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MINI-REVIEW

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The nitrile-degrading enzymes: current status and future prospects

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Abstract Nitrile-converting enzymes are becoming commonplace in the synthesis of pharmaceuticals and commodity chemicals. These versatile biocatalysts have potential applications in different fields including synthetic biocatalysis and bioremediation. This review attempts to describe in detail the three major classes of nitrile-converting enzymes, namely nitrilases, nitrile hydratases and amidases. Various aspects of these enzymes including their occurrence, mechanism of action, characteristics and applicability in different sectors have been elaborately elucidated. Cloning of genes related to nitrile-converting enzymes is also discussed.

Introduction

Nitrile compounds are widespread in the environment. In nature they are mainly present as cyanoglycosides, which are produced by plants (Conn 1981). Plants also produce other nitrile compounds such as cyanolipids, ricinine, phenylacetonitrile, etc. Chemical industries make extensive use of various nitrile compounds for manufacturing a variety of polymers and other chemicals. For example, acrylonitrile and adiponitrile are required for the production of polyacrylonitrile and nylon-66 polymers. In general, different nitrile compounds are used as feedstock, solvents, extractants, pharmaceuticals, drug intermediates (chiral synthons), pesticides (dichlobenil, bromoxynil, ioxynil, buctril), etc. They are also important intermediates in the organic synthesis of amines, amides, amidines, carboxylic acids, esters, aldehydes, ketones and heterocyclic compounds.

Most nitriles are highly toxic, mutagenic and carcinogenic in nature (Pollak et al. 1991). The general toxicities of nitriles in humans are expressed as gastric problems, vomiting (nausea), bronchial irritation, respiratory distress, convulsions, coma and osteolathrysm, which leads to lameness and skeletal deformities. Nitriles inactivate the respiration system by binding tightly to cytochrome-c-oxidase (Solomonson and Spehar 1981). Microbial degradation has been considered as an efficient way of removing highly toxic nitriles from the environment. Biological methods are more acceptable because of their eco-friendly nature. Different enzymes are responsible for the metabolism of nitriles in microbes. These involve hydrolysis [nitrile hydratase (NHase), amidase, nitrilase], oxidation (oxygenase) (Sawyer et al. 1984) and reduction (nitrogenase) (Liu et al. 1997) of nitriles.

From the synthetic viewpoint, nitriles represent a widely applicable chiral synthon, which can be employed for the homologation of a carbon framework; on the other hand, further transformations of the nitriles thus obtained are impeded due to the harsh reaction conditions required for their hydrolysis. In this context, the chemo-selective biocatalytic hydrolysis of nitriles represents a valuable alternative because it occurs at ambient temperature and near physiological pH. Many reactions catalyzed by nitrile-metabolizing enzymes are either already operated in large scale or have the potential to become so. The success of nitrile-converting organisms such as *Rhodococcus* N-774, *Pseudomonas chlororaphis* B23 and *R. rhodochrous* J1 in the kiloton production of acrylamide and synthesis of nicotinamide and nicotinic acid from 3-cyanopyridine (Mathew et al. 1988) has demonstrated the commercial viability of these enzymes. In recent years the use of these nitrile-converting enzymes, especially nitrilase and NHases, has increased tremendously. In this review we report the occurrence of nitrile-degrading enzymes, various enzymatic systems associated with nitrile metabolism in microorganisms, the mechanism of catalysis, reaction characteristics, cloning of genes related to various nitrile-converting enzymes and current applications.

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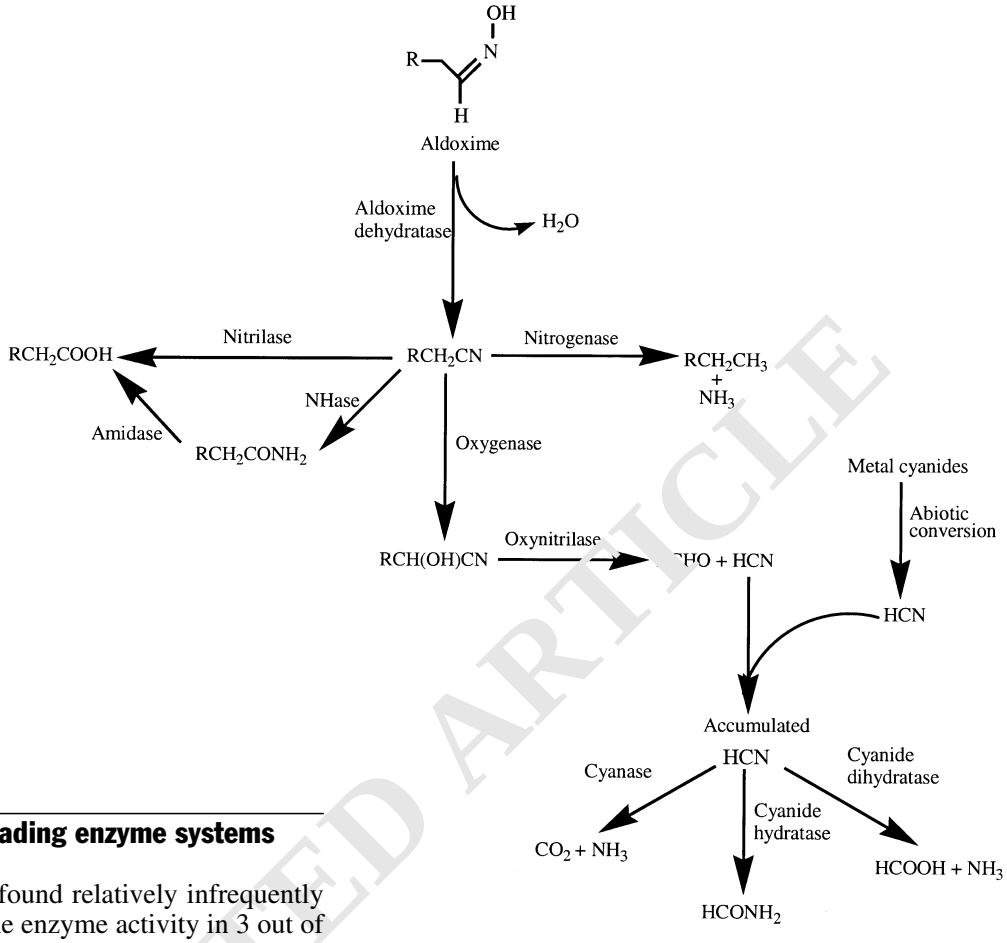

Fig. 1 Different pathways of nitrile metabolism

Distribution of nitrile-degrading enzyme systems

Nitrile-degrading activity is found relatively infrequently in nature. The existence of the enzyme activity in 3 out of 21 plant families (Gramineae, Cruciferae, and Musaceae) (Thimann and Mahadevan 1964) and in a limited number of fungal genera (*Fusarium*, *Aspergillus*, *Penicillium*) (Harper 1977a) indicates the relative rarity of this activity. Nitrile-degrading activity is more frequent in bacteria, though without extensive screening it is almost impossible to assess the actual distribution frequency. A number of bacteria (*Acinetobacter*, *Corynebacterium*, *Arthrobacter*, *Pseudomonas, Klebsiella*, *Nocardia*, *Rhodococcus*, etc.) are known to metabolize nitriles as sole source of carbon and nitrogen. The physiological role of nitrile-degrading enzymes in microorganisms is not clear. In plants, such activities are implicated in nutrient metabolism, particularly in the degradation of glucosinolates (Bestwick et al. 1993) and in the synthesis of indole acetic acid (Bartel and Fink 1994). In some higher plants, nitrile-degrading activity is also required for cyanide detoxification (Piotrowski et al. 2001). It has also been suggested that nitrile-degrading enzymes form components of complex pathways controlling both production and degradation of cyanogenic glycosides and related compounds where aldoximes are the key intermediates. While such a role is yet to be established in a microbial system, it is noted that some of the upstream enzyme activities, in particular the activity of aldoxime dehydratase, are responsible for the formation of nitriles from aldoximes (Kato et al. 2000), which further undergo hydrolysis, oxidation, reduction, etc. by various enzymes (Fig. 1).

Enzymology of nitrile metabolism

Hydrolysis is the most common pathway for the microbial metabolism of nitriles. Nitrilases catalyze the conversion of organic nitriles to corresponding acids and NH_3, while NHases catalyze the formation from nitriles of amides, which are subsequently converted to acids and NH_3 by amidases. HCN abiotically formed from various metal cyanides leads to various products via the action of different enzymes, e.g., cyanase produces CO_2 and NH_3 (Dorr and Knowles 1989), cyanide hydratase leads to formamide (Wang et al. 1992), and cyanide dihydratase produces formic acid and NH_3 (Ingovorsen et al. 1991). Many plants and insects use oxygenases to oxidize some of the nitriles to cyanohydrins (α-hydroxynitriles), which are further converted to an aldehyde and HCN by oxynitrilases (hydroxynitrile lyases) (Johnson et al. 2000). This type of enzymatic system is almost unknown in microorganisms. However, a fungus (*Trichoderma* sp.) has been reported to degrade diaminomalenonitrile, releasing HCN (Kuwahara and Yanase 1985). Nitrogenases present in nitrogen-fixing organisms are capable of reducing nitrile compounds to the corresponding hydrocarbons and NH_3 (Liu et al. 1997).

Table 1 Characteristics of some nitrilases from different microorganisms

Microorganism	Formation type	Properties of nitrilase							Reference
		Molecular weight (MW) (kDa)	No. of subunits and MW (kDa)	Optimum		Stability		Substrate specificity	
				pH	*T* (°C)	pH	*T* (°C)		
Bacteria									
Nocardia sp. NCIB 11216	Inducible	560	– (45)	8.0	–	–	–	Aromatic nitriles	(Harper 1977b)
Nocardia sp. NCIB 11215	Inducible	560	12 (46)	8.0	40	7.0–9.5	10–50	Aromatic and heterocyclic	(Harper 1985)
Rhodococcus rhodochrous J1	Constitutive	78	2 (41.5)	7.5	45	–	Below 50	Aliphatic and aromatic nitriles	(Kobayashi et al. 1989)
R. rhodochrous K22	Constitutive	650	15–16 (41)	5.5	50	6.0–8.0	Below 50	Aliphatic nitriles	(Kobayashi et al. 1990)
R. rhodochrous PA-34	Inducible	45	Monomer	7.5	35	6.0–9.0	30–50	Aliphatic nitriles	(Bhalla et al. 1992)
Rhodococcus sp. ATCC 39484	Inducible	560	– (40)	7.5	30	5.5–9.5	25–40	Aromatic nitriles	(Stevenson et al. 1992)
R. rhodochrous NCIMB 11216	Inducible	45.8	–	8.0	30	–	–	Aromatic nitriles	(Hoyle et al. 1998)
Arthrobacter sp. J1	Inducible	30	1	8.5	40	–	–	Aromatic nitriles	(Bandopadhayay et al. 1986)
Alcaligenes faecalis JM3	Inducible	260	6	7.5	45	5.8–9.3	20–50	Arylacetonitriles	(Nagasawa et al. 1990)
Acinetobacter sp. AK226	Inducible	580	–	8.0	50	5.8–8.0	Up to 60	Aliphatic and aromatic nitriles	(Yamamoto and Komatsu 1991)
Alcaligenes faecalis ATCC 8750	Inducible	32	–	7.5	40–45	6.5–8.0	Below 50	Arylacetonitriles	(Yamamoto et al. 1992a)
Comamonas testoteroni	–	–	Oligomeric (38)	7.0	25	–	–	Adiponitrile	(Levy-Schill 1995)
Pseudomonas fluorescens DSM 7155	Inducible	–	2 (40, 38)	9.0	55	–	–	Arylacetonitriles	(Layh et al. 1998)
Bacillus pallidus Dac 521	Inducible	600	– (41)	7.6	65	6.0–9.0	Below 70	Aromatic nitriles	(Almatawah et al. 1999)
Klebsiella ozaenae	Constitutive	37	2 (–)	9.2	35	–	–	Bromoxynil	(Stalker et al. 1988a)
Fungi									
Fusarium solani	Inducible	620	8 (76)	–	–	7.8–9.1	–	Aromatic nitriles	(Harper 1977a)
Fusarium oxysporum	Constitutive	550	Oligomeric (27)	–	4	6.0–11.0	–	Aliphatic and aromatic	(Goldhust and Bohak 1989)
Cryptococcus sp. UFMG-Y28	Inducible	–	–	–	–	–	–	Benzonitrile	(Rezende et al. 2000)

Nitrilase

Nitrilase, the first nitrile-metabolizing enzyme to be discovered almost 40 years ago, is known to convert indole-3-acetonitrile to indole-3-acetic acid (an auxin) in plants (Thimann and Mahadevan 1964). Later, a number of microorganisms possessing nitrilase activity were isolated that have the capability to metabolize several natural and synthetic nitriles. Based on their substrate specificity, microbial nitrilases are differentiated into three different categories. Some nitrilases hydrolyze aromatic or heterocyclic nitriles to the corresponding acids and ammonia. Others are known to preferentially hydrolyze either aliphatic nitriles or arylacetonitriles to their respective carboxylic acids. Table 1 summarizes the biochemical properties of some nitrilases reported in the literature. Nitrilases are generally inducible enzymes composed of one or two types of subunits of different size and number. *Nocardia* sp. nitrilase was reported to be induced by benzonitrile (Collins and Knowles 1983). Acetonitrile has been used as an inducer for the formation of nitrilase in *Fusarium oxysporum* (Goldhust and Bohak 1989). The use of isobutyronitrile or isovaleronitrile enhanced the production of benzonitrilase in *R. rhodococcus* J1 (Nagasawa et al. 1988). It has been observed in most cases that different subunits of nitrilase self-associate to convert the enzyme to the active form. The monomers (47 kDa) of *Nocardia* sp. NCIB 11216 nitrilase associate to produce a 560 kDa dodecamer due to benzonitrile-induced activation (Harper 1977b). This association is accelerated by temperature and enzyme concentration. It has been reported that a nitrilase from

Table 2 Characteristics of some nitrile hydratases (NHases) from different microorganisms. *PQQ* Pyrroloquinoline quinone

Microorganism	Formation type	Properties of nitrile hydratase									Reference
		Metal and PQQ	MW (kDa)	No. of subunits and MW (kDa)	Optimum		Stability		Substrate specificity	Photo-activation	
					pH	*T* (°C)	pH	*T* (°C)			
Bacteria											
R. rhodochrous J1											(Okada et al.
a) Low molecular mass enzyme	Inducible	Co (+)	101	18–20 (α: 26) (β: 29)	8.8	40	–	–	Aromatic nitriles	–	1997)
b) High molecular mass enzyme	Inducible	Co (+)	505	4–5 (α: 26) (β: 29)	6.5	35–40	6.0–8.5	50	Aliphatic nitriles	–	
Rhodococcus sp. N 774	Constitutive	Fe (+)	70	2 (α: 28.5) (β: 29)	7.7	35	7.0–8.5	–	Aliphatic nitriles	+	(Endo and Watanabe 1989)
Rhodococcus sp. N 771	Constitutive	Fe (+)	70	2 (α: 27.5) (β: 28)	7.8	30	6.0–8.0	below 35	Aliphatic nitriles	+	(Yamada and Kobayashi 1996)
Rhodococcus equi A4	Inducible	– (–)	60	2 (α: 25) (β: 25)	–	–	–	–	Aliphatic nitriles	–	(Prepechalova et al. 2001)
Rhodococcus erythropolis	–	–	–	–	–	–	–	–	Steroidal nitriles	–	(Kaufmann et al. 1999)
Rhodococcus sp. YH3–3	Inducible	Co (–)	130	2 (α: 27.1) (β: 34.5)	–	–	2.5–11.0	40–60	Aliphatic and aromatic nitriles	–	(Kato et al. 1999)
Pseudonocardia thermophila JCM3095	Constitutive	Co (–)	–	– (α: 29) (β: 25)	–	60	–	60	Acrylonitrile	–	(Yamaki et al. 1997)
Agrobacterium tumefaciens IAMB-261	Inducible	Co and Fe	102	4 (25)	7.5	–	6.5–9.5	–	Indole 3-acetonitrile	–	(Kobayashi et al. 1995)
Agrobacterium tumefaciens d3	Inducible	Fe (–)	69	4 (27)	7.0	40	7.0–10.0	50	2-arylpropio-nitrile	–	(Bauer et al. 1994)
Arthrobacter sp. J1	Inducible	–	420	2 (24)	7.0–7.2	35	–	–	Aliphatic nitrile	–	(Asano et al. 1982a)
Brevibacterium sp. R 312	Constitutive	Fe (+)	85	3–4 (α: 26) (β: 27.5)	7.8	25	6.5–8.5	–	Aliphatic nitrile	–	(Nagasawa et al. 1986)
Pseudomonas chlororaphis B 23	Constitutive	Fe (+)	100	4 (α: 25) (β: 25)	7.5	20	6.0–7.5	20	Aliphatic nitrile	–	(Nagasawa et al. 1987)
Pseudomonas putida NRRL-18668	Constitutive	Co (+)	54, 95	– (α: 23) (β – 25)	–	–	–	–	Aliphatic nitrile	–	(Fallon et al. 1997)
Brevibacterium imperalis CBS489–74	Constitutive	– (–)	–	– (–)	6.0	–	5.8–7.4	–	Acrylonitrile	–	(Alfani et al. 2001)
Corynebacterium sp. C5	Constitutive	Fe (+)	61.5	2 (–)	8.0–8.5	–	–	–	Aliphatic dinitrile	–	(Yamamoto et al. 1992b)
Bacillus sp. RAPc8	–	Fe (–)	–	4 (α: 28) (β: 29)	7.0	60	–	Above 50	Alkylnitrile	–	(Pereira et al. 1998)
Fungi											
Myrothecium verrucaria	Inducible	Zn (–)	170	6 (27.7)	7.7	55	–	–	Cyanamide	–	(Maier-Greiner et al. 1991)

R. rhodochrous cells is converted to the active form by subunit association when incubated with substrate (Stevenson et al. 1992), or in the presence of higher concentration of enzyme, salt or organic solvent (Nagasawa et al. 2000). The hydrophobic effect resulting from the presence of the above-mentioned conditions might change the conformation of the enzyme, exposing hydrophobic sites thereby enabling subunit assembly and enzyme activation.

Nitrilases, unlike NHases, do not show the presence of any metal co-factor or prosthetic group. They are reported to have catalytically essential cysteine residues at, or

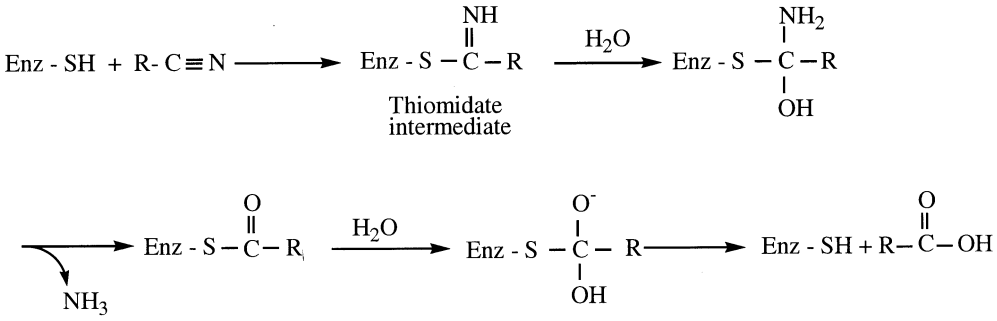

Fig. 2 Mechanism of nitrilase-catalysed reaction

near, the active site (Kobayashi et al. 1992a). Nitrilase from *R. rhodococcus* K22 was found to have a cysteinyl residue (Cys 170) in the active site (Kobayashi et al. 1992b). A possible mechanism for the nitrilase-catalyzed reaction indicates a nucleophilic attack by a thiol group on the carbon atom of the nitrile with concomitant protonation of nitrogen to form a tetrahedral thiomidate intermediate. Subsequent steps involve attack by two water molecules and protonation of the nitrogen atom, which is lost as ammonia. In some cases the tetrahedral intermediate formed can break down anomalously to produce amide instead of the normal acid product (Stevenson et al. 1992). Figure 2 shows the mechanism of the nitrilase-catalyzed reaction. Recently, it was observed that nitrilase from *R. rhodochrous* J1 catalyzes the hydrolysis of amides to acids and NH_3 (Kobayashi et al. 1999). This suggests the existence of a common tetrahedral intermediate in the reaction, involving nitriles or amides as substrates.

NHase

NHase is a key enzyme in the bi-enzymatic pathway for conversion of nitriles to acids, which converts nitriles to corresponding amides. A number of microorganisms with NHase activity have been isolated; the enzymes have been purified and characterized (Table 2). All these revealed wide-ranging physiochemical properties and substrate specificities. Although the enzyme has α and β subunits in equimolar amounts, the amount varies for enzymes from different microbial sources. These are metalloenzymes containing either cobalt or iron. On the basis of the metal ion present, NHases can be classified into two broad groups: ferric NHases and cobalt NHases. There may be two main reasons for having metal in the active site: (1) metal ions are very good catalysts for -CN hydration, and (2) they are required for the folding of the enzyme. In addition to their function in the active center, the metal ions may play a role in enhancing the folding or the stabilization of the subunit polypeptides of the enzyme.

Ferric NHases

The NHases from *Rhodococcus* R312 (formerly known as *Brevibacterium* R312) and *P. chlororaphis* B23 are the first examples of non-heme iron enzymes containing a low spin Fe(III) ion (Sugiura et al. 1987). The activity of NHase has unique features when exposed to light (Endo et al. 1999; Popescu et al. 2001). The chromophore involved in the photo-activation is an iron complex in the β subunit; light irradiation of the complex induces a conformational change of the subunit. Because of this, the endogenous NO molecule that is bound to the non-heme iron (III) center in the inactive NHase is released, resulting in the recovery of the original NHase activity. Three-dimensional analysis of *Rhodococcus* R312 NHase showed that it contains a novel iron center (Huang et al. 1997). All the metal ion-protein ligands are contained within the α subunit. Three cysteine thiolates and two main chain nitrogen atoms are ligands. These five iron ligands (Cys 110, Cys 113, Cys 115, Ser 114, and Cys 115) are located on five vertices of an octahedron and the sixth position is likely to be occupied by a hydroxide ion. Furthermore, Cys 112 and Cys 114 (corresponding to Cys 113 and Cys 115 in the *Rhodococcus* R312 NHase) are post-translationally oxidized to cysteine-sulfinic and sulfenic acids, respectively, in the *Rhodococcus* N-771 NHase. A possible reaction mechanism for NHase catalysis might be: (1) the nitrile substrate approaches a metal-bound hydroxide ion, which can act as a nucleophile attacking the nitrile carbon atom (Fig. 3, mechanism I) or (2) a metal-bound hydroxide ion acting as a general base activates a water molecule, which then attacks on nitrile carbon (Fig. 3, mechanism II), resulting in the formation of an imidate. The imidate finally tautomerizes to form an amide (Fig. 3).

Cobalt NHase

In the presence of cobalt ions, the actinomycete *R. rhodochrous* J1 (Komeda et al. 1996b) produces two NHases, depending on the inducer. When cultured in a medium containing urea and cyclohexane carboxamide, high and low molecular weight NHases (H- and L-NHases) are selectively induced (Yamada and Kobayashi 1996). H-NHases (Nagasawa et al. 1991) act preferentially on aliphatic nitriles, whereas L-NHases (Komeda et al. 1996b) exhibit higher affinity for aromatic nitriles. H- and L-NHases have been used for the industrial production of acrylamide and nicotinamide from acrylonitrile and 3-cyanopyridine, respectively.

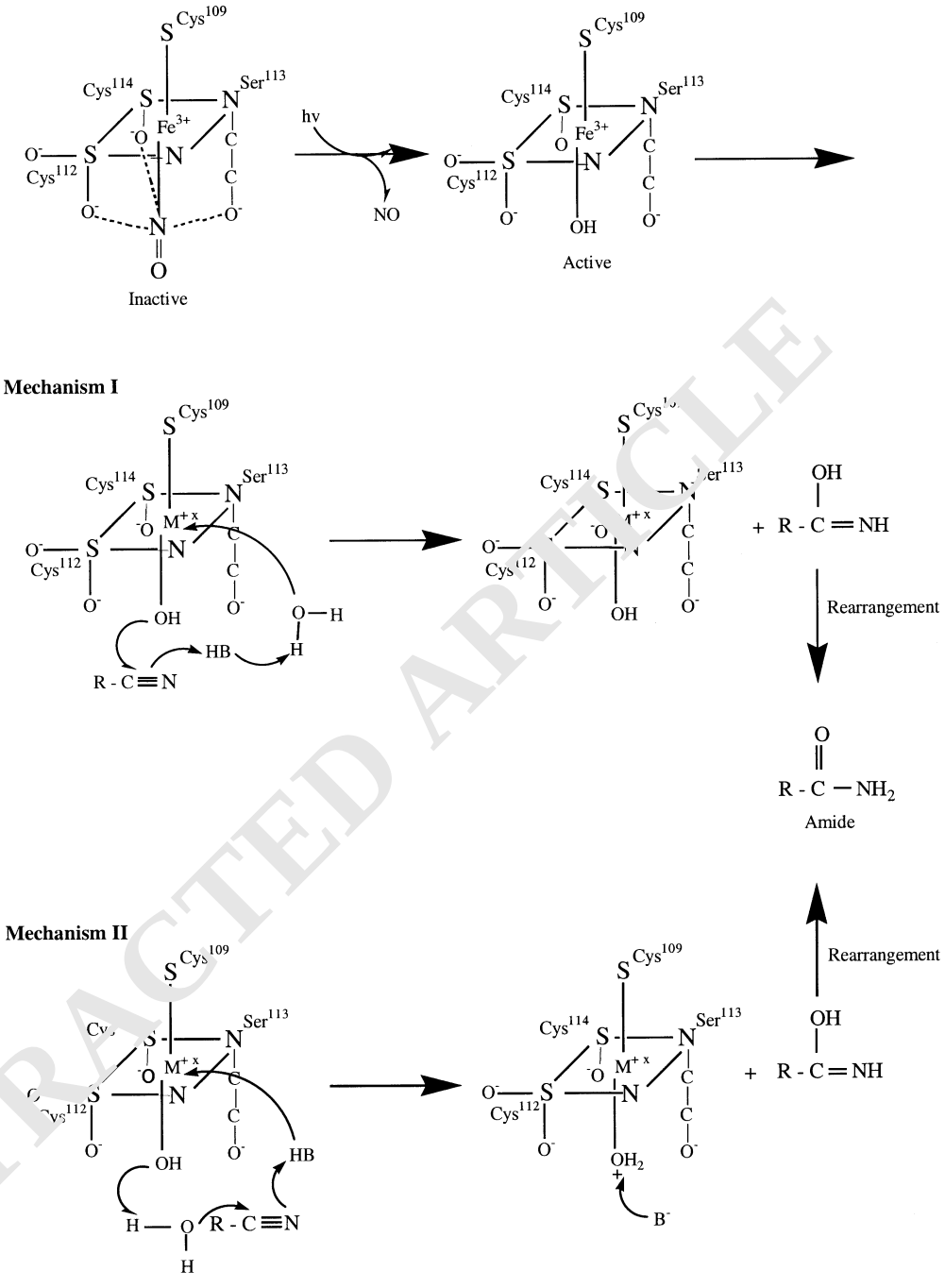

Fig. 3 Mechanism of nitrile hydratase (NHase) photoactivation and enzymatic catalysis

Both the purified NHases contain cobalt as co-factor. The cobalt in H-NHase exists as a low-spin Co ion in a tetragonally distorted octahedral ligand field. The similarities of the pre-edge and extended X-ray absorption fine structure spectrum suggests that the ligand environments of the metal ions in the cobalt- and iron-containing NHases are similar. The cobalt NHases have threonine in the -V-C-(T/S)-L-C-S-C- sequence as the active site (Payne et al. 1997), whereas the ferric NHases have serine. The difference in the metal co-factors may be ascribed to the different amino acid residues at this position. The crystal structure of Co(III)-containing NHase from *Pseudonocardia thermophila* JCM3095 revealed that both Co(III) and Fe(III) reside in similar environments. A tryptophan residue (Trp 72), which may be involved in substrate binding, in the Co(III)-containing enzyme replaces the tyrosine residue in the Fe(III)-containing enzyme. This is probably responsible for the preference of cobalt NHases for aromatic rather than aliphatic nitriles (Miyanaga et al. 2001).

Amidase

Amidases catalyze the hydrolysis of amides to free carboxylic acids and ammonia. These enzymes are involved

Table 3 Characteristics of some amidases from different microorganisms

Microorganism	Formation Type	Properties of amidase							Reference
		MW (kDa)	No. of subunits and MW (kDa)	Optimum		Optimum		Substrate specificity	
				pH	*T* (°C)	pH	*T* (°C)		
Bacteria									
Arthrobacter sp. J1	Inducible	320	8 (39)	7.0	35	7.0–9.0	30–45	Aliphatic amides	(Asano et al. 1982a)
Brevibacterium sp. R312	Inducible	120	2 (46)	–	–	–	–	Aryloxypropionamides	(Mayaux et al. 1990)
Klebsiella pneumoniae NCTR1	Inducible	62	Monomer	7.0	65	5.0–8.5	30–65	Aliphatic amides	(Nawaz et al. 1996)
Pseudomonas chlororaphis B23	Inducible	105	2 (54)	7.0–8.6	50	5.9–9.9	25–50	Aliphatic amides	(Ciskainik et al. 1995)
Ochrobactrum anthropi SV3	Inducible	40	– (–)	8.5–9.5	45	6.5–11.0	35–50	Amino acid	(Komeda and Asano 2000)
Agrobacterium tumefaciens d3	Inducible	490	– (63)	–	–	–	–	Aromatic amides	(Trott et al. 2001)
Bacillus stearothermophilus BR388	Inducible	–	–	7.0	55	–	–	Wide spectrum amidase	(Cheong and Oriel 2000)
Rhodococcus sp.	Inducible	118	2 (–)	–	–	–	–	Arylpropionamide	(Mayaux et al. 1991)
Rhodococcus sp.	Constitutive	360	– (44.5)	8.5	40	–	–	Aliphatic amide	(Nawaz et al. 1994)
Rhodococcus erythropolis MP50	Inducible	480	– (–)	7.5	55	6.0–9.0	40–60	Aromatic amide	(Hirrlinger et al. 1996)
Rhodococcus rhodochrous M8	Constitutive	150	4 (43)	7.0	55–60	5.0–8.0	40–65	Aliphatic amide	(Kotlova et al. 1999)

in nitrogen metabolism in both prokaryotic and eucaryotic cells. Characteristics of amidases from different sources are given in Table 3. Some are specific for aliphatic amides (Asano et al. 1982b), others cleave amides of aromatic acids (Hirrlinger et al. 1996) and still others hydrolyze amides of α- or ω-amino acids (Stelkes-Ritter et al. 1995). Stereoselectivity has been reported to be generally associated with the amidases in the bi-enzymatic pathway (Mayaux et al. 1990).

Unlike NHases, the association of amidases with metals such as cobalt or iron is reported only in case of *K. pneumoniae* (Nawaz et al. 1996). *Rhodococcus* sp. R312 was reported to have a wide spectrum of amidases; an α-amino acid amidase specific for L-α-amino amides (Kieny-L' Homme et al. 1981), an aliphatic amidase (Kieny-L' Homme et al. 1981), an enantioselective amidase hydrolyzing aryloxy propionamides (Mayaux et al. 1990), a novel amidase hydrolyzing dinitriles (Moreau et al. 1993), etc. The other amidases from *P. chlororaphis* B23, *Rhodococcus* sp. N-774, *R. rhodochrous* J1, etc. belong to a group of amidases containing a GGSS signature in their amino acid sequence (Chebrou et al. 1996). Kobayashi et al. (1997) showed that these amidases contain Asp 191 and Ser 195 in the active site in place of the more familiar cysteine residues (Novo et al. 1995). The substrate specificity and biological functions of these enzymes vary widely including carbon/nitrogen metabolism in prokaryotes through hydrolysis of amides, the generation of properly charged $tRNA^{Gln}$ in eubacteria through transfer of NH_3 from glutamine (Curnow et al. 1997) and the degradation of neuromodulatory fatty acid amides in mammals (Cravatt et al. 1996). The mammalian enzyme belongs to "amidase signature family" defined by a conserved stretch of approximately 130 amino acids termed the "amidase signature sequence". Studies revealed that this group of amidases represents a large class of serine-lysine catalytic dyad hydrolases (Patricelli and Cravatt 2000) and more specifically resemble serine hydrolases (Boger et al. 2000). Surprisingly, the amidase of *R. rhodochrous* J1 is found to catalyze the hydrolytic cleavage of the functional nitrile group to an acid and ammonia stoichiometrically (Kobayashi et al. 1998). Thus, the reaction mechanisms of both the nitrilase- and amidase-catalyzed reactions are analogous, but the active nucleophile present in the enzymes differs. Figure 4 shows the reaction mechanism of amidase, which also involves nitrile hydrolysis. The carbonyl group of amide undergoes a nucleophilic attack, resulting in the formation of a tetrahedral intermediate, which is converted to acyl-enzyme with the removal of ammonia and subsequently hydrolyzed to acid. All of the different amidases also exhibit acyl transfer activity in the presence of hydroxylamine (Fournand et al. 1998). Recently, a novel amidase involved in bacterial cyclic imide metabolism was purified from *Blastobacter* sp. strain A17p-4 (Soong et al. 2000). Physiologically, the enzyme functions in the second step of cyclic imide degradation, i.e., the hydrolysis of monoamidated dicarbox-

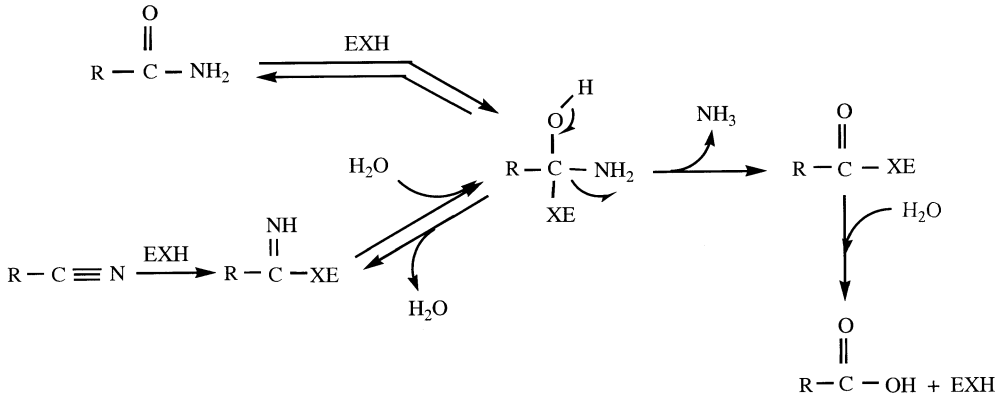

Fig. 4 Mechanism of catalysis by amidase

ylates (half-amides) to dicarboxylates and ammonia. Enzyme production was enhanced by cyclic imides such as succinimide and glutarimide but not by amide compounds, which are conventional substrates and inducers of known amidases.

Applications

Synthetic biocatalysis

Biotransformation of nitriles provides great potential for synthetic chemists. The ability of the enzyme system to convert a cyano functionality to either an acid or an amide is, in itself, of great use. Traditional chemical methods for conversion of nitriles to acids or amides have several drawbacks: (1) reactions need to be carried out in strongly acidic (6 M HCl) or basic (2 M NaOH with reflux) conditions (Vogel 1989), (2) higher reaction temperature, (3) formation of byproducts such as toxic HCN or large amounts of salt, etc. Biocatalytic conversion of nitriles is attractive due to its ability to effect reactions in a "greener" manner and the potential for carrying out chemo-, regio-, and enantio-selective transformations. All the nitrile-metabolizing enzymes hydrolyze a number of structurally diverse nitriles. Several commercially important organic compounds, such as *p*-aminobenzoic acid, benzamide, acrylamide, nicotinic acid, pyrazinoic acid, thiophenamide, etc., have been prepared from the corresponding nitriles using microbial cells.

Acrylamide is produced by a third generation biocatalyst, *R. rhodochrous* J1 (Yamada and Nagasawa 1994), and productivity has increased up to 30,000 tonnes per annum. Recently, surfacial nitrile groups of acrylic fibers have been converted to the corresponding amides by *R. rhodochrous* NCIMB 11216 NHase (Tauber et al. 2000). Due to enzymatic modification, the acrylic fibers became more hydrophilic and thus adsorption of dyes is enhanced. Conversion of 3-cyanopyridine to nicotinamide as well as nicotinic acid is obtained by using *R. rhodochrous* J1 (Mathew et al. 1988). One of the most attractive features of nitrile-metabolizing enzymes is their ability to selectively convert one cyano group of a polynitrile, which is virtually impossible using conventional chemical methods. *R. rhodochrous* K22 catalyzes the conversion of adiponitrile to 5-cyanovaleric acid, which is an intermediate in the synthesis of nylon-6 (Godtfredsen et al. 1985). Tranexamic acid, a homeostatic drug, is obtained by selective mono-hydrolysis of trans 1,4-dicyano cyclohexane by *Acremonium* sp. (Nishise et al. 1987). Recently *ortho*-, *meta*-, and *para*-CH_2CN substituted compounds were biotransformed using whole cell suspensions of the bacterium *R. rhodochrous* LL100–21 (Dadd et al. 2001). Comparison of the initial rates of conversion of the aliphatic cyano side chain of 2-(cyanomethyl) benzonitrile and other substituted benzonitriles indicate that electronic effects did not affect the initial rate of the reaction.

Another important aspect of nitrile biocatalysis is the ability of these enzymes to carry out stereoselective transformations. The optically active compounds can have a considerable impact on the market of high value pharmaceuticals (non-steroidal anti-inflammatory drugs), agricultural chemicals and ferroelectric crystals (Layh et al. 1998). Some laboratory-scale studies have revealed that hydrolysis of (R, S)-(±)-ibuprofen nitrile by *Acinetobacter* sp. AK226 leads to the formation of the non-steroidal anti-inflammatory drug, (S)-(+)-ibuprofen with an ee of 95% (yield 45%) (Yamamoto et al. 1990). Different racemic α-substituted nitriles were also enantioselectively hydrolyzed by these enzymes. For example, (R)-(–)-mandelic acid was produced in very high yields from the corresponding racemic mandelonitrile using R-enantioselective nitrilase from *Alcaligenes faecalis* ATCC 8750 (Yamamoto et al. 1991). Optically active amino acids are also produced from the respective 2-aminonitriles. The amino acids produced are in the L-form with the exception of alanine, which showed a reverse stereoselectivity (Bhalla et al. 1992). In bi-enzymatic systems, it has been generally observed that enantiomeric discrimination occurs during amide hydrolysis. Recently, it has been demonstrated that some NHases are also stereoselective in nature. An (S)-selective nitrile hydratase from *Agrobacterium tumefaciens* d3 (Bauer et al. 1994) and *Pseudomonas putida* NHase (Fallon et al. 1997), both of which convert (±)-2-arylpropionitriles specifically to the corresponding (S)-amides, have been

purified and characterized. Recently, a stereoselective NHase that converts phenylglycine nitrile to phenylglycine, a precursor for semisynthetic cephalosporins and penicillins, has been isolated (Wegman et al. 2000).

Bioremediation

Waste treatment

Synthetic nitrile compounds are widespread in the environment in the form of industrial wastewater. Most of these are toxic, carcinogenic and mutagenic in nature (Pollak et al. 1991) and thus there is a need to control their release into the environment. A mixed culture of bacteria containing different nitrile hydrolyzing enzymes (including NHase, nitrilase and amidase) that metabolize effluent containing acrylonitrile, fumaronitrile, succinonitrile, etc. are grown in batch and continuous culture on these components of waste (Wyatt and Knowles 1995). These authors also reported the biodegradation of effluent from acrylonitrile manufacturing industries using mixed cultures of bacteria, with a 75% reduction in COD value and 99% removal of detectable toxic components (Wyatt and Knowles 1995). The use of specialized consortia of microorganisms to degrade toxic wastes could be a viable alternative approach to the classical activated sludge system. It has also been reported by Battistel et al. (1997) that acrylonitrile decontamination of polymer emulsions can be efficiently obtained by enzymatic (NHase) treatment of raw aqueous latexes under mild reaction conditions.

Herbicide degradation

Prolonged exposure to nitrile herbicides [dichlobenil (2,6-dichlorobenzonitrile), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile)] results in symptoms of weight loss, fever, vomiting, headache and urinary problems (Freyssinet et al. 1996). Nitrile-metabolizing enzymes efficiently degrade these cyano group-containing herbicides and prevent them from entering the food chain. *Agrobacterium radiobacter*, a bromoxynil-degrading soil bacterium, is used for the degradation of the herbicide under nonsterile batch and continuous conditions. The bromoxynil concentration in a column reactor decreases to 65% after 5 days. The efficacy of degradation is enhanced by addition of ferrous, cobaltous or cupric ions (Muller and Gabriel 1999). A gene encoding the nitrile-degrading enzyme has been cloned and used to raise herbicide-resistant plants (Stalker et al. 1988b). Bromoxynil-resistant transgenic plants resulting from the introduction of microbial bromoxynil-specific nitrilase genes into tomato or tobacco could be on the market in the near future (Freyssinet et al. 1996). Similarly, other nitrile-degrading enzymes could also be potential candidates for molecular manipulation of bio-degradative systems in plant biotechnology.

Cloning of nitrile-degrading genes

The first nitrilase gene cloned in *Escherichia coli* encoded a bromoxynil-degrading activity from *Klebsiella pneumoniae* subsp. *ozaenae* (Stalker and McBride 1987). Several other nitrilase genes from different sources have also been cloned [the *nit*A gene from *R. rhodochrous* K22 into plasmid pNK21 (Kobayashi et al. 1992a), the *nitA* gene (750 bp) from *R. rhodochrous* in *E. coli* plasmid pNJ1 (Kobayashi et al. 1992b), *Alcaligenes faecalis* JM3 in *E. coli* (Kobayashi et al. 1993), *Gordona terrae* MA-1 in *E. coli* (Yu 1999)]. Komeda et al. (1996a) reported the presence of a downstream region (1.4 kb) in *R. rhodochrous* J1 that is important for the induction of nitrilase synthesis. Sequence analysis shows the existence of an open reading frame (*nitR*) of 957 bp that codes for a positive transcriptional regulator for *nitA* expression. Recently, the nitrilase AtNIT1 from *Arabidopsis thaliana*, a higher plant, was overexpressed in *E. coli*. The recombinant AtNIT1 has properties similar to those of the native enzyme and the nitrilase from *Brassica napus* (Osswald et al. 2002).

In the case of NHase, both the H- and L-NHase genes have been cloned from the industrial strain *R. rhodochrous* J1 (Kobayashi et al. 1991). Both these genes showed significant similarity, in the β subunit's N-terminal sequence, with the NHase gene of *Rhodococcus* sp. N-774 (Ikehata et al. 1989). Expression of H- and L-NHase genes under the control of a *lac* promoter in *E. coli* is possible only in medium supplemented with $CoCl_2$. Although *E. coli* is the best-known cloning vehicle, expression of NHase genes from *Rhodococcus* sp. N-774 and *R. rhodochrous* J1 in *E. coli* resulted in accumulation of an insoluble polypeptide with minimal activity. Thus, NHase and amidase genes of *Rhodococcus* sp. N-774 have been introduced into *R. rhodochrous* ATCC 12674, where they are expressed in active form (Hashimoto et al. 1992). The close spacing of five genes (amidase, α and β-subunit of NHase, P47 K and *OrfE*) in *P. chlororaphis* B23 suggests that these proteins are translated from a single mRNA species. *E. coli* transformants carrying pPCN4, where this 6.2 kb region is placed under the control of a *lac* promoter, expressed NHase (10% of total soluble protein) (Nishiyama et al. 1991). The structural genes encoding the α and β-subunits of the stereoselective cobalt NHase from *P. putida* NRRL 18668 have been cloned and sequenced (Payne et al. 1997). A 6-fold over-production of the enzyme has been obtained by the co-expression of a novel downstream gene encoding protein P14 K that appears to be part of an operon that includes the structural genes for the α and β subunits of NHase. Another NHase gene from *Pseudonocardia thermophila* JCM 3095 has been cloned, and the expressed enzyme shows high thermal stability (Yamaki et al. 1997). Mayaux et al. (1990) cloned the amidase and α-subunit of the NHase gene from *Brevibacterium* sp. R312. Recently, a D-stereospecific amino acid amidase gene (*daaA*) was cloned from *Ochrobactrum anthropi* SV3 (Komeda and Asano 2000). A wide-spectrum amidase gene from *B. stearothermophilus* BR388 was cloned and over-

expressed in *E. coli* (Cheong and Oriel 2000). All this leads to a better understanding of, and improvements in, enzyme function for biotechnological applications.

Conclusions

The versatile biocatalytic nature and applications of nitrile-converting enzymes are now increasingly recognized for the production of several pharmaceutically important compounds and fine chemicals. By virtue of their capability to eliminate highly toxic nitriles, these nitrile-degrading enzymes also play a significant role in protecting the environment. The advances in biosynthetic regulation, genetics and better understanding of the structure and reaction mechanism of nitrile-metabolizing enzymes will lead to improved properties such as higher enzyme activity, higher tolerance to substrate and products and higher thermostability of the biocatalysts used in commercial processes. With the advancement in techniques related to screening, cultivation, protein and genetic engineering, it is possible to isolate novel enzymes with extremophilic characteristics. Though recent developments have broadened the scope of the potential application of these versatile biocatalysts, further application-oriented studies are required to fully exploit their biotechnological potential.

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