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Influence of point mutations near the active site on the catalytic properties of fungal arylacetonitrilases from *Aspergillus niger* and *Neurospora crassa*

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ABSTRACT

Arylacetonitrilases from *Aspergillus niger* CBS 513.88 and *Neurospora crassa* OR74A (NitAn and NicNc, respectively) were expressed in recombinant *Escherichia coli* JM109. The respective whole-cell catalysts preferentially hydrolyzed (*R*)-enantiomers of (*R*,*S*)-mandelonitrile and (*R*,*S*)-2-phenylpropionitrile. NitNc also formed significant amounts of mandelamide from (*R*,*S*)-mandelonitrile (40% of total product), mainly found as the (*S*)-enantiomer. At pH 4.5 and 5.0, the cells retained more than 40 and 60% of their nitrilase activity at pH 7, respectively. Nitrilase variants generated by site-directed mutagenesis carried amino acid replacements in the vicinity of the catalytically active cysteine residues (C162 and C167 in NitAn and NitNc, respectively). The conversions of (*R*,*S*)-mandelonitrile and (*R*,*S*)-2-phenylpropionitrile by the nitrilase variants were compared to the wild-type nitrilases in terms of activity, enantioselectivity, and acid/amide ratio of the products formed. Thus the W168A variant of NitNc was identified, which formed significantly increased amounts of mandelamide and 2-phenylpropionamide, and which demonstrated an almost complete inversion of enantioselectivity for the conversion of (*R*,*S*)-2-phenylpropionitrile (from *R*- to *S*-selectivity).

1. Introduction

Arylacetonitrilases are studied as useful tools for manufacturing valuable pharmaceutical products or intermediates such as hydroxy and amino acids (for a review, see [1]). The nitrilase-catalyzed hydrolysis of nitriles often proceeds with a significant preference for a particular substrate enantiomer.

For instance, the enzymatic hydrolysis of (*R*,*S*)-mandelonitrile into (*R*)-mandelic acid (general resolving agent, intermediate of the semisynthetic antibiotic cefamandol, *etc.*) was demonstrated with bacterial nitrilases from *Alcaligenes faecalis* JM3 [2], *A. faecalis* ATCC 8750 [3], *Alcaligenes* sp. ECU0401 [4,5], *Pseudomonas putida* [6,7], *Microbacterium paraoxydans* or *Microbacterium liquefaciens* [6], which are characterized by their high selectivity for (*R*)-mandelonitrile. Variants of the *A. faecalis* JM3 enzyme were constructed, *e.g.* one that is highly active at pH 4.5, conditions suitable for the enantioretentive conversion of (*R*)-2-chloromandelonitrile [8]. A number of nitrilases selective for (*R*)-mandelonitrile were also found among recombinant strains expressing metagenomic nitrilase genes [9]. Another arylacetonitrilase from *Pseudomonas fluorescens* EBC191 [10] differed from the aforementioned enzymes in its lower enantioselectivity toward (*R*,*S*)-mandelonitrile [11]. Recently, new mandelonitrile-hydrolyzing nitrilases from *Bradyrhizobium japonicum* [12–14] and *Burkholderia xenovorans* [12] were obtained by rational genome mining. The nitrilase from *B. xenovorans* is not enantioselective for (*R*,*S*)-mandelonitrile [12]. Hence, the enzymes from *P. fluorescens* and *B. xenovorans* may also be used to prepare (*S*)-mandelic acid if the enantiomerically pure substrate is available [11,12], similarly as nitrilases from *B. japonicum* with no or moderate enantioselectivity [13,14].

The enzymes from both *A. faecalis* strains and from *P. putida* are identical in their amino acid sequences [8] and differ from the enzyme from *Alcaligenes* sp. in only 13 amino acid residues (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The enzymes from *B. japonicum*, *B. xenovorans* and *P. fluorescens* are only distantly related to the nitrilases from the *Alcaligenes* genus and *P. putida* (with amino acid identitites of about 50%; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

The strong tendency of the nitrilase from *P. fluorescens* to produce amides might be useful for their synthesis [11], especially if it is enhanced with appropriate mutations [15]. Nitrile hydratases are well-known catalysts of this reaction and have already found commercial use (for a review, see [16]). Nevertheless, they are often less stable than nitrilases and exhibit lower enantioselectivities. The use of nitrilases as nitrile hydratases is enabled by a less common cleavage of the reaction intermediate to yield free enzyme and amide instead of acyl enzyme and ammonia [11]. The amide-forming

