetic analysis of ^{14}C-labeled 4-CBA uptake by NTB-1 cells. Cells were incubated under aerobic conditions in 50 mM potassium phosphate (pH 7.0) and the initial rate of ^{14}C-labeled 4-CBA uptake was determined from time points of label uptake taken within 1 min. The 4-CBA concentration was varied between 0.9 and 12 μM

The specificity of the 4-CBA transport system was assessed from the extent of inhibition of ^{14}C-labeled 4-CBA transport by a 30-fold excess of a wide range of substrate

analogs. The transport system appeared to be specific for *para*substituted mono- and dihalobenzoates (Table 1). 4-CBA uptake is blocked by other halobenzoates in the following order of efficiency: 4-CBA ≈ 4-bromobenzoate > 4-iodobenzoate > 3,4-dichlorobenzoate > 4-fluorobenzoate (FBA) > 2,4-dichlorobenzoate (Table 1). 4-HBA did not inhibit uptake of 4-CBA. The carboxyl-group on the benzene nucleus seems to be essential for recognition by the 4-CBA uptake system.

Inhibitor	**Residual activity** (%)[a]
Benzoate	80
3-Chlorobenzoate	82
4-Fluorobenzoate	26
4-Chlorobenzoate	0
4-Bromobenzoate	0
4-Iodobenzoate	1
4-Hydroxybenzoate	100
4-Nitrobenzoate	90
4-Methoxybenzoate	30
4-Aminobenzoate	100
4-Chloro 3,5-dinitrobenzoate	100
2,4-Dichlorobenzoate	39
3,4-Dichlorobenzoate	16
2,5-Dichlorobenzoate	82
4-Chlorophenylacetate	100

[a]Inhibitor and 4-chloro[^{14}C-]benzoate were present at concentration of 100 and 3.1 μM, respectively.

Table 1. Inhibition of ^{14}C-labeled 4-CBA uptake by structural analogs

DISCUSSION

The main objective of this study was to obtain information about the mechanism of 4-CBA uptake by the coryneform bacterium NTB-1 and the mechanism of energy coupling. Several lines of evidence are consistent with the involvement of an Δ p-driven uptake system for this haloaromatic compound, and argue against a passive diffusion

mechanism: (i) the imposition of Δ p under anaerobic conditions resulted in a transient accumulation of 4-CBA, (ii) the uptake of 4-CBA under anaerobic conditions was dependent solely on the presence of a suitable electron acceptor, (iii) the uptake of 4-CBA under aerobic conditions was sensitive to ionophores, and uptake is electrogenic at alkaline pH values, (iv) the rate of 4-CBA uptake showed saturation kinetics with a high apparent affinity, (v) the substrate specificity of the transport system was rather narrow. To our knowledge, this is the first report of a specific transport system for halogenated aromatic compounds in bacteria.

Lipophilic compounds usually enter the cytoplasmic membrane by passive diffusion. For a lipophilic weak acid, such as 4-CBA, with a high permeability coefficient (log P 2.65) (Fujita et al. 1964), passive diffusion is the most obvious mechanism for uptake. Uptake by such a mechanism would, however, be coupled solely to the Δ pH and the inwardly directed concentration gradient which is sustained by metabolism. The dramatic effects of nigericin and valinomycin at low pH values are consistent with this model. However, at alkaline pH, uptake is almost completely abolished by valinomycin which indicates that uptake of 4-CBA is an electrogenic process. This implies that translocation takes place in symport with more than one proton. At this stage, it is not possible to determine the value of the 4-CBA/H^+ stoichiometry. It is possible that uptake of 4-CBA can be regulated by the intracellular pH, as has been demonstrated for uptake of solutes in lactococci (Poolman et al. 1987). With the prevailing metabolism, a steady state which not necessarily represents a thermodynamic equilibrium with the existing Δ p is reached.

Intact cells require an electron acceptor for the dehalogenation of 4-CBA (Shimao et al. 1989; van den Tweel et al. 1986), whereas studies with cell extracts indicate that the enzymatic reaction itself is independent of molecular oxygen or any other electron acceptor (Shimao et al. 1989). Our results show that the inability of cells to dehalogenate 4-CBA under anaerobic conditions is due to a lack of activity of the transport system. Activity of 4-CBA uptake is restored when a Δ p is generated by the addition of an alternative electron acceptor (i.e. nitrate) or the imposition of diffusion gradients. Similar phenomena have been observed for the degradation of pentachlorophenol by Rhodococcus chlorophenolicus. This organism was isolated on pentachlorophenol and requires a hydrolytic dehalogenase for the degradation of polychlorinated phenols (Apajalahti and Salkinoja-Salonen 1987). Intact cells were unable to dehalogenate any of the polychlorinated phenols during anaerobiosis while with cell extracts did not needed molecular oxygen. The suggestion that oxygen is essential for generating energy or for restoring the activity of the enzymes or cofactors used in the dehalogenation/hydroxylation reaction has been made. The analogy with the oxygen-requirement of dehalogenation of 4-CBA by the coryneform bacterium NTB-1 indicates that an active transport system may be involved in uptake of polychlorinated aromatic compounds by

Rhodococcus chlorophenolicus.

NTB-1 cells are able to use 4-HBA as sole carbon and energy source (van den Tweel et al. 1986, 1987). Cells grown on halobenzoates, rapidly oxidize 4-HBA at a rate comparable to the rate of 4-CBA oxidation (van den Tweel et al. 1986). 4-HBA is not a substrate for the halo-benzoate carrier, which may indicate that a separate transport system for 4-HBA is present in NTB-1 cells. On the other hand, 4-FBA efficiently inhibits uptake of 4-CBA, and rapid counterexchange between both substrates can be observed under anaerobic conditions (data not shown) indicating that 4-FBA is efficiently translocated. Growth of NTB-1 cells is not supported by this compound (van den Tweel et al. 1986, 1987). 4-FBA is not oxidized or dehalogenated by a washed cells suspension or a cell-free extract (Groenewegen et al. 1992).

The results presented here demonstrate that the first enzymatic step of the degradation of 4-CBA, and other halobenzoates, is the carrier-mediated uptake of these compounds. Similar experiments with other microorganisms capable of converting (halo)aromatic compounds are essential for a understanding of the kinetics and regulatory mechanisms involved in these degradative processes.

Acknowledgement

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CHAPTER 3

Anaerobic bioformation of 4-hydroxybenzoate from 4-chlorobenzoate by the coryneform bacterium NTB-1

Peter E.J. Groenewegen, Will J.J. van den Tweel, and Jan A.M. de Bont

ABSTRACT

Resting cells of the coryneform strain NTB-1, previously incorrectly classified as *Alcaligenes denitrificans* NTB-1, quantitatively converted 4-chlorobenzoate (4-CBA) to 4-hydroxybenzoate (4-HBA) under strict anaerobic conditions in the presence of ferricyanide or nitrate. 4-HBA formation was enhanced by supplying anaerobic cells with glucose as an energy source. Using permeabilized cells it was shown that energy is not only needed to drive the energy-dependent uptake of 4-CBA but also to convert 4-CBA into 4-HBA. In cell-free extracts it was subsequently demonstrated that a Coenzyme A-thioester of 4-CBA is involved in the metabolism of 4-CBA.

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INTRODUCTION

Hydroxylated aromatic compounds are widely used in the pharmaceutical and petrochemical industries. Chemical introduction of a hydroxyl group on the aromatic ring involves laborious and expensive reactions because no direct preparative chemical reactions are available for aromatic hydroxylation (van den Tweel et al. 1986). Therefore biotechnological preparation of hydroxylated aromatic compounds can be an alternative. Bioformation can be accomplished by direct hydroxylation of the benzene nucleus, or by replacement of a substituent by a hydroxyl group. In the present work, microbial formation of a hydroxylated aromatic compound from a halogenated compound was investigated. Halo-aromatics are easily prepared by chemical synthesis.

Previously, it was shown by van den Tweel et al. (1986) that a mixed culture containing the coryneform strain NTB-1 produced 4-hydroxybenzoate (4-HBA) from 4-chlorobenzoate (4-CBA) under O_2-limited conditions. Anaerobic 4-HBA formation from 4-CBA by whole cells of strain NTB-1 did not occur (van den Tweel et al. 1987a), which is surprising in view of the presumably hydrolytic nature of the first reaction step. This hydrolytic nature of the dehalogenase reaction has been established in two other 4-CBA degrading organisms by showing that the oxygen atom of the hydroxyl group of 4-HBA is derived from water and not from molecular O_2 (Marks et al. 1984b, Müller et al. 1984). Recently, however, the involvement of ATP and CoA in the reaction have been suggested (Elsner et al. 1991b).

In a previous paper (Groenewegen et al. 1990), we showed that in the coryneform bacterium NTB-1 an energy-dependent uptake system of 4-CBA is present. The uptake of 4-CBA in whole cells was coupled to the proton motive force, suggesting a proton symport mechanism. Under anaerobic conditions, no uptake of 4-CBA was observed unless an alternative electron acceptor was present.

In this paper anaerobic production of 4-HBA from 4-CBA, but in the presence of alternative electron acceptors, has been studied in more detail. Furthermore, evidence is provided for the involvement of the CoA ester of 4-CBA rather than free 4-CBA in the dehalogenation reaction.

MATERIAL AND METHODS

Media and culture conditions

The coryneform strain NTB-1 (NCIB 12617) was routinely grown in a 2-l chemostat (working volume 1 l, $D = 0.02\ h^{-1}$) under carbon-limited conditions. The cells were grown

at pH 7.0, at 50 % of air saturation, and at 30°C on a medium containing 4-CBA (15 mM), K_2HPO_4 (1.55 gl^{-1}), NaH_2PO_4 (0.85 gl^{-1}), $(NH_4)_2SO_4$ (3.0 gl^{-1}), $MgSO_47H_2O$ (0.1 gl^{-1}, yeast extract 0.1 gl^{-1}, and 0.2 ml of a trace solution (Vishniac and Santer 1957). The chemostat containing 2 mM 4-CBA was inoculated with a batch culture precultivated on 2 mM 4-CBA. Cells able to use glucose as an alternative energy source were grown on the same medium with glucose (9.25 gl^{-1}) added to it. Cells used in anaerobic experiments were induced for using nitrate as alternative electron acceptor by precultivating under low controlled oxygen concentrations (5% of air saturation) and in the presence of KNO_3 (2.0 gl^{-1}).

Experiments with whole cells

In anaerobic experiments potassium ferricyanide (10 mM) was added as alternative electron acceptor (Adlercreutz 1987). The anaerobic experiments were performed in 100 ml serum bottles, with washed concentrated cell suspensions. After flushing the bottles for more than 15 min with O_2 free N_2 gas the reaction was initiated with 4-CBA (flushed with N_2). The bottles were incubated in a shaking waterbath (30°C, 1 Hz, amplitude 0.02 m), and samples were periodically withdrawn from the incubation mixture and cells were directly removed by centrifugation.

Anaerobic formation of 4-HBA in the presence of nitrate (60 mM) and glucose (50 mM) was tested using the same procedure with cells precultivated in a chemostat at low oxygen concentration in the presence of nitrate.

The effect of nitrite was studied by preincubation of these concentrated cell suspensions in 100 ml serum bottles flushed with oxygen free N_2 gas for two hours in the presence of different amounts of nitrite. After this preincubation nitrate (60 mM) and 4-CBA (2 mM), flushed with N_2 gas were added, and the initial rate of 4-HBA formation was measured during two hours.

Permeabilized cells were obtained by storing concentrated cell suspensions in 50 mM phosphate buffer with 0.1 % triton X-100 for 24 h at -20°C (Miozarri et al. 1978).

Analytical methods

The samples were subjected to reversed-phase high pressure liquid chromatography to measure the amount of 4-CBA and 4-HBA, as described by Marks et al. (1984a), with a modified mobile phase consisting of acetonitrile, water, acetic acid (ratio by volume, 40:59:1). The concentrations of fumarate and malate were determined by HPLC at 210 nm using reversed phase C-18 columns (200 by 3 mm, Chrompack , Middelburg, The Netherlands) eluated with 25 mM phosphate buffer (pH 7.0) with 2mM octylamine. For quantification of 4-CBA-CoA an aliquot was hydrolysed with 0.05 M NaOH and subjected to HPLC analysis according to the method of Merkel et al. (1989). Nitrate was measured colorimetrically using diphenylamine sulfonic acid chromogene (Szechrome, NAS reagent; Polysciences Inc., Warrington, USA). Nitrite was measured colorimetrically, with the

Griess-Romijn reagent (Griess-Romijn van Eck, 1966). Protein contents of whole cells or cell-free extracts were determined by the Lowry method (1951). Oxygen concentrations in the serum bottles were determined with a Packard 427 gas chromatograph (Packard Instrument Co. Dawners Grove, Ill., U.S.A.), fitted with a molecular sieve; the column temperature was 100°C and helium was the carrier gas.

Preparation of cell-free extracts

Cell extracts were prepared by freeze pressing cells grown aerobically in a chemostat. A frozen washed cell suspension (5 ml), in 50 mM potassium phosphate buffer containing 5 mM Dithiothreitol (DTT) (pH 7.0), was subjected to 2 passages through a prechilled X-Press [(BIOX X-Press (AB Biox, Götenborg, Sweden.)]. DNAse I (Gibco, Breda, The Netherlands) was added to the cell homogenate (10 μg nuclease g^{-1} cell paste and the broken cell suspension was diluted with 2.5 ml potassium phosphate buffer (pH 7.0) before centrifugation at 30,000 g for 20 min at 4°C.

For the preparation of cell-free extracts, two other methods were used, ultrasonic disintegration (8x20 s) with power input of 10 W at 0°C (Branson Sonic Power Company, Danbury, USA), and by using a prechilled French press (4°C, 100 Mpa).

Dehalogenase assay

The cell-free extract was assayed for 4-CBA dehalogenase activity in 50 mM potassium phosphate buffer containing 5 mM DTT and 1 mM $MnCl_2$ (Marks et al. 1984a). Before starting the reaction with the addition of 0.5 mM 4-CBA the assay mixture and stock solution of 4-CBA were flushed with N_2 gas after incubation of the samples at 30°C for 10 min, the reaction was terminated by addition of 6 M HCl to a final concentration of 0.6 N HCL. The reaction mixture was extracted once with 10 volumes of diethyl ether. The ether phase was evaporated to dryness in a stream of air. The dried extract was redissolved in the mobile phase used for HPLC analysis and subjected to high pressure liquid chromatography to determine the 4-CBA and 4-HBA concentrations.

A low relative molecular weight fraction (Mr) from the extract was obtained by using a centriprep concentrator 10 (Amicon, Danvers, Mass., USA).

Chemicals

2,4-Dichlorobenzoate, 4-CBA, and 4-HBA were obtained from Janssen Chimica (Beerse, Belgium), and were used without further purification. 4-CBA Coenzyme A was synthesized according to the method of Merkel et al. (1989). All other chemicals were of the highest purity commercially available.

RESULTS

The strain NTB-1 used in this paper was motile, catalase positive, oxidase negative,

and Gram positive. The strain was further characterized by the National Collections of Industrial and Marine Bacteria (Aberdeen, UK), who showed by chemotaxonomic analyses that the cell wall diamino acid was lysine, that no mycolic acids were present and that the fatty acid profile had a high concentration of C16:0. On this basis the strain was classified as a coryneform bacterium (N.C.I.B. 12617). Strain NTB-1 is a member from a biculture previously incorrectly thought to be a pure culture and classified as *Alcaligenes denitrificans* NTB-1 (van den Tweel et al. 1986, 1987a,b). After separating this biculture by streaking it onto yeast glucose agar plates, it was found that the coryneform strain grew on 2,4-diCBA, 4-CBA, 4-HBA, 4-bromo-(4-BBA), and 4-iodobenzoate (4-IBA). The *Alcaligenes* strain obtained from the biculture did not grow on any of these compounds. Experiments reported previously on the bioformation of 4-HBA from 4-CBA using the biculture (van den Tweel et al. 1986, 1987a, 1987b) were repeated with the coryneform pure culture, and the same results were obtained.

Bioformation of 4-HBA by washed cell suspensions in the absence of oxygen

It was tested if anaerobic bioformation of 4-HBA by 4-CBA-grown whole cells is possible by using alternative electron acceptors and by supplying glucose as an additional

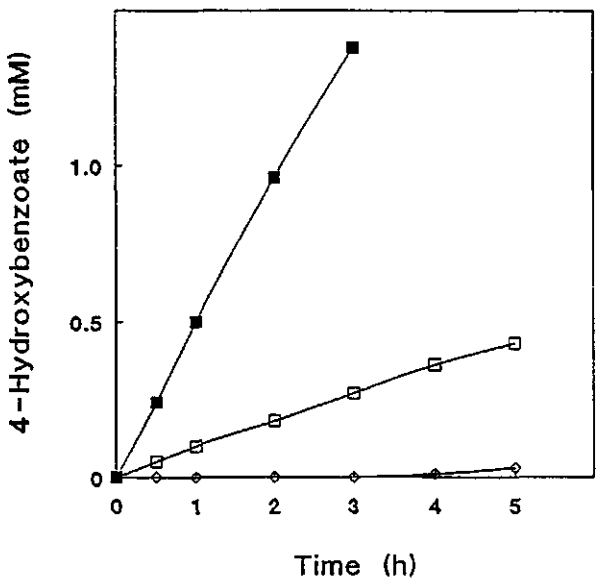

Fig. 1. Anaerobic formation of 4-HBA from 4-CBA by cells grown on a mixture of glucose and 4-CBA. Accumulation of 4-HBA in the absence of glucose with (□) or without (◇) ferricyanide; (■) glucose and ferricyanide both present. The reaction mixture contained 2.3 mg protein

energy source. Upon addition of ferricyanide (10 mM) anaerobic bioformation did occur and a quantitative conversion of 4-CBA to 4-HBA and Cl^- was detected.
The rate of 4-HBA formation was 5 nmol min^{-1} mg^{-1} protein. Using cells precultivated on glucose and 4-CBA the addition of ferricyanide and glucose resulted in a higher rate of 4-HBA formation (24 nmol min^{-1} mg^{-1} protein) (Fig. 1).
The 4-HBA formation in the presence of ferricyanide proceeded at a constant rate for several hours. No 4-CBA was converted in the presence of ferricyanide whenever cells were absent or when cells were boiled.

The coryneform bacterium NTB-1 did not grow on glucose under anaerobic conditions in the absence of an electron acceptor but poor anaerobic growth on glucose was observed when nitrate was added as an electron acceptor. Induction of nitrate reductase was also possible in cells growing on 4-CBA by cultivating cells in a 4-CBA-limited chemostat at low and controlled O_2 concentrations. With such cells a quantitative conversion of 4-CBA into 4-HBA was obtained under anaerobic conditions when nitrate was added. In the absence of nitrate no product was formed (Fig. 2). During the anaerobic conversion of 4-CBA to 4-HBA in the presence of nitrate the rate of nitrite formation was approximately 250 nmol min^{-1} mg^{-1} protein.

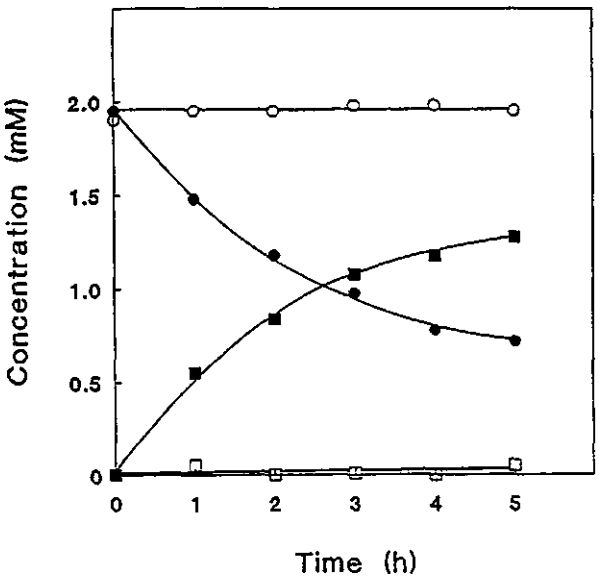

Fig. 2. Nitrate-dependent anaerobic formation of 4-HBA from 4-CBA by cells grown at low O_2 concentrations in the presence of nitrate. Accumulation of 4-HBA in the absence (□) or presence (■) of nitrate and degradation of 4-CBA in the absence (○) and presence (●) of nitrate. The reaction mixture (10 ml) contained 4.8 mg of protein

Other halogenated aromatics (4-BBA, 4-IBA, and 2,4-diCBA) were also anaerobically converted to 4-HBA in the presence of nitrate (results not shown). However upon prolonged incubation for several hours the anaerobic activity with nitrate levelled off in all incubations. This phenomenon cannot be explained by the accumulation of either 4-HBA or chloride during the incubation since adding either one of these compounds (2 mM) at the start of an anaerobic incubation did not effect the initial anaerobic activity. Therefore the energy requirement of cells upon prolonged incubation was also studied. Strain NTB-1 was now grown in a C-limited chemostat culture with both 4-CBA and glucose as carbon sources nitrate was given as an additional electron acceptor while the O_2 concentration was kept below 5 % of air saturation. Under this condition, the culture at steady state completely metabolized glucose and 4-CBA while a small amount of the nitrate was recovered as nitrite (4 mM). The addition of glucose to washed cell suspensions from this culture had no inhibitory effect on the initial 4-CBA conversion rate. These cells could therefore anaerobically be energized by glucose and nitrate while the conversion of 4-CBA was not repressed. With these cells, glucose as an additional energy source did not enhance the initial rate of 4-HBA formation but it prolonged the formation of 4-HBA under anaerobic conditions in the presence of nitrate (Fig. 3).

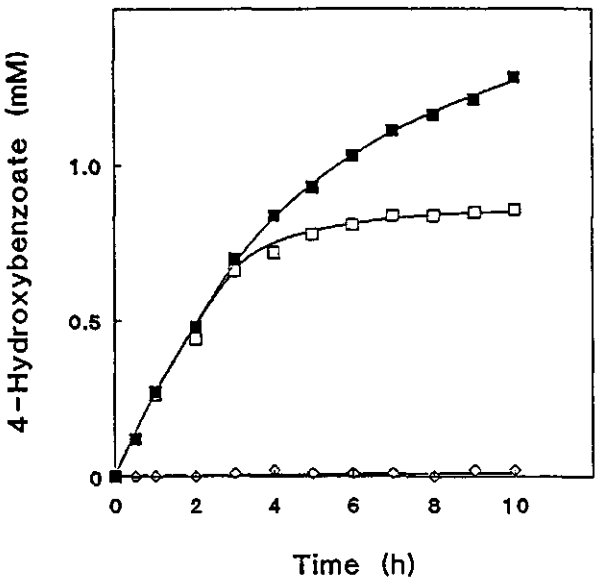

Fig. 3. Effect of glucose and nitrate on the anaerobic 4-HBA formation from 4-CBA. Accumulation of 4-HBA in the presence of nitrate and glucose (■), in the presence of nitrate (□) and absence of nitrate and glucose (◇). The reaction mixture (10 ml) contained 3.7 mg of protein.

However, also in the presence of glucose the 4-HBA formation rate levelled off. Subsequently it was studied if nitrite in the course of the reaction is inhibitory to the cells. Addition of nitrite in addition to nitrate during an anaerobic incubation indeed resulted in a nitrite-dependent inhibition of 4-HBA formation. The effect of nitrite on the anaerobic formation of 4-HBA was studied in more detail using different concentrations of nitrite. At the start of an anaerobic incubation a strong inhibition was found by concentrations of nitrite above 2mM nitrite (Fig. 4).

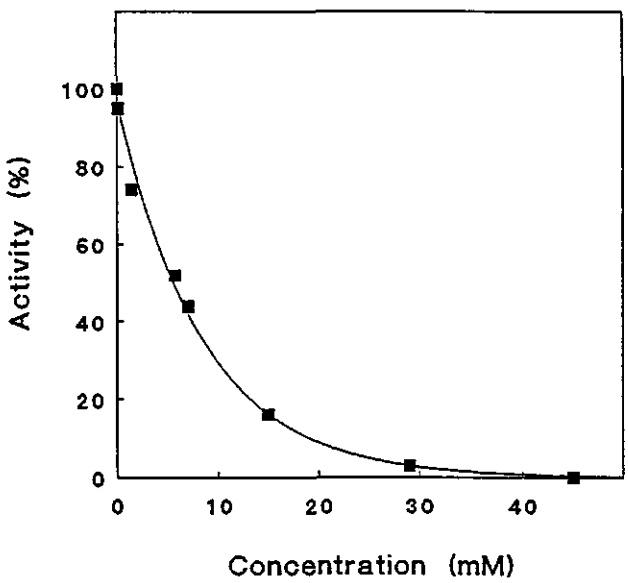

Fig. 4. Effect of nitrite on the initial rate of 4-HBA formation by cells grown at low oxygen concentrations in the presence of nitrate. (100% = 15 nM 4-HBA min^{-1} mg^{-1} protein)

Bioformation of 4-HBA by permeabilized cells

The above experiments demonstrate energized cells produce 4-HBA anaerobically from 4-CBA. By using permeabilized cells it was investigated if the energy requirement is exclusively associated with 4-CBA transport by using permeabilized cells. Several permeabilisation methods were used including triton X-100 treatments. The effect of the permeabilisation procedures was tested by measuring the anaerobic formation of malate from fumarate by whole cells. Cells resuspended in phosphate buffer (pH 7.0) with 0.1 % triton X-100 exhibited an high anaerobic fumarase activity (120 nmol min^{-1} mg $protein^{-1}$), whereas cells resuspended in phosphate buffer exhibited no detectable anaerobic

fumarase activity. Cells suspended in the triton X-100 containing buffer degraded 4-CBA under aerobic conditions, albeit at reduced rates. Under anaerobic conditions, however, such permeabilized cell suspensions did not form 4-HBA from 4-CBA.

Dehalogenase activity in cell-free extracts

The lack of anaerobic 4-HBA formation of the permeabilized cells was not consistent with the hypothesis that transport of 4-CBA is the only energy-dependent process involved in 4-HBA formation. The 4-CBA dehalogenase reported in other organisms is an O_2-independent enzyme with no requirement for cofactors containing energy rich bonds (Marks et al. 1984a, Müller et al. 1988), although very recently the involvement of ATP and CoA has been implied (Elsner et al. 1991b). It was therefore investigated in cell extracts whether the enzyme from NTB-1 is also a hydrolytic enzyme or whether other cofactors are involved.

Cell-free extracts of NTB-1 were prepared either by freeze pressing, by sonification or by using a French press. In cell-free extracts prepared by freeze pressing, dehalogenase activity was detectable, albeit at very low specific activities of 0.12 nmol 4-HBA min^{-1} mg^{-1} protein. The specific activity in cell-free extracts was only 2 % of the specific activity of whole cells. No dehalogenase activity was detected whenever the two other methods of cell disintegration were applied.

Under anaerobic conditions the dehalogenase appeared to be more stable than under aerobic conditions. In addition to 4-CBA, also 4-iodo-, 4-bromo- and 2,4-dichlorobenzoate were converted to 4-hydroxybenzoate. The specific activities were 0.11 ,0.14 and 0.04 nmol product min^{-1} mg^{-1} protein formed for 4-IBA, 4-BBA, and 2,4-diCBA, respectively. The dehalogenation of 2,4-diCBA is presumably due to another dehalogenase present in the cell-free extract. No activity was found with other substituted aromatic compounds tested as for instance 4-fluoro-, 3,4-dichloro-, 4-nitro-, and 4-methoxybenzoate, 4-chlorophenylacetate and 4-chloromandelate. The temperature optimum for dehalogenase activity was 30°C. At 15°C only 50 % of the activity was found and above 50°C no activity remained. The 4-CBA dehalogenase in NTB-1 is an inducible enzyme because no activity was found in glucose-grown cells.

To study the cofactor requirement of the dehalogenase the cell extract was dialysed overnight in phosphate buffer in the presence of 1 mM DTT. Dialysis resulted in a complete loss of activity, also under anaerobic conditions. Addition of Mn^{2+}(1mM) and FAD (1mM) had no effect on the dehalogenase activity. However, addition of a low mass M_r fraction obtained from crude cell extract partly restored the dehalogenase activity. Several cofactors were tested to replace this low molecular weight fraction in restoring activity. Only the addition of ATP resulted in restored activity and this activity was further enhanced by also adding CoA (Table 1).

Conditions			Activity*
ATP (2 mM)	Mn^{2+} (1 mM)	CoA (1 mM)	
+	-	-	1.3
+	+	-	1.3
+	+	+	4.8
+	-	+	4.9
-	-	-	0.0

* nmol 4-HBA min^{-1} mg^{-1} protein

Table 1. The effect of ATP and CoA on 4-CBA degradation in cell-free extracts

Addition of Mn^{2+} or Mg^{2+} did not effect the 4-HBA formation rates in cell extracts. The addition of AMP or ADP (5 mM) to incubation mixtures containing ATP and CoA resulted in 40% loss of activity. The finding that ATP and CoA are neccesary for 4-CBA degradation in cell extracts suggest a 4-CBA-CoA ester as a new intermediate in the metabolic pathway of 4-CBA in NTB-1.

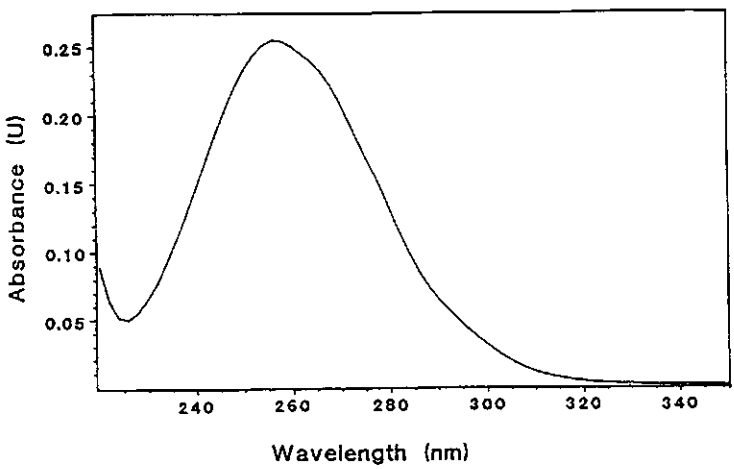

Fig. 5. UV-absorbtion spectrum of purified 4-CBA:CoA. The spectrum was recorded in 0.2 M ammonium acetate buffer (pH 5.0)/acetonitril (80/20); U, units

To prove the involvement of this 4-CBA-CoA ester the compound was chemically synthesized. Formation of 4-CBA-CoA from 21 mg CoA (Li salt) and 23 mg 4-chlorobenzoate was followed by HPLC and the product was purified by preparative HPLC. The UV spectrum of the purified 4-CBA-COA is shown in Fig. 5. In dialysed cell extract this 4-CBA-CoA was rapidly degraded and 4-HBA and CoA were formed in almost stoichiometric amounts (Fig. 6). Some of the 4-CBA-CoA was apparently hydrolyzed since traces of 4-CBA were also detected.

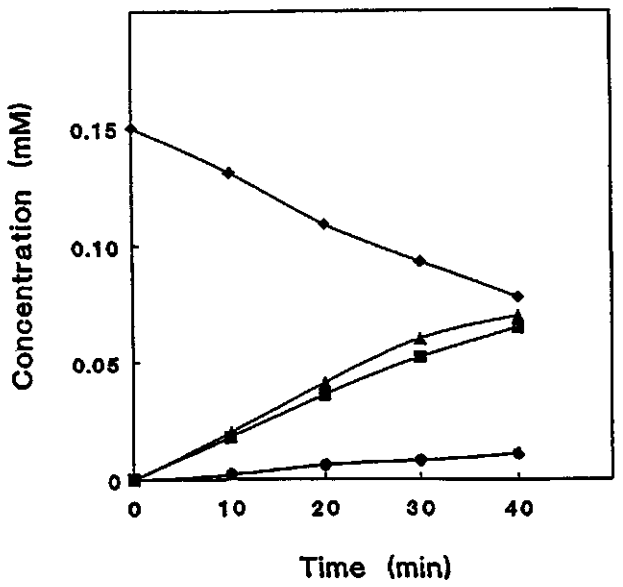

Fig. 6. Degradation of 4-CBA:CoA in dialyzed cell extracts 4-CBA:CoA (♦), Coenzyme A (▲), 4-HBA (■), 4-CBA (●). The reaction mixture (1 ml) contained 0.05 mg of protein

DISCUSSION

The potential use of a halogenated aromatic compound as starting material for bioformation of hydroxylated aromatic compounds was investigated. An example of such a bioconversion was discovered in the degradation of 4-CBA (Keil et al. 1983, Marks et al. 1984a, van den Tweel 1986, Zaitsev et al 1985). 4-CBA is an intermediate in the degradation of chlorinated biphenyls (e.g. 4-chlorobiphenyl) (Adriaens et al. 1989, Sylvestre et al. 1985). Several bacterial species have been isolated with the ability to use this 4-CBA as sole carbon and energy source, e.g. an *Arthrobacter* sp. (Marks et al.

1984a), a *Pseudomonas* sp. (Keil et al. 1983), a *Corynebacterium* (Zaitsev et al. 1985), and an *Acinetobacter* sp. (Adriaens et al. 1989). In these strains 4-CBA is dehalogenated to 4-HBA followed by oxidation of 4-HBA to protocatechuate.

Under anaerobic conditions whole cells did not convert 4-CBA to 4-HBA. Similar observations have been reported for a 4-CBA degrading *Arthrobacter* sp.(Shimao et al. 1989) and a *Rhodococcus chlorophenolicus* that hydrolytically dechlorinated polychlorinated polyphenols (Apajalahti and Salkinoja-Salonen 1987, Häggblom et al. 1988).

Interestingly an *Acinetobacter* sp. quantitatively converted 4-CBA to 4-HBA (Adriaens et al. 1989) upon anaerobic incubation. In this Gram-negative 4-CBA degrading strain no carrier may be no involved in the uptake of 4-CBA, and also the metabolism of 4-CBA may differ from the CoA-ester dependent metabolism observed in strain NTB-1.

Addition of the alternative electron acceptors nitrate or ferricyanide to NTB-1 cells resulted in anaerobic production of 4-HBA and it was even possible to prolong anaerobic 4-HBA formation by adding glucose as an energy source. Quantitative conversions of halo-substituted to hydroxylated aromatic compounds could thus be obtained under strictly anaerobic conditions.

In initial experiments permeabilized cells of NTB-1 were used to circumvent transport problems. Although in such cells no barrier for 4-CBA uptake should be present, no anaerobic 4-HBA formation occurred. This observation can be explained by assuming ATP is needed to degrade 4-CBA which cannot be generated under these conditions.

In the coryneform strain NTB-1, a dehalogenase is present in cell-free extracts when cultivated on 4-CBA, but not when grown on glucose. The dehalogenase from NTB-1 had a restricted substrate specificity and thus resembled the *Arthrobacter* S.U. DSM 20407 dehalogenase (Müller et al. 1984). In contrast to the *Arthrobacter* TM-1 dehalogenase (Marks et al. 1984a), no 4-fluorobenzoate was converted by NTB-1 cell-free extracts. The finding that 2,4-diCBA is also dehalogenated to 4-HBA in cell-free extract may indicate that also a reductive 2,4-diCBA dehalogenase is present in NTB-1 cell-free extracts. In the absence of ATP and CoA, activities were extremely low but comparable with activities found in extracts from other bacterial species. Recently it has been shown that in a *Pseudomonas* sp. the 4-CBA dehalogenase is a multicomponent enzyme. Two components were stable proteins whereas a third component was highly unstable with molecular weight below 10,000 (Elsner et al. 1991a). Furthermore, it has been suggested that this low molecular component can be replaced by ATP, CoA and Mg^{2+} (Elsner et al. 1991b). In Table 1 similar results are presented for the coryneform strain NTB-1 since in dialysed extracts dehalogenase activity was fully dependent on ATP and CoA. It was subsequently investigated if these observations are compatible with the involvement of the CoA-thioester of 4-CBA in the degradative route of 4-CBA. To this the ester was chemically synthesized. The absorbtion spectrum of the CoA-ester (Fig. 5) resembled the

spectrum published for 4-HBA-CoA (Webster et al. 1974). It was indeed observed in dialysed cell-extracts this 4-CBA-CoA is rapidly degraded to 4-HBA with the concomitant release of CoA (Fig. 6). This specific activity of 28 nmol min^{-1} mg^{-1} protein for 4-HBA formation was approximately equal to the activity for 4-CBA degradation exhibited by whole cells from the batch the extract was prepared. However, from these results the mechanism of dehalogenation hydroxylation is still not completely elucidated. For instance it remains unclear if 4-HBA-CoA is an intermediate in the degradation route. These investigations will require the chemical synthesis of 4-HBA-CoA. Further work is also hampered by the low growth rate of the coryneform bacterium NTB-1 on 4-CBA, the toxicity of 4-CBA for the organism and furthermore specific enzyme activities are not always consistent and may vary from batch to batch.

Activation of aromatic acids to their CoA derivatives before breakdown has been observed in the degradation of benzoate and 4-hydroxybenzoate by *Rhodopseudomonas palustris* (Harwood and Gibson 1986, Geissler et al. 1988, Merkel et al. 1989), of 2-aminobenzoate by a *Pseudomonas* strain (Ziegler et al. 1987), and in aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini* (Schnell and Schink 1991). Recently is has also been shown that the aerobic metabolism of phenylacetate (Martinez-Blanco et al. 1990) in *P. putida* also may proceed *via* a CoA thioester.

The present work gives the first example of the involvement of a CoA-ester in the degradation of a halogenated aromatic compound and it clarifies the observations of Elsner et al. (1991b) who suggested the involvement of ATP and CoA in 4-CBA degradation.

Acknowledgement

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CHAPTER 4

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

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ABSTRACT

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-free 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.

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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 microorganisms. In the first pathway, the nitro substituent is directly removed as nitrite, as demonstrated for the metabolism of *o*- and *p*-nitrophenol (Zeyer and 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 and Müller 1991, Kinouchi and 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 and Müller 1991).

In addition, the involvement of nitroreductases in the complete degradative pathways of nitro-aromatic compounds has also been reported (Durham 1956, Germanier and Wuhrmann 1963, Haller and Finn 1978). In the aerobic metabolism of 2- and 4-nitrobenzoate, reduction of the nitro-group via nitroso- and hydroxylaminobenzoate was demonstrated (Cain 1966a,b, Cartwright and Cain 1959a,b). The amino intermediate transiently accumulated during growth on 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 1966a, Ke et al. 1958, Cain and 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 oxygen consumption rates of whole cells also an intermediary role for 4-hydroxybenzoate (4-HBA) in *Nocardia erythropolis* was also suggested (Cartwright and Cain 1959a,b).

In this paper, evidence is presented for an 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 (N.C.I.M.B., Aberdeen, U.K.) as a strain of *Comamonas acidovorans*. Strain NBA-10 was routinely grown in a chemostat ($D = 0.1\ h^{-1}$) 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 16,000 *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 an 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 the 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 (10,000 *g*) and the supernatant was then analyzed by HPLC.

Experiments with cell-free extracts

Cell-free extracts were prepared by disrupting washed cell suspensions by ultrasonic disintegration (Branson Sonic Power) for 8x15 s with a power input of 10 W at 0° C. The resulting homogenate was centrifuged at 30,000 *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 the 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 analyzed by HPLC.

4-NBA reductase assay

4-NBA consumption by cell-free extracts or partly 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 10 000 *g* (15 min at 4° C) dissolved in 10 ml Tris HCl (50 mM, pH 8.0), dialyzed against the same buffer and applied to a DEAE-Sepharose 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 sulfate, and dialyzed as above. This concentrated fraction was applied onto a FPLC MonoQ column (Pharmacia), and the enzyme was eluted with a linear gradient of NaCl (0-0.5 M).

Chemicals

4-HABA was synthesized chemically (Bauer and Rosenthal, 1944) and was free of 4-ABA as determined by HPLC analysis. 4-NOBA was synthesized by the method described by Cartwright and 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 analyzed 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 an 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 reverse phase HPLC on a C_{18} column (200 x 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/0.01 N 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 NBA-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 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).

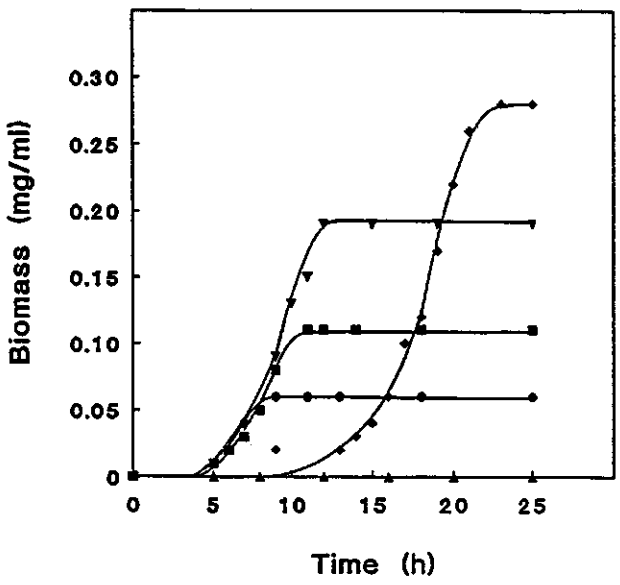

Fig. 1. Growth of *C. acidovorans* NBA-10 at various concentrations of 4-NBA. The initial concentrations of 4-NBA (mM) were 0.5 (●); 1.0 (■); 2.0 (▼); 4.0 (♦); and 5.0 (▲)

Substrate tested	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

* Rates of oxygen uptake are expressed in nmol O_2 consumed min^{-1}(mg protein)$^{-1}$ after correction for the endogenous oxygen uptake rate

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

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

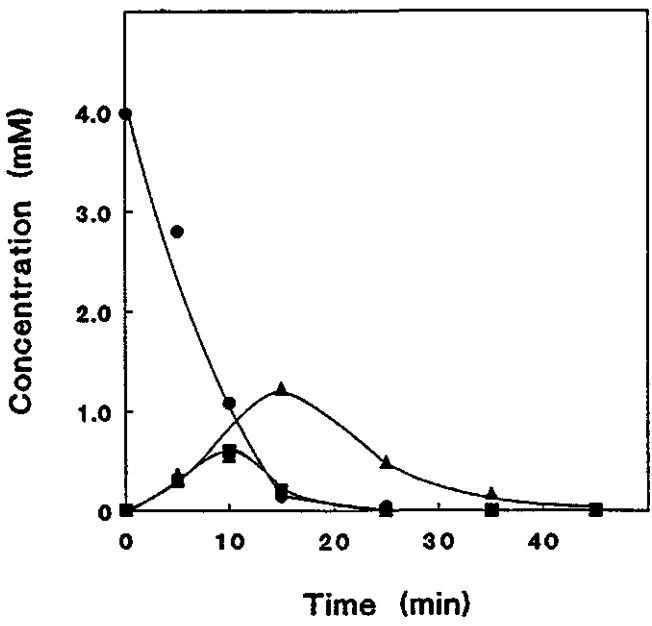

Fig. 2. Degradation of 4-NBA (●) and formation of 4-HABA (▲) and 3,4-diHBA (■) by resting cells of *C. acidovorans* NBA-10 grown on 4-NBA. The cell suspensions (5.6 mg protein in 10 ml) were incubated in the presence of phenanthroline (0.1 mM) under oxygen-limited conditions

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).

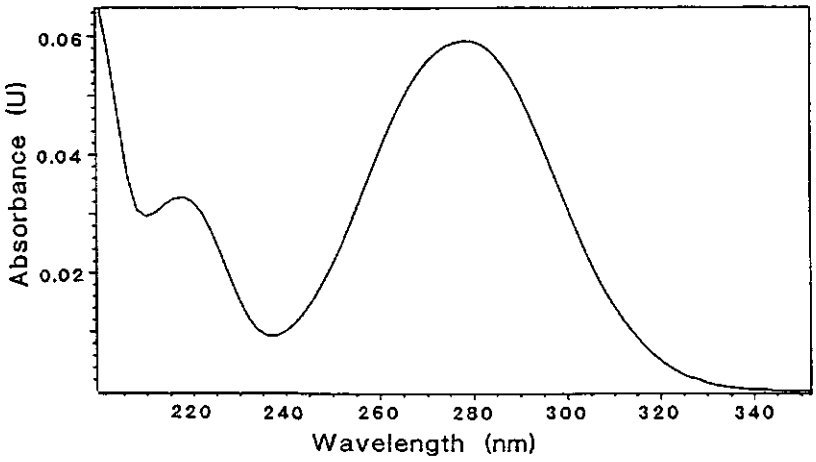

Fig. 3. Absorption spectrum of chemically synthesized 4-hydroxylaminobenzoate

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).

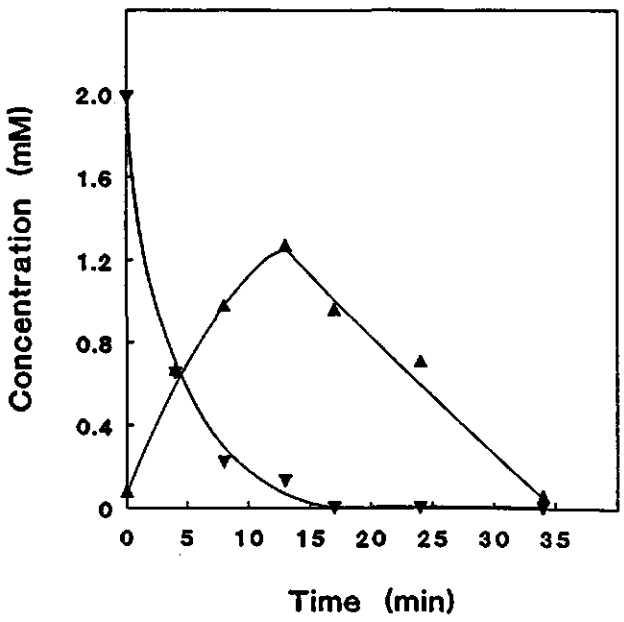

Fig. 4. Degradation of 4-NOBA (▼) to 4-HABA (▲) by resting cells of *C. acidovorans* NBA-10 grown on 4-NBA. The cell suspensions (0.8 mg protein in 10 ml) were incubated in the presence of phenanthroline (0.1 mM) under oxygen-limited conditions

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 catalyzed 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 cells 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 nmol min^{-1} (mg protein)$^{-1}$ and it was 550 nmol 4-NBA min^{-1}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 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-free extract was recovered.

Gel filtration, hydroxyapatite chromatography, and Cibracon 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 μg) 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 retained at 40° C. Enzyme 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 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 stoichiometrical 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.

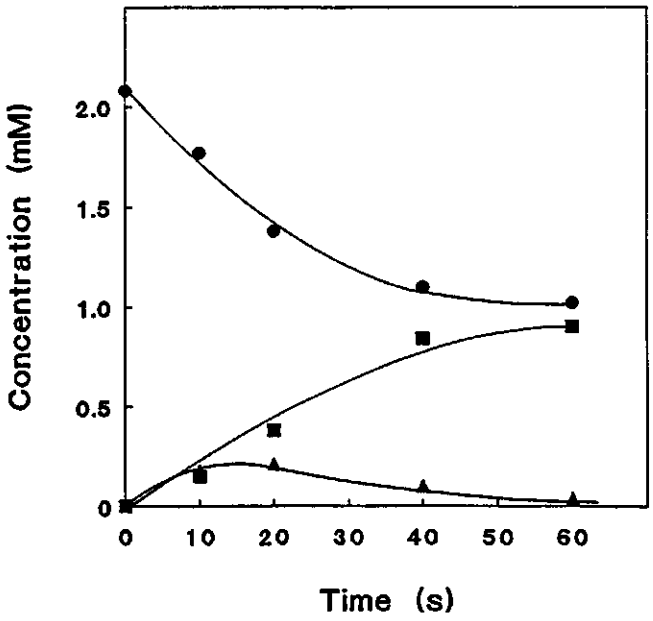

Fig. 5. Anaerobic degradation of 4-NBA (●) and formation of 4-HABA (▲) and 3,4-diHBA (■) in cell extracts. The incubations contained 2.1 mg protein in 1 ml

Purification step	Vol. (ml)	Total act. (U*)	Protein mg(ml)$^{-1}$	Sp.act. U*(mg)$^{-1}$	Yield (%)	Purification fold
Cell-free extract	41	580	22.4	0.63	100	1
$(NH_4)_2SO_4$ 35-55 %	10	312	49.6	0.63	54	1
DEAE	30	182	1.1	5.60	31	8.9
MonoQ (FPLC)	5	18	0.3	11.60	3.2	18.4

* 1 Unit is 1 µmol 4-NBA(min)$^{-1}$ degraded.

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

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 and Cain 1959b) and for *Nocardia erythropolis* (Cartwright and Cain 1959a,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 mol 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 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 also 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-hydroxylaminobenzoate was detected as well as methyl-4-aminobenzoate also the dialysed NAD(P)H-dependent *Escherichia 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 and Onishi 1983).

Fractionation by anion-exchange chromatography of the 4-NBA reductase from *C. acidovorans* NBA-10 resulted in a 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 antioxidans (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 dialyzing extracts.

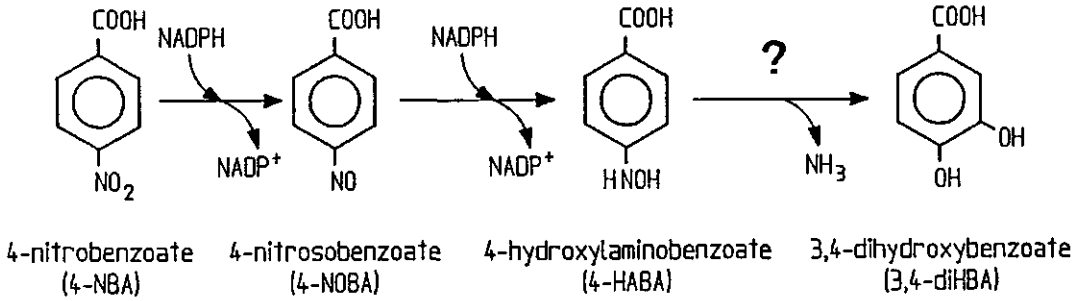

Fig. 6. Proposed degradative pathway of 4-nitrobenzoate by *C. acidovorans* NBA-10

Acknowledgement

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CHAPTER 5

Degradation of 4-nitrobenzoate via 4-hydroxylaminobenzoate and 3,4-dihydroxybenzoate in Comamonas acidovorans NBA-10

Peter E.J. Groenewegen and Jan A.M. de Bont

ABSTRACT

Comamonas acidovorans NBA-10 was previously shown to degrade 4-nitrobenzoate *via* 4-hydroxylaminobenzoate and 3,4-dihydroxybenzoate. Washed cells, grown on a mixture of 4-nitrobenzoate and ethanol, stoichiometrically produced ammonium and 3,4-dihydroxybenzoate from 4-nitrobenzoate under anaerobic conditions provided ethanol was present. In cell-free extracts 4-hydroxylaminobenzoate was degraded to ammonium and 3,4-dihydroxybenzoate, but this activity was lost upon dialysis. No requirement for a cofactor was found, but rather reduced incubation conditions were necessary to restore enzyme activity. The 4-hydroxylamino-degrading enzyme was purified and the role of this novel type of enzyme in the degradation of nitro-aromatic compounds is discussed.

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INTRODUCTION

Aerobic microbial degradation of homocyclic aromatic compounds is generally initiated by (mono)oxygenases introducing hydroxyl groups into the aromatic nucleus (Smith 1990). Only in specific cases as for instance in the metabolism of 4-chlorobenzoate (Müller et al. 1984, Marks et al. 1984, Elsner et al. 1991, Groenewegen et al. 1992a) are these hydroxyl groups derived from water rather than from molecular oxygen. Subsequent ring cleavage of hydroxylated aromatics is by oxygen-dependent reactions.

Aerobic degradation of heterocyclic aromatic compounds often follows a different pattern. Dehydrogenases rather than (mono)oxygenases introduce water-derived hydroxyl groups into the aromatic nucleus (Kretzer and Andreesen 1991) before the ring is opened.

Under anaerobic conditions, aromatic compounds are degraded as well. Many types of oxygen-independent reactions have been detected in various organisms as recently summarized (Dangel et al. 1991).

Aerobic metabolism of homocyclic nitro-aromatics seemingly follows the general degradation pattern of homocyclic aromatics. The nitro compounds are converted into hydroxylated aromatics, either in an initial oxygenation reaction also yielding nitrite, or in a sequence of reactions yielding ammonium rather than nitrite. Examples of the first approach are the aerobic degradation of 2-nitrophenol by *Pseudomonas putida* B2 *via* a nitrophenol oxygenase and a catechol 1,2-dioxygenase (Zeyer et al. 1986) and of 4-nitrophenol by a *Moraxella* sp. which resulted in hydroquinone as intermediate (Spain et al. 1979). Also the degradation of 2,6-dinitrophenol yielded nitrite as an end-product (Bruhn et al. 1987). Recently, it was demonstrated that a *Pseudomonas* sp. degraded 2,4-dinitrotoluene *via* a dioxygenase resulting in nitrite and 4-methyl-5-nitrocatechol which was further metabolized with removal of the second nitro group again as nitrite (Spanggord et al. 1991). A *Rhodococcus* sp. isolated on 1,3-dinitrobenzene as sole nitrogen source converted the dinitro compound in a similar way. Both nitro-groups were released as nitrite presumably as a result of the action of dioxygenases (Dickel and Knackmuss 1991).

Many examples of the second approach have been described (Durham 1958, Ke et al. 1959, Germanier and Wuhrman 1963, Cartwright and Cain 1959a,b, Cain 1966, Zeyer and Kearney 1984) but the long standing question (Cain and Cartwright 1960) still remains whether or not amines are intermediates in the aerobic degradation of nitro-aromatics. From this older literature it furthermore is not clear if molecular oxygen participates in the formation of intermediary hydroxylated aromatics. Involvement of amines is often implied (Zeyer 1988, Schackmann and Müller 1991) and is mainly based on the detection of aromatic amines as products accumulating from nitro-aromatics in bacterial cultures.

It should be noted, however, that organisms growing on 2-nitrobenzoate were not simultaneously adapted to 2-aminobenzoate (Ke et al. 1959, Cain 1966), and that it was not possible to show an involvement of 3-aminophenol in the ammonium-yielding degradation of 3-nitrophenol (Zeyer and Kearney 1984).

Under anaerobic conditions, nitro-aromatic compounds may be used as a terminal electron acceptor to yield the amine as end-product (Oren et al. 1991). Several nitroreductases have been described reducing the nitro group of aromatic compounds *via* nitroso and hydroxylamino to amino groups (Kitamura et al. 1983, Rafii et al. 1991). The resulting amines can be further degraded by other organisms either aerobically (Fuchs et al. 1991) or anaerobically (Ziegler et al. 1987, Schnell and Schink 1991).

Recently we described the degradation of 4-nitrobenzoate (4-NBA) by a *Comamonas acidovorans* NBA-10 in which under fully aerobic conditions a novel, incomplete reduction of the nitro substituent was detected. 4-Nitrosobenzoate and 4-hydroxylaminobenzoate (4-HABA) were identified as intermediates. But contrary to results obtained by others studying various other organisms, we were not able to obtain any accumulation of 4-aminobenzoate (4-ABA) by strain NBA-10 (Groenewegen et al. 1992b). Furthermore, evidence was obtained that 4-HABA, also in the absence of oxygen, in a puzzling fashion was further degraded via 3,4-dihydroxybenzoate (3,4-diHBA). The degradative pathway for 4-NBA consequently does not follow the general pattern of aerobic metabolism of homocyclic aromatic compounds since no involvement of molecular oxygen was implied in the formation of 3,4-diHBA.

In this article we report further details of the conversion of 4-NBA *via* 4-HABA and 3,4-diHBA in *Comamonas acidovorans* NBA-10. The enzyme involved in the degradation of 4-HABA was purified and has been initially characterized.

METHODS

Media and culture conditions

Comamonas acidovorans NBA-10 was grown in a 4-NBA-limited chemostat as reported previously (Groenewegen et al. 1992b). Cells grown on a mixture of 4-NBA and ethanol were cultivated carbon-limited in a chemostat supplied with mineral salts medium containing 20 mM 4-NBA and 5 mM ethanol.

Experiments with resting cells

Cells grown in a chemostat were harvested by centrifugation and washed in potassium phosphate buffer pH 7.0 (50 mM) and resuspended in the same buffer. Experiments for following substrate utilization by washed cell suspensions (10 ml) were done in 100-ml serum flasks. Before starting anaerobic experiments the bottles were flushed for more

than 15 min. with N_2 gas (oxygen free). The reaction was started by adding substrate with a gas-tight syringe from a N_2-flushed concentrated solution. Samples (0.5 ml) were periodically withdrawn from the incubation mixture with a gas-tight syringe and were immediately centrifuged (10,000 *g*) and analyzed for ammonium and for aromatic compounds.

Experiments with cell-free extracts

Cell extracts were prepared by disrupting washed cell suspension by ultrasonic disintegration (Branson Sonic Power Company) (8x15 s) with power input of 10 W at 0° C. The resulting homogenate was centrifuged at 30,000 *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 with cell extracts were performed in 5-ml tubes with gas-tight rubber septa. Anaerobic experiments in these tubes were performed as described above for the serum bottles. Samples (0.3 ml) were periodically withdrawn from the incubation mixture with a gas-tight syringe and the reaction was terminated by the addition of 6 M HCl to a final concentration of 0.6 M. After removal of the protein by centrifugation the samples were analyzed for ammonium and for aromatic compounds.

Enzyme assays

The 4-HABA-degrading enzyme was assayed by measuring the depletion of 4-HABA and the production of 3,4-diHBA. The standard assay contained 50 mM Potassium phosphate and 1 mM NADH, 2 mM 4-HABA and enzyme in 1 ml volume. The reaction was assayed at 30° C under strictly anaerobic conditions in 5 ml tubes with gas-tight rubber septa. The reaction was started adding NADH and 4-HABA from N_2 stock solutions and terminated by addition of 6 M HCl to a final concentration of 0.6 M. Details of HPLC analysis are described below. The purified 4-HABA-degrading enzyme was assayed in the presence of 5 mM dithiotreitol with no precautions taken to exclude oxygen.

Activity of 3,4-diHBA oxygenase was measured by means of a polarographic oxygen probe. (Marks et al. 1984).

Partial purification of the 4-HABA-degrading enzyme

The crude cell-free extract was loaded onto a DEAE-Sepharose Cl-6B column equilibrated with 50 mM Tris HCL (pH 8.0). The column was washed with 200 ml of this buffer and run with a linear gradient of 0-0.25 M NaCl in Tris HCl (50 mM, pH 8.0). Fractions of 10 ml were collected and active fractions were pooled and concentrated by pressure filtration with Amicon (YM 10) (Grace Company, Danvers, U.S.). This concentrated fraction was applied on a Sephacryl S-300 column and this column was eluted with 50 mM Potassium phosphate buffer (pH 7.0) and 50 mM NaCl. Fractions with highest activity were pooled again, concentrated by pressure filtration and desalted by Gel filtration (disposable PD-10 column Pharmacia). The protein solution was injected

onto a Mono Q column (Pharmacia fast protein liquid chromatography system, FPLC) equilibrated with 10 mM Bis Tris HCL (pH 7.0). The enzyme was eluted with a step-and-linear gradient of sodium chloride (percentage of 1 M NaCl in volume of 10 mM BisTris: 0%, 5 ml; 0 to 30%, 10 ml linear gradient; 30 to 100%, 5 ml linear gradient; 100%, 5ml; 0%, 3 ml) After concentration this fraction with amicon the active fraction was supplied onto Sepharose 12 prepacked HR 10/30 column (FPLC). The enzyme was eluted with 20 mM BisTris buffer containing 50 mM NaCl. The column was calibrated with mixture of proteins for determination the molecular weight of the 4-HABA-degrading enzyme.

Analytical methods

Concentrations of 4-NBA, 4-HABA and 3,4 diHBA were routinely determined by HPLC analysis using reversed phase C-18 column (200 by 3 mm, Chrompack, Middelburg, the Netherlands). For determinations 4-HABA and 3,4-diHBA acetonitrile:0.01 N sulphuric acid (v/v 10:90) were used as mobile phase. The concentrations of NADP, NADPH, NAD and NADH were determined by HPLC using C-18 columns with 100 mM phosphate buffer(pH 7.0):acetonitrile (v/v 96:4) as mobile phase.

Protein contents of whole cells and cell extracts were determined by the method of Bradford (1976) using crystalline bovine serum albumin as a standard.

Ammonium was determined with Nessler reagent (N.E.N. 6.1.1 1972).

Chemicals

4-HABA was chemically synthesized and its identity and purity were checked as reported previously (Groenewegen et al. 1992b). The compound was free of 4-ABA as determined by HPLC analysis.

All other chemicals were of the highest purity commercially available.

RESULTS

Degradation of 4-NBA and of 4-HABA by resting cells

Washed cells of *Comamonas acidovorans* NBA-10, grown on 4-NBA, degraded 4-HABA both under aerobic and anaerobic conditions at rates of 345 and 280 nmol min^{-1} mg^{-1}protein, respectively. In the absence of oxygen 3,4-diHBA and ammonium accumulated (Fig. 1A) but no 4-ABA was detected. These cells degraded 4-NBA at 220 nmol min^{-1} mg^{-1}protein under aerobic conditions, but in the absence of oxygen no 4-NBA was degraded. This inability to anaerobically degrade 4-NBA is not caused by a requirement for molecular oxygen since washed cells cultivated on a mixture of 4-NBA and ethanol did degrade 4-NBA anaerobically, provided ethanol was included in the reaction mixture. Under these conditions a quantitative conversion of 4-NBA to 3,4-diHBA and ammonium occurred at a rate of 130 nmol min^{-1} mg^{-1}protein (Fig. 1B), while no 4-ABA was formed.

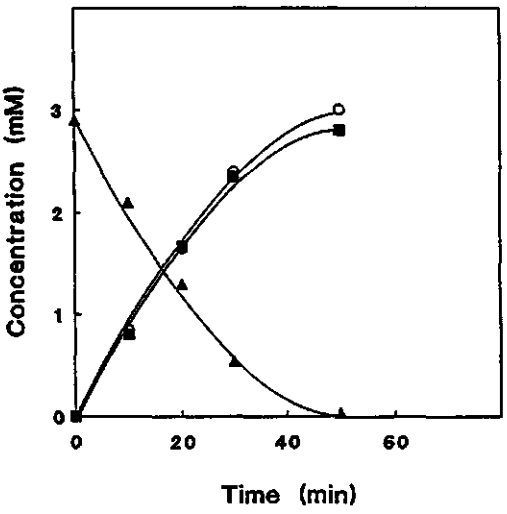

Fig. 1a. Anaerobic formation of 3,4-diHBA (○) and ammonium (■) from 4-HABA (▲) by washed cells grown on 4-NBA. Incubations contained 0.3 mg protein.ml^{-1}

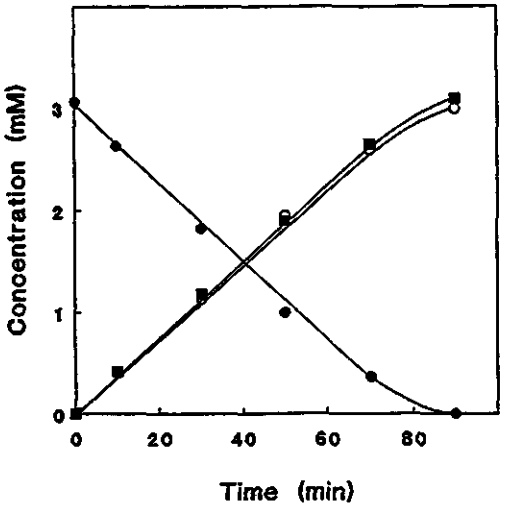

Fig. 1b. Anaerobic formation of 3,4-diHBA (○) and ammonium (■) from 4-NBA (●) by washed cells grown on a mixture of 4-NBA and ethanol. Incubations (0.3 mg protein.ml^{-1}) contained initially 5 mM ethanol

Degradation of 4-HABA and of 3,4-diHBA in cell-free extracts

In crude extracts, under anaerobic conditions and without any external cofactor added, a stoichiometric conversion of 4-HABA to 3,4-diHBA and ammonium was detected (Fig. 2). The specific enzyme activity was 170 nmol.min^{-1}.mg^{-1}protein which is comparable to the degradation rate for 4-HABA detected in whole cells. The reaction was enzymatic because without extract or with boiled extract no 4-HABA was degraded.

Dialysis of the crude extract, either under aerobic or anaerobic conditions, resulted in complete loss of activity both under aerobic and anaerobic incubation conditions. Several known cofactors and metal ions were tested to restore activity. Surprisingly, addition of NADH or NADPH restored activity provided anaerobic incubation conditions were applied. Under aerobic incubation conditions no activity was restored by either NADH or NADPH.

Cell-free extracts of 4-NBA-grown cells contained 3,4-diHBA oxygenase activity (45 nmol min^{-1} mg^{-1}protein), whereas succinate-grown cells contained less than 5 nmol min^{-1} mg^{-1}protein of the enzyme.

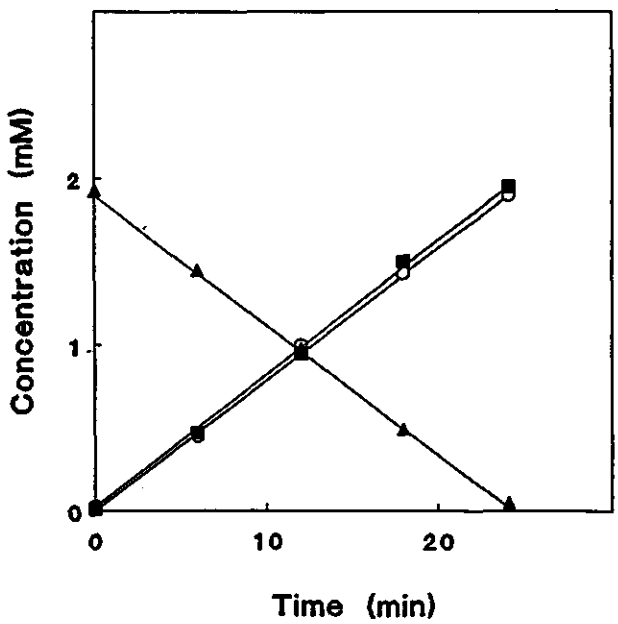

Fig. 2. Formation of 3,4-diHBA (○) and ammonium (■) from 4-HABA (▲) by cell extracts. Incubations contained 0.5 mg protein.ml^{-1}

Purification of the 4-HABA-degrading enzyme

The 4-HABA-degrading enzyme was partly purified to investigate the role of NADH or NADPH in the reaction. Activity assays during purification of the enzyme were by incubating fractions under anaerobic conditions in the presence of 4-HABA and NADH. In the activity assays both degradation of 4-HABA and formation of 3,4-diHBA was monitored by HPLC. The purification scheme contained 4 steps and resulted in a 105 fold increase of the specific activity relative to the crude cell extract (Table 1).

Purification step	Volume (ml)	Protein (mg)	Specific activity (nmol.min^{-1}.mg^{-1}protein)	Recovery (%)
Cell-free extract	60	1020	155	100
DEAE-cellulose	70	51	3720	73
Sephacryl S-300	90	8.7	9455	55
Mono Q (FPLC)	6	2.34	13950	41
Superose 12 (FPLC)	1.5	1.85	16275	35

Table 1. Partial purification of the 4-HABA degrading enzyme.

Role of NAD(P)H in the reaction

Since no net reduction should takes place in the transformation of 4-HABA into 3,4-diHBA and ammonium, it was difficult to envisage what role NAD(P)H has in the degradation of 4-HABA. By showing NAD(P)H levels were constant in incubation mixtures using purified enzyme it was verified no net consumption of NADH or NADPH occurred during the reaction. Nevertheless, NADH or NADPH might be substrates in the reaction catalyzed by the 4-HABA-degrading enzyme with the reduced nucleotides regenerated by the same enzyme. This hypothesis was studied by incubating the enzyme in the presence of both NADH and NADP. NAD and NADPH would then be formed if regeneration of the cofactor takes place. However, under such conditions no formation of NAD or NADPH was found and NADH and NADP levels remained constant (Fig. 3). From these results it was concluded that NADH or NADPH are not substrates in the reaction but either activate or stabilize the enzyme.

Since enzyme activity in the presence of NADH (16.2 μmol min^{-1} mg^{-1} protein) or NADPH (16.3 μmol min^{-1} mg^{-1}protein) was only observed under anaerobic conditions,

it was investigated if these compounds acted as stabilizing agents by lowering the redox potential. Indeed, other compounds with reducing power as for instance ascorbate, cysteine and dithiotreitol also restored enzyme activity at rates of 8.2, 11.2 and 16.2 μmol.min^{-1}.mg^{-1}protein, respectively. In the presence of dithiotreitol it was possible to assay the purified 4-HABA-degrading enzyme also under aerobic conditions provided incubations were stationary.

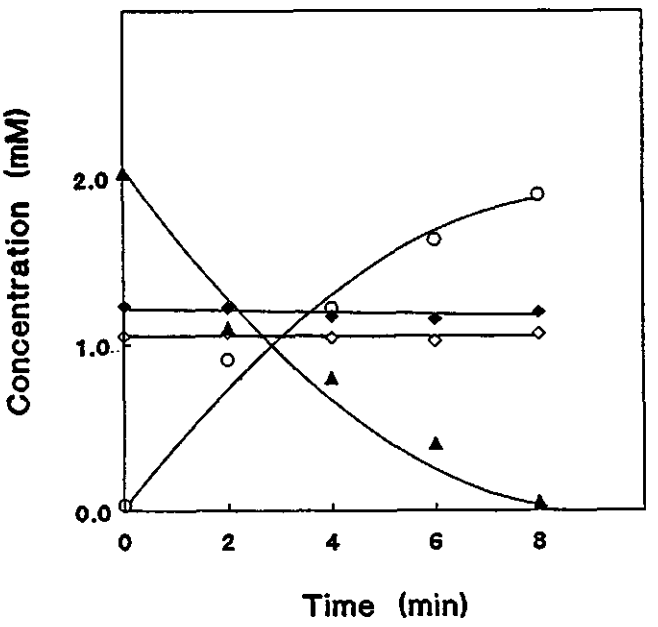

Fig. 3. Formation of 3,4-diHBA (○) from 4-HABA (▲) in the presence of NADH (◆) and NADP (◇) by partial purified enzym. No formation of NAD or NADPH was detected. Incubations contained 0.015 mg protein ml^{-1}

Enzyme properties

The optimal pH for 4-HABA-degrading activity was 7.0 with at pH 6.0 and 8.0 activities of 35% and 25% respectively of the activity at pH 7.0. The optimal temperature of the enzyme activity was at 32° C.

With 4-HABA as substrate, the 4-HABA-degrading enzyme followed Michaelis

Menten kinetics with Km and Vm values for 4-HABA of 1.3 mM and 13.5 μmol/min/mg of protein, respectively.

For gel filtration the protein eluted as a single peak on a Superose 12 column with a retention volume of 14 ml, which was the same as the retention volume of ovalbumin (14 ml). The molecular weight of the 4-HABA-degrading enzyme was estimated to be about 45,000.

DISCUSSION

Previously we described the reductive degradation of 4-NBA to 4-HABA in *Comamonas acidovorans* NBA-10 (Groenewegen et al. 1992b). In the present study the further metabolism of 4-HABA was examined. Whole cells under anaerobic conditions were able to excrete 3,4-diHBA and ammonium from both 4-NBA and 4-HABA (Fig. 1) and in extracts it was also possible to demonstrate anaerobic formation of 3,4-diHBA and ammonium from 4-HABA (Fig. 2). The metabolism of 4-NBA is therefore rather unusual since in most other pathways in aerobic organisms molecular oxygen participates in the formation of catechols.

Initial attempts to study the enzyme involved in the formation of 3,4-diHBA from 4-HABA were not successful since activity was lost upon dialysis. Surprisingly, it was found NADH or NADPH restored activity of dialysed extracts provided incubations were in the absence of oxygen. It was considered that a concerted oxidation/reduction of NAD(P)H and NAD(P) might occur during the enzyme-catalyzed reaction. In this way participation of a dehydrogenase in the formation of a hydroxylated aromatic might be envisaged, as has been observed in the degradation of heterocyclic aromatic compounds (Kretzer and Andreesen 1991). However, since no formation of NAD or NADPH was found in incubations with both NADH and NADP (Fig. 3) and especially since reducing agents as dithiotreitol were able to replace NAD(P)H, it appeared a cofactor-independent enzyme is responsible for the formation of 3,4-diHBA from 4-HABA.

The suggested pathway for the degradation of 4-NBA in *Comamonas acidovorans* NBA-10 as summarized in Fig. 4 is a route not previously described, also not in higher organisms. Metabolism of nitro-aromatics and especially of nitrated polycyclic aromatic hydrocarbons has been studied extensively in animals (Tokiwa and Ohnishi 1986). The compounds may be reduced to amines by the intestinal flora or may undergo various transformations by the animals, including formation of hydroxylamine-containing polycyclic aromatics (Delclos et al. 1990). In rats, 4-NBA may be conjugated at the carboxylic group with glycine or glucuronic acid or is reduced to 4-ABA (Gardner and Renwick 1978). 4-HABA as an intermediate was not reported, but metabolism in the rat

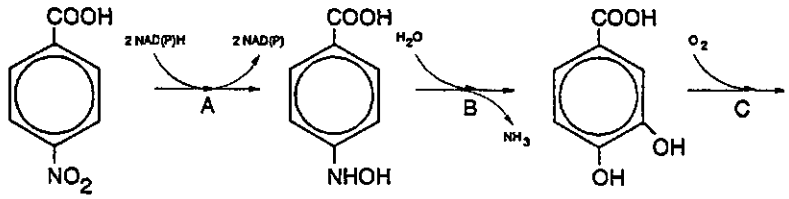

Fig. 4. Proposed pathway for the degradation of 4-NBA in *Comamonas acidovorans* NBA-10. [(A): 4-nitrobenzoatereductase, (B): 4-hydroxylaminobenzoate degrading enzyme, (C): 3,4-dihydroxybenzoatedioxygenase]

of other aromatic hydroxylamines was studied by others (Sternson and Gammans 1975). These authors studied and discussed the mechanism of the enzymatic rearrangement of aromatic hydroxylamines to aminophenols catalyzed by a hydroxylamine isomerase present in liver homogenates. Formation of an aminophenol from an aromatic hydroxylamine was also observed in a yeast (Corbett and Corbett 1981). The 4-HABA-degrading enzyme in our bacterium is clearly different from the reported isomerases since it liberated ammonium and yielded a catechol instead of an aminophenol. Since the enzyme has a rather low molecular weight, the mechanism of the reaction catalyzed by it might be rather simple, possibly with an arylnitrene as intermediate as postulated for chemical or enzymatic isomerization of hydroxylamines to aminophenols (Shine, 1967; Sternson and Gammans 1975). The amount of 4-HABA degraded by the purified enzyme was always equal to the amount of 3,4-diHBA formed, also during incubations at various temperatures and at various pH-values.

Although no involvement of an enzyme converting an aromatic hydroxylamine into a catechol has been reported previously, it seems likely many other organisms may employ the same type of enzyme. The *Flavobacterium* sp. studied by Ke et al. (1959) when grown on 2-nitrobenzoate was simultaneously adapted to 2-hydroxylaminobenzoate but not to 2-aminobenzoate. *Nocardia opaca* (Cain 1966) when growing on 2- nitrobenzoate transiently accumulated 2-aminobenzoate under oxygen-limited conditions, but not when aeration was good. The organism was only adapted to 2-aminobenzoate during growth on 2-nitrobenzoate under conditions allowing accumulation of 2-aminobenzoate. It was consequently concluded the amino compound was not an intermediate on the pathway of 2-nitrobenzoate metabolism in the organism (Cain 1966). Similarly it was concluded

Nocardia erythropolis (Cartwright and Cain 1959b) does not degrade 4-NBA *via* 4-ABA although the organism produced 4-ABA from 4-NBA and was also able to grow on the amine. From these results it seems likely these organisms degraded the nitrobenzoates via the same route as in our organism. The organisms differ from our strain in that they were able to grow on the aminobenzoates and because they produce amines rather than 3,4-diHBA under oxygen-limited conditions. They consequently contain another type of nitroaromatic dehydrogenase than *Comamonas acidovorans* NBA-10, possibly along with a hydroxylamino-yielding dehydrogenase.

A similar aromatic hydroxylamine-degrading enzyme may be present in *Pseudomonas putida* degrading 3-nitrophenol while releasing ammonium since the organism failed to degrade 3-aminophenol and since it was simultaneously adapted to 3-nitrophenol and 1,2,4-benzenetriol but not to 3-aminophenol (Zeyer and Kearney 1984). Based on their results the organism might reduce 3-nitrophenol to 3-hydroxylaminophenol which would then be further degraded to 1,2,4-benzenetriol and ammonium.

From an applied point of view, the novel type of hydroxylamine-degrading enzyme is interesting because it forms oxygen-labile catechols in the absence of oxygen. It might allow the production of valuable catechols not only from aromatic hydroxylamines but also from relatively cheap nitro-aromatics using whole cells containing an adequate aromatic nitroreductase.

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CHAPTER 6

CONCLUDING REMARKS

In this thesis microbial degradative pathways of substituted benzoates were studied to obtain insight in the bioformation of hydroxylated aromatic compounds in the absence of oxygen. The work resulted in the detection of novel enzymatic mechanisms involved in the replacement of a substituent on the aromatic nucleus by a hydroxyl group. Furthermore, it was shown that hydroxylated aromatics could be obtained from both 4-chlorobenzoate (4-CBA) and 4-nitrobenzoate (4-NBA).

Degradation of 4-chlorobenzoate

The degradative pathway of 4-CBA enzyme was analyzed in the coryneform bacterium NTB-1. Characterization of the dehalogenase revealed an energy-dependent sequence of reactions, since ATP and CoA were utilized for the formation of 4-CBA:CoA, which was further degraded to 4-hydroxybenzoate. This 4-CBA-dehalogenase is the first example of a CoA-depended enzyme involved in the degradation of halogenated aromatic compounds. Surprisingly, it was also shown that 4-CBA enters the cell by a carrier-mediated transport mechanism.

Degradation of 4-nitrobenzoate

In the degradative pathway of 4-NBA in *Comamonas acidovorans* NBA-10 two completely novel enzymes were characterized. A NADPH-dependent nitroreductase was described forming hydroxylaminobenzoate rather than aminobenzoate. The subsequent formation of 3,4-dihydroxybenzoate from 4-hydroxylaminobenzoate was rather suprising, also from a chemical point of view, since phenylhydroxylamines are normally chemically rearranged to aminophenols.

Applied aspects

Chemical synthesis of hydroxyaromatics is rather difficult and consequently alternative, biotechnological preparation methods may be relevant. In this respect, mostly oxygenases or peroxidase have been investigated. However, application of these enzymes is not simple in view of cofactor regeneration and lack of regioselectivity, while the presence of molecular oxygen or hydrogen peroxide leads to further undesired chemical oxidation of the product.

Organisms studied in this thesis contained enzymes converting substituted aromatic compounds into hydroxy-aromatic compounds in the absence of oxygen or hydrogen peroxide. Both halogenated and nitrosubstituted aromatic compounds were utilized as starting materials. Optimization of the reaction conditions utilizing whole cells eventually led to systems producing 4-hydroxybenzoate from 4-chlorobenzoate and 3,4-dihydroxybenzoate from 4-nitrobenzoate. In both cases, anaerobic product accumulation was achieved only when a cosubstrate was included in the reaction mixture. The 4-chlorobenzoate system required an electron acceptor whereas the 4-nitrobenzoate system required an electron donor. The role of nitrate as electron acceptor in the 4-CBA conversion was attributed to energy-requiring reactions in both the uptake of 4-CBA and in its metabolism via a CoA-derivative. Ethanol as electron acceptor in the 4-NBA system was obviously required to regenerate NAD(P)H consumed in the reduction of 4-NBA to 4-hydroxylaminobenzoate. The fundamental aspects of these reactions have been dealt with in the preceding chapters. However, several other aspects should be taken into account when considering applied aspects of the two types of reactions studied.

Application of hydrolytic dehalogenase

The **availability** of 4-CBA or other halogenated aromatic compounds as a starting compound for biochemical hydroxylation/substitution reactions is no problem, since these compounds are rather cheap and can be produced chemically rather easily. Since halogenated aromatic compounds are rather toxic for many biological systems, the effect of the high concentration of 4-CBA is of special interest. Although growth of 4-CBA-degrading bacteria is strongly inhibited by concentrations of 4-CBA above 4 mM, under resting cell conditions no inhibition of the coryneform bacterium NTB-1 was found up to 20 mM 4-CBA. Apart from the **toxicity of the substrate**, also the effect of product produced is of great importance. Several starting concentrations of 4-hydroxybenzoate were tested and under resting cell conditions no **product inhibition** was detected up to 40 mM. The **activity** of whole cells of strain NTB-1 was rather poor. Other organisms capable of hydroxylation of 4-CBA exhibited specific activities in the same order of magnitude. However, these activities can probably be enhanced rather easily, since recently the genes of the 4-CBA dehalogenase from a *Pseudomonas* sp. were cloned. Another problem using whole cells for the anaerobic bioformation of hydroxylated aromatic compounds from halogenated aromatic compounds using whole cells containing hydrolytic dehalogenases is the need for an **alternative electron acceptor**. In this thesis, nitrate was used to obtain anaerobic activity, however the nitrite formed during the reaction caused strong inactivation of the biocatalyst. The **stability** of the whole cells has not been studied in detail. The **substrate specificity** of the 4-CBA-dehalogenase of the

coryneform bacterium NTB-1 was rather limited; apart from 4-CBA only 4-iodo and 4-bromobenzoate were dehalogenated. Other 4-CBA degrading organisms exhibited the same limited substrate specificity. Since the **substrate specificity** of the hydrolytic dehalogenases is rather narrow, during this research various attempts were made to isolate microorganism capable of converting other halogenated aromatic compounds e.g. 4-fluorophenylglycine and 4-chloromandalic acid. A great number of microorganisms capable of degrading halogenated aromatic compounds was isolated but no organism was obtained containing a hydrolytic dehalogenases or yielding other interesting hydroxylated aromatic compounds.

Application of nitroreductase/hydroxylaminolyase systems

In search for other novel enzymes yielding hydroxyaromatics also enzymes involved in the degradation of nitroaromatic compounds were studied. Nitroaromatic compounds are also rather **cheap substrates** for the production of hydroxylated aromatic compounds, and these compounds are already applied in chemical syntheses of many chemical preparation methods. A *Comamonas acidovorans* was isolated on 4-nitrobenzoate. With whole cells, 3,4-dihydroxybenzoate could be produced from 4-nitrobenzoate under strictly anaerobic conditions. In this anaerobic process, ethanol was needed as a **cosubstrate** for cofactor regeneration. A great advantage of this system is that the reaction can be performed in the absence of oxygen, so that chemical oxidation of the dihydroxyaromatic compound formed cannot take place. The **toxicity** of nitroaromatic compounds was also a problem when using growing cells. However, under resting cell conditions activity was still found with concentrations above 50 mM 4-nitrobenzoate. In an initial experiment with whole cells, formation of about 4 grams of 3,4-dihydroxybenzoate per liter could be reached. Also under these conditions the **activity** of this biocatalyst was rather high (120 nmol min^{-1} mg^{-1} of protein). The **operational stability** of the system was not investigated but cells remained active after 20 h of incubation. Unfortunately, the **substrate specificity** of the reductase system was rather narrow since apart from 4-nitrobenzoate no other nitroaromatic compounds were reduced to hydroxylaminobenzoates. During this work no other hydroxylaminoaromatics were available and consequently the substrate specificity of the hydroxylaminobenzoate-lyase unfortunately could not be tested.

SUMMARY

In the introduction of this thesis several aspects of the production of hydroxylated aromatic compounds are described. These compounds are applied in the production of pharmaceuticals, polymers, flavors and dyes, but their chemical synthesis is rather difficult in preparative organic chemistry. Therefore, biotechnological production might be an alternative approach, in particular in the case of specialty chemicals. It is discussed that an attractive way to accomplish the formation of a hydroxylated aromatic is by replacing a substituent on the aromatic nucleus by a hydroxyl group. In this research nitro and halogen substituted aromatic compounds were used as starting material for the biosynthesis of hydroxy-aromatics and the microbial degradation of these compounds is discussed extensively in **chapter 1**.

In the **chapters 2** and **3** the potential of the conversion of 4-chlorobenzoate to 4-hydroxybenzoate was investigated. In cell-free extracts of the coryneform bacterium NTB-1 a reaction was detected resulting in the formation of 4-hydroxybenzoate from 4-chlorobenzoate in the absence of oxygen. Surprisingly, although the hydroxyl donor in this reaction is water, with whole cells no conversion took place under anaerobic conditions. After this unexpected result the uptake system of 4-chlorobenzoate in this bacterium was studied in detail and a very specific energy-dependent uptake mechanism for 4-chlorobenzoate was detected. The uptake of 4-chlorobenzoate was shown to be coupled to the proton-motive force, suggesting a proton symport mechanism. The presence of this uptake system seemed to explain the lack of activity with whole cells under anaerobic conditions. After these findings, cells were permeabilized and anaerobic formation of 4-hydroxybenzoate was measured. Surprisingly, however, still no dehalogenase activity by whole cells was detected. But addition of alternative electron acceptors did result in a stoichiometrical conversion of 4-chlorobenzoate to 4-hydroxybenzoate. From these data it was concluded that apart from the energy-dependent uptake system also energy is required for regeneration of cofactors involved in the dehalogenase reaction. Examination of the mechanism involved in the dehalogenation of 4-chlorobenzoate revealed a novel mechanism in the degradation of halogenated aromatic compounds. In strain NTB-1 two enzymatic steps were involved in the conversion to 4-hydroxybenzoate: in the first step 4-chlorobenzoate was activated in the presence of ATP and CoA to 4-chlorobenzoyl:CoA. In the second step the 4-chlorobenzoyl:CoA ester was hydrolyzed to yield 4-hydroxybenzoate.

In the second part of this thesis, enzymes involved in the conversion of nitro substituted to hydroxylated aromatic compounds were investigated (**chapters 4** and **5**).

After isolation of several organisms capable of degrading 4-nitrobenzoate, one strain designated NBA-10 was studied in detail. The degradation of 4-nitrobenzoate by *Comamonas acidovorans* NBA-10 followed a completely novel degradative pathway. In the first degradation steps, the 4-nitrobenzoate was reduced by a 4-NBA-reductase via 4-nitrosobenzoate to 4-hydroxylaminobenzoate. This reaction differed from published degradative pathways of nitro-aromatics involving release of ammonia. In such pathways formation of amino aromatic intermediates is often implied. However, in strain NBA-10 the involvement of 4-aminobenzoate as intermediate in the degradation of 4-nitrobenzoate was ruled out. The 4-NBA reductase was purified and it was demonstrated this hydroxylamine-forming enzyme was NADPH-dependent. The reductase displayed a narrow substrate specificity and it was not effected by flavine nucleotides. From these properties it was concluded that the reductase is quite distinct from other purified nitroaromatic reductases. In **chapter 5** it was shown the intermediate 4-hydroxylaminobenzoate is further metabolized to 3,4-dihydroxybenzoate. Formation of 3,4-dihydroxybenzoate was catalyzed by only one enzyme and also took place in the absence of oxygen. Purification of the enzyme revealed that apart from a reduced environment no cofactors were necessary for this reaction. The finding of this novel 4-hydroxylaminobenzoate degrading enzyme also elucidates various results found by others in the degradation of other nitroaromatic compounds.

In **chapter 6** it is discussed whether biotechnological processes are feasible in the formation of hydroxylated aromatics by replacing aromatic halogen or nitro groups. It was shown that anaerobical formation of 3,4-dihydroxybenzoate from 4-nitrobenzoate with strain NBA-10 can be achieved with resting cells provided a cosubstrate is present. Since this novel type of hydroxylaminobenzoate-degrading enzyme forms catechols in the absence of oxygen this type of bioformation might be interesting for the production of valuable catechols.

SAMENVATTING

In de inleiding van dit proefschrift is een aantal aspecten van de produktie van gehydroxyleerde aromaten beschreven. Deze verbindingen worden bijvoorbeeld gebruikt bij de produktie van pharmaceutische produkten, polymeren, geurstoffen en kleurstoffen. Isolatie uit natuurlijke bronnen (olie en planten) is zeker als het gaat om fijnchemicaliën onvoldoende mogelijk. Daarnaast is chemische synthese van gehydroxyleerde aromaten nogal lastig omdat door de extreme reactiecondities de gehydroxyleerde aromaten vaak verder worden geoxideerd. Daarom zou biotechnologische produktie, zeker als het gaat om fijnchemicaliën, een alternatieve produktiemethode kunnen bieden. In het verleden is een groot aantal enzymen (oxygenasen, peroxidasen) gekarakteriseerd dat in staat is een aromaat te oxideren tot een gehydroxyleerde aromaat. Deze enzymen zijn echter ook afhankelijk van zuurstof en toepassing van dit soort enzymsystemen resulteert meestal in verdere oxidatie van de gevormde gehydroxyleerde aromaten. Een andere biochemische methode is het gebruik van enzymen die onafhankelijk van zuurstof een substituent op de aromatische ring vervangen door een hydroxylgroep. In dit proefschrift is onderzocht in hoeverre nitro- en chloro-aromaten, gesubstitueerde aromaten waarvan de chemische synthese relatief eenvoudig is, als uitgangsstoffen kunnen dienen voor de biosynthese van gehydroxyleerde aromaten. De microbiële afbraak van deze gesubstitueerde aromaten en de verschillende enzymen die bij deze afbraak een rol spelen wordt uitvoerig besproken in **hoofdstuk 1**.

In **hoofdstuk 2** en **3** wordt een aantal aspecten van de omzetting van 4-chloorbenzoaat naar 4-hydroxybenzoaat beschreven. In celvrij extract van de coryneforme bacterie NTB-1 kon een enzym aangetoond worden dat zonder gebruik te maken van zuurstof de vorming van 4-hydroxybenzoaat uit 4-chlorobenzoaat katalyseert. Hoewel de hydroxyldonor in deze reactie water is, kon in een zuurstofloos milieu met hele cellen geen omzetting worden gemeten. Deze waarneming leidde tot onderzoek naar het 4-CBA opnamesysteem in deze bacterie. In deze stam werd een erg specifiek energie-afhankelijk 4-chloorbenzoaat opnamesysteem aangetoond. De opname van 4-chloorbenzoaat bleek afhankelijk te zijn van de protonengradiënt. De aanwezigheid van dit opnamesysteem was aanvankelijk een verklaring voor het uitblijven van activiteit in hele cellen onder anaerobe condities. In hele cellen, die permeabel waren gemaakt voor 4-chloorbenzoaat, was echter anaeroob geen activiteit meetbaar. Toevoeging van een alternatieve electronenacceptor resulteerde wel in een stoichiometrische omzetting van 4-chloorbenzoaat naar 4-hydroxybenzoaat. Uit deze resultaten is af te leiden dat zowel energie nodig is voor de opname van 4-chloorbenzoaat
als voor de regeneratie van cofactoren die betrokken zijn bij de enzymatische

dehalogenering/hydroxylering. Onderzoek aan het 4-chloorbenzoaat dehalogenase in de bacterie NTB-1 leverde een nieuw mechanisme van dehalogenering op, dat nog niet eerder was aangetoond in de afbraak routes van andere gechloreerde aromaten. De omzetting van 4-chloorbenzoaat naar 4-hydroxybenzoaat bestaat uit twee enzymatische stappen. In de eerste stap wordt 4-chloorbenzoaat geenergetiseerd door ATP en Coenzyme A tot 4-chloorbenzoyl:CoA, en in de tweede stap wordt deze CoA ester gehydrolyseerd tot 4-hydroxybenzoaat.

In het tweede gedeelte van dit proefschrift is onderzocht of het mogelijk is nitroaromaten biochemisch om te zetten in gehydroxyleerde aromaten (**hoofdstuk 4** en **5**). Na de isolatie van een aantal bacteriën, dat in staat was om 4-nitrobenzoaat af te breken, werd één bacterie in detail onderzocht. 4-Nitrobenzoaat wordt door *Comamonas acidovorans* NBA-10 afgebroken via een compleet nieuwe afbraakroute. In de eerste stappen van de afbraakroute wordt 4-nitrobenzoaat gereduceerd door het 4-nitrobenzoaatreductase via 4-nitrosobenzoaat tot 4-hydroxylaminobenzoaat. In de studies naar de afbraak van 4-nitrobenzoaat door andere bacteriën is reeds eerder gesuggereerd dat 4-hydroxylaminobenzoaat een intermediair in de afbraakroute is. In tegenstelling tot andere beschreven afbraakroutes voor nitroaromaten vindt er in NBA-10 geen reductie plaats tot 4-aminobenzoaat. In de afbraakroute van 4-nitrobenzoaat in deze bacterie is aangetoond dat 4-aminobenzoaat geen intermediar is in de afbraakroute van 4-nitrobenzoaat. Het 4-nitrobenzoaatreductase is gezuiverd en blijkt inderdaad een NADPH-afhankelijke omzetting van 4-nitrobenzoaat tot 4-hydroxylaminobenzoaat te katalyseren. Dit enzym reduceert slecht een beperkt aantal nitroaromaten en is niet afhankelijk van flavine. Met name deze eigenschappen van het 4-nitrobenzoaatreductase geven aan dat dit enzym verschilt van andere reductases, betrokken bij de afbraak van nitroaromaten. Het intermediair 4-hydroxylaminobenzoaat wordt verder afgebroken tot 3,4-dihydroxybenzoaat (**hoofdstuk 5**). De vorming van 3,4-dihydroxybenzoaat werd gekatalyseerd door één enzym en vond ook plaats in de afwezigheid van zuurstof. De vondst van een enzym, dat in staat is anaeroob 4-hydroxylaminobenzoaat om te zetten in 3,4-dihydroxybenzoaat, verklaart een aantal resultaten uit andere studies, die tot nog toe onverklaarbaar waren.

In **hoofdstuk 6** worden kort enkele aspecten besproken, die een rol spelen bij de biotechnologische toepassing van de gekarakterizeerde enzymen. Met name de vorming van het 3,4-dihydroxybenzoaat uit 4-nitrobenzoaat is interessant, omdat de activiteit relatief hoog is en onder goed controleerbare reactiecondities een hoge opbrengst haalbaar is. Verder onderzoek zal uit moeten wijzen of het ook mogelijk is met dit enzymsysteem andere meer waardevolle catecholen te produceren.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 4 april 1960 geboren te Delft. In 1978 behaalde hij zijn gymnasium β diploma aan het St. Stanislas college in Delft. In 1979 is hij voor een periode van 16 maanden ingelijfd bij de Koninklijke Landmacht. In 1980 begon hij met zijn studie Biologie aan de Rijksuniversiteit Leiden. In 1986 deed hij doctoraal examen in de microbiële fysiologie. In datzelfde jaar startte hij een promotieonderzoek bij de sectie Industriële Microbiologie van de Landbouwuniversiteit, dat leidde tot dit proefschrift.
Vanaf 1 maart 1992 is hij werkzaam bij KNOLL B.V. in Amsterdam.