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reaction needs to be suppressed during the manufacture of carboxylic acids, preferably by choosing a suitable enzyme. For instance, the *R*-selective nitrilases from the *Alcaligenes* genus [2–6] and *P. putida* [6,7] are suitable for producing (*R*)-mandelic acid that is not contaminated with mandelamide. Similarly, the non-selective nitrilase from *B. xenovorans*, which does not produce any mandelamide either, may be suitable for the production of (*S*)-mandelic acid from (*S*)-mandelonitrile [12].

The nitrilases from *Aspergillus niger* CBS 513.88 and *Neurospora crassa* OR74A (NitAn and NitNc, respectively) were the first fungal arylacetonitrilases to be expressed in *Escherichia coli* [17] and subsequently purified [18]. These enzymes are only distantly related to the aforementioned bacterial arylacetonitrilases, with approximately 40% amino acid identities (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). (*R*,*S*)-Mandelonitrile was found to be a good substrate for both fungal enzymes, while (*R*,*S*)-2-phenylpropionitrile was hydrolyzed by them at a lower rate [18]. The aim of this work is to compare the potential of these enzymes with known bacterial nitrilases in terms of their enantioselectivities and their amide-forming capacities in conversions of (*R*,*S*)-mandelonitrile and (*R*,*S*)-2-phenylpropionitrile. In addition, the potential activity of these enzymes with a bulky substrate (acetophenone cyanohydrin, which was transformed by the nitrilase from *P. fluorescens* [19]) was also examined.

In bacterial nitrilases, some amino acid residues in the immediate vicinity of the catalytically active cysteine residue were identified (especially at position 165 in the nitrilase from *P. fluorescens* EBC191 and at the corresponding 164 position in the nitrilase from *A. faecalis*), which were important for enzyme enantioselectivity and amide production [20]. This prompted us to study mutants of fungal nitrilases carrying modifications at the corresponding positions. In addition, the effects of some other mutations in the vicinity of the active center, which have not been studied in bacterial nitrilases, were examined with the fungal enzymes.

2. Experimental

2.1. Chemicals

The source of (*R*,*S*)-2-phenylpropionamide has been described previously [21]. (*R*,*S*)-2-Hydroxy-2-phenylpropionic acid (atrolactic acid) was purchased from abcr GmbH (Karlsruhe, Germany). Acetophenone cyanohydrin and (*R*,*S*)-2-hydroxy-2-phenylpropionamide (atrolactamide) were synthesized as described previously [19]. All other chemicals were of analytical grade and purchased from commercial suppliers.

2.2. Nitrilase expression

NitNc (GenBank: CAD70472) and NitAn (GenBank: XP_001397369) were expressed in recombinant *E. coli* JM109 [22] from synthetic genes whose codon frequency was optimized for expression in *E. coli* (GenBank: JN012232 and JN012230, respectively; synthesized by GeneArt, Regensburg, Germany). The nitrilase genes were cloned into the relevant *Nde*I and *Bam*HI sites of the expression vector pJOE2775 [23]. The recombinant strains were grown in LB broth with ampicillin (100 μg mL^{-1}) at 30 °C, nitrilase expression was induced by the addition of rhamnose (0.2%) and cells were harvested by centrifugation after a further 18 h of cultivation. The analysis of cellular proteins was performed by SDS–PAGE as described previously [24].

2.3. Construction of nitrilase mutants

Nitrilase mutants were prepared using a QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. For primer sequences, see Supplemental Table S1.

2.4. Nitrilase assay

Resting *E. coli* cells were resuspended in Tris/HCl buffer (50 mM, pH 7.0 or 7.5) or sodium citrate buffer (100 mM, pH 4.5 or 5.0) to give 0.24–0.27 and 1–2.4 mg of dry cell weight mL^{-1} for wild-type and mutant enzyme producers, respectively. After a 10-min preincubation at 30 °C, the reactions were started by adding 10 mM (*R*,*S*)-mandelonitrile or (*R*,*S*)-2-phenylpropionitrile (from 200 mM stock solutions in methanol). The reaction of 10 mM acetophenone cyanohydrin was carried out in the same way with 28 mg of dry cell weight mL^{-1} at pH 4.5. The reactions were terminated after various time periods by the addition of 1 M HCl (1/10 of sample volume). After removal of the precipitate by centrifugation, the concentration of substrates and reaction products in the supernatant were analyzed by HPLC (see below). The specific activities were calculated from the amount of mandelic acid formed after a 10-min reaction. The dry cell weight content in whole cell suspensions was calculated from optical density measurements (OD_{600} = 1 corresponding to approx. 0.3 mg of dry cell weight mL^{-1}).

2.5. Analytical HPLC

(*R*,*S*)-2-Phenylpropionitrile, (*R*,*S*)-mandelonitrile and their reaction products (acids, amides) were analyzed using a Lichrosphere RP8 column (Trentec Analysentechnik, 125 mm × 4 mm, 5 μm particles) and a solvent system consisting of CH_3OH (40%) and H_3PO_4 (0.3%) in water at 0.6 mL min^{-1}.

The separation of acetophenone cyanohydrin and its products was achieved by using a Lichrospher RP18 column (Trentec Analysentechnik, 250 mm × 4 mm, 5 μm particles) and a solvent system consisting of CH_3CN (40%) and H_3PO_4 (0.2%) in water at 0.7 mL min^{-1} [19].

The enantiomers of (*R*,*S*)-2-phenylpropionic acid, (*R*,*S*)-mandelic acid and the corresponding amides were analyzed as described previously [15].

3. Results and discussion

3.1. Effect of pH on nitrilase activity of recombinant E. coli cells

2-Hydroxynitriles are interesting substrates for arylacetonitrilases, but suffer from chemical instability at neutral pH-values. It was previously demonstrated that whole cells of *E. coli* JM109(pIK9) which expressed the nitrilase from *P. fluorescens* EBC 191 were able to convert mandelonitrile efficiently at pH 5.0 [26]. In order to compare the acid tolerance of the fungal nitrilases and their bacterial counterpart, resting cells of recombinant *E. coli* JM109 producing NitAn or NitNc were incubated at pH 4.5, pH 5.0 or pH 7.0 with (*R*,*S*)-mandelonitrile or (*R*,*S*)-2-phenylpropionitrile (10 mM each). Under these conditions, the whole cell catalysts synthesizing these fungal nitrilases exhibited the highest rates of mandelic acid formation at pH 7.0. The specific activities of the recombinant cultures (non-optimized) were typically in the range of 0.7–1.3 and 1–1.5 U mg^{-1} of dry cell weight in strains expressing NitAn and NitNc, respectively. The activities of both strains decreased only moderately when the pH was decreased from 5 to 4.5 (Table 1). In contrast, the cells of *E. coli* JM109 synthesizing the nitrilase from *P. fluorescens* at pH 4.5 exhibited only 25% of their activity at pH 5 [26].

Table 1
Effect of pH on the specific activities of resting cells of *Escherichia coli* JM109 expressing NitAn and NitNc.

pH	Relative activity (%)			
	(*R*,*S*)-2-Phenylpropionitrile[a]		(*R*,*S*)-Mandelonitrile[b]	
	NitAn	NitNc	NitAn	NitNc
4.5	41	47	42	51
5	59	73	61	68
7	100	100	100	100

NitAn = nitrilase from *A. niger*.
NitNc = nitrilase from *N. crassa*.
The enzyme activity was assayed as described in Section 2.

[a] The activities of NitAn and NitNc at pH 7 for (*R*,*S*)-2-phenylpropionitrile were taken as 100% (0.067 and 0.011 μmol 2-phenylpropionic acid min^{-1} mg^{-1} of dry cell weight, respectively).

[b] The activities of NitAn and NitNc at pH 7 for (*R*,*S*)-2-mandelonitrile were taken as 100% (0.79 and 1.04 μmol mandelic acid min^{-1} mg^{-1} of dry cell weight, respectively). The production of mandelamide by *N. crassa* nitrilase was not significant in the initial reaction stage, which was used for activity calculations.

3.2. Conversion of (R,S)-mandelonitrile and (R,S)-2-phenylpropionitrile by fungal nitrilases

The previously described bacterial nitrilases convert (*R*,*S*)-mandelonitrile to different amounts of mandelamide as the side product. This is exemplified by the nitrilases from *A. faecalis* and *P. fluorescens* EBC191 which formed <0.5 and 19% of mandelamide, respectively, from the total amount of (*R*,*S*)-mandelonitrile converted [24]. The recombinant cells synthesizing NitAn produced almost no mandelamide at pH 5 (Fig. 1a), while the *R*-acid was formed with a high enantiomeric excess (Fig. 1c).

In contrast, the end product of (*R*,*S*)-mandelonitrile transformation by NitNc at pH 5 consisted of 40% mandelamide (relative to the total amount of products formed). Surprisingly, it was observed that the production of mandelamide was delayed compared to the production of mandelic acid. The formation of significant amounts of mandelamide only occurred when about half of the racemic mandelonitrile had been converted (Fig. 1b).

With some caution, we compared the use of this enzyme on (*R*,*S*)-mandelonitrile with the conversion of enantiopure (*R*)- and (*S*)-mandelonitrile by the nitrilase from *P. fluorescens*, which converted these substrates into (*R*)-mandelic acid and (*S*)-mandelamide as the major products, respectively [11,26]. This indicated that the acid/amide ratio was not only dependent on the presence of electron-withdrawing or electron-donating groups in the substrate molecule and its bulkiness, but also on the stereochemistry of the molecule [11]. NitNc produced the same products from racemic mandelonitrile; this appeared to be due to the higher enantioselectivity of this enzyme compared to the nitrilase from *P. fluorescens*. Hence, during the initial stage of the reaction, the enzyme preferentially consumed (*R*)-mandelonitrile, converting it into (*R*)-mandelic acid with a high e.e. (91% at 30% conversion). The remaining enantioenriched (*S*)-mandelonitrile was then transformed into (*S*)-mandelamide (97% e.e. at 90% nitrile conversion).

The enzyme from *P. fluorescens* converted (*R*)- or (*S*)-mandelonitrile to end products consisting of the acid and amide in ratios of 93:7 and 54:46, respectively, at pH 5.0 [26]. If (*R*,*S*)-mandelonitrile was used under the same conditions, the ratios of *R*-acid/*R*-amide and *S*-acid/*S*-amide in the end product were the same or similar, that is 93:7 and 57:43, respectively [25]. NitNc produced almost no (*R*)-amide, while the ratio of *S*-acid/*S*-amide was lower (16:84) than in *P. fluorescens* (Fig. 1d).

Under conditions of rapid racemization (pH 7.0–7.5), both enzymes formed (*R*)-mandelic acid with an excellent e.e. (>99%), since the preferred (*R*)-enantiomer was available over the entire reaction time. NitNc also produced some mandelamide under these conditions but in lower amounts (15% of the total reaction product) and with a lower *S*-selectivity (e.e. 51%). Thus the variability of both nitrilases in terms of stereo- and chemoselectivity was more

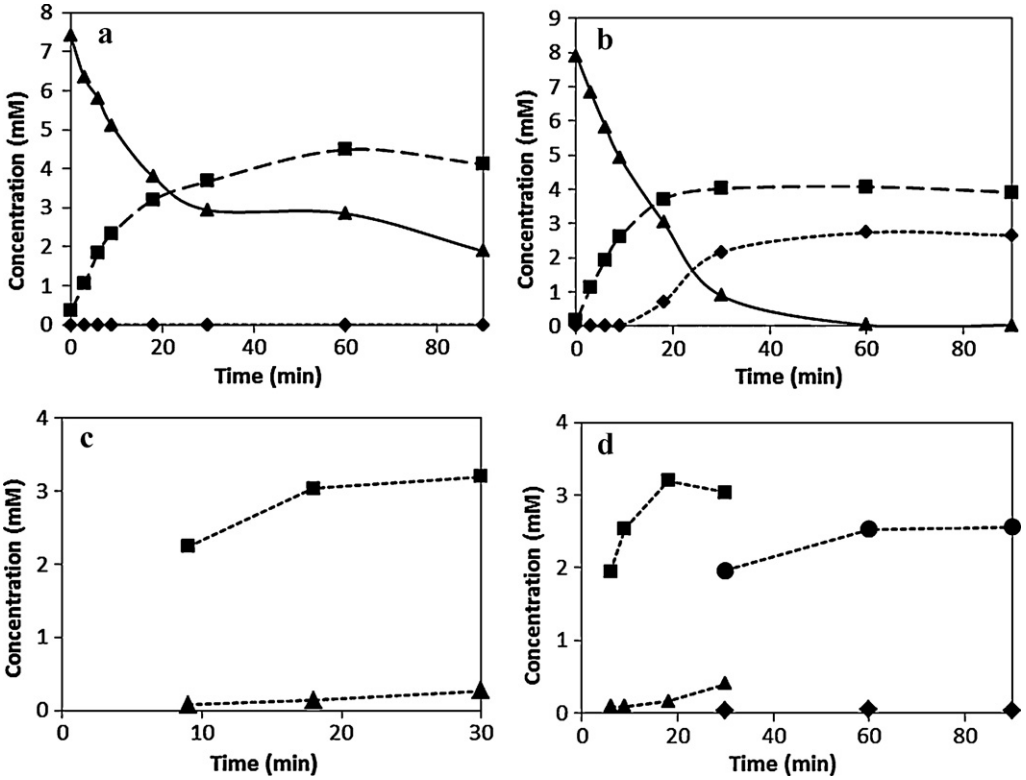

Fig. 1. Conversion of 10 mM (*R*,*S*)-mandelonitrile at pH 5.0 by *E. coli* JM109 cells expressing NitAn (a and c); OD_{600} = 0.9) and NitNc (b and d; OD_{600} = 0.8). (a and b): Mandelonitrile (▲), mandelic acid (■), and mandelamide (♦); (c and d): (*R*)-Mandelic acid (■), (*S*)-mandelic acid (▲), (*R*)-mandelamide (♦), and (*S*)-mandelamide (•).

Table 2
Comparison of NitAn, NitNc and their variants in terms of their (*R*,*S*)-mandelonitrile conversion at pH 5.

	NitAn	NitAn W163A	NitAn H165N	NitNc	NitNc W168A	NitNc H170N
Activity ($\mu mol_{MA}\ min^{-1}\ mg^{-1}_{dcw}$)	0.775	0.045	0.409	1.039	0.034	0.148
Activity ($\mu mol_{MAA}\ min^{-1}\ mg^{-1}_{dcw}$)	–	0.022	0.007	0.512	0.257	0.006
MAA (% in total product)	–	33	10	40	85	16
e.e. MA (%)/conversion (%)	93 (*R*)/31	18 (*R*)/36	94 (*R*)/40	91 (*R*)/28	78 (*R*)/25	94 (*R*)/28
e.e. MAA (%)/conversion (%)	–	99 (*S*)/36	70 (*S*)/40	97 (*S*)/90[a]	32 (*S*)/25	53 (*S*)/28

The enzyme activity was assayed as described in Section 2.
MA, mandelic acid; MAA, mandelamide; dcw, dry cell weight; –, not detected.
[a] Production of MAA is delayed compared to MA

Table 3
Comparison of NitAn, NitNc and their variants in terms of their (*R*,*S*)-2-phenylpropionitrile conversion at pH 7.5.

	NitAn	NitAn W163A	NitAn H165N	NitNc	NitNc W168A	NitNc H170N
Activity ($\mu mol_{PPA}\ min^{-1}\ mg^{-1}_{dcw}$)	0.050	0.006	0.016	0.011	0.010	0.004
Activity ($\mu mol_{PPAA}\ min^{-1}\ mg^{-1}_{dcw}$)	–	–	–	–	0.004	–
PPAA (% in total product)	–	–	–	–	31	–
e.e. PPA (%)/conversion (%)	90 (*R*)/28	63 (*S*)/30	94 (*R*)/33	35 (*R*)/35	98 (*S*)/35	18 (*R*)/26
e.e. PPAA (%)/conversion (%)	–	–	–	–	90 (*S*)/35	–

NitAn = nitrilase from *A. niger*
NitNc = nitrilase from *N. crassa*
The enzyme activity was assayed as described in Section 2.
PPA, 2-phenylpropionic acid; PPAA, 2-phenylpropionamide; dcw, dry cell weight; –, not detected.

significant in a slightly acidic than in a neutral medium. Therefore, a comparison of the wild-type nitrilases and their variants was also done at pH 5.0 (see Section 3.4).

The specific activities of NitAn and NitNc for (*R*,*S*)-2-phenylpropionitrile were one and two orders of magnitude lower, respectively, for (*R*,*S*)-2-phenylpropionitrile than for (*R*,*S*)-mandelonitrile (comp. Tables 2 and 3). Neither of the enzymes produced 2-phenylpropionamide from (*R*,*S*)-2-phenylpropionitrile. NitAn produced (*R*)-2-phenylpropionic acid with an e.e.-value of 90% at 28% conversion. In contrast, NitNc exhibited only a low degree of enantioselectivity toward this substrate (e.e. 35% for the (*R*)-acid at 35% conversion). Thus, in terms of their enantiopreference, both fungal nitrilases resembled the enzyme from the bacterium *A. faecalis*, which also demonstrated a high degree of enantioselectivity for the formation of (*R*)-mandelic acid (e.e. 96% at about 30% conversion) and an (*R*)-selectivity for the hydrolysis of (*R*,*S*)-2-phenylpropionitrile (e.e. 64% for the *R*-acid at about 30% conversion). In contrast, the enzyme from *P. fluorescens* converted (*R*,*S*)-mandelonitrile to (*R*)-mandelic acid with a lower degree of selectivity (e.e. 31% at about 30% conversion) and hydrolyzed (*R*,*S*)-2-phenylpropionitrile preferentially to (*S*)-2-phenylpropionic acid [20].

The specific activities of the purified highly enantioselective nitrilases from *P. putida* [27], *A. faecalis* strains JM3 [2] and ATCC 8750 [3], and *Alcaligenes* sp. ECU0401 [5] were 3.26, 12.4, 3.1 and 19.0 U mg^{-1} of protein, respectively, for (*R*,*S*)-mandelonitrile and those of purified NitNc and NitAn were also in this range with 6.1 and 7.7 U mg^{-1} of protein, respectively, for the same substrate (unpublished results). The specific activity of the non-selective nitrilase from *B. xenovorans* was the lowest (1.52 U mg^{-1} of protein) [12]. In contrast, the slightly enantioselective enzyme from *P. fluorescens* and a nitrilase from *B. japonicum* (with no enantioselectivity) exhibited the highest activities of 32.8 and 24.4 U mg^{-1} of protein, respectively [13,26]. On the other hand, K_m-values were the lowest in the nitrilases from *B. xenovorans* and *B. japonicum* (0.084 and 0.26 mM, respectively [12,13]), while those of the enantioselective nitrilases were one to three orders of magnitude higher (3.4, 5.75, 11.4, 13.39 and 21.8 mM in nitrilases from *N. crassa* [18], *A. faecalis* [3], *A. niger* [18], *P. putida* [27] and *Alcaligenes* sp. [5], respectively). However, the kinetic parameters must be compared with some caution, as differing enzyme assays (based on mandelic acid or ammonia determination) were used with different enzymes.

3.3. Conversion of acetophenone cyanohydrin by fungal nitrilases

The arylacetonitrilase from *P. fluorescens* EBC191, in addition to mandelonitrile and 2-phenylpropionitrile, also converts acetophenone cyanohydrin [19]. Therefore, it was tested whether the fungal arylacetonitrilases were also able to convert this sterically highly demanding substrate. It was found that acetophenone cyanohydrin is indeed a substrate for both fungal nitrilases, although only very low reaction rates were found (Fig. 2). NitNc gave a higher acid:amide ratio from this substrate (5:1 in the end product) than the nitrilase from *P. fluorescens* (3.4:1) [19], while NitAn did not produce any significant amount of the corresponding amide. Thus this enzyme may be promising for the production of atrolactate; the enantioselectivity of this reaction remains to be examined.

3.4. Site-directed mutagenesis of tryptophan residue directly adjacent to catalytically active cysteine residue in fungal nitrilases

Sequence alignments of several nitrilases demonstrated that the amino acid residue positioned next to the catalytically active cysteine residue (toward the C terminus) is a tryptophan residue in almost all bacterial, plant and fungal nitrilases [24,28–30]. In contrast, the nitrilases from *P. fluorescens* EBC191 [24], *Klebsiella ozaenae* [31], *B. xenovorans* and *B. japonicum* [12] carry an alanine residue at the corresponding positions.

In NitAn and NitNc, the tryptophan residues at the relevant positions (W163 and W168, respectively) were replaced with alanine residues. The enzyme variants were subsequently compared with the respective wild-type enzymes. The whole-cell catalysts synthesizing the nitrilase variants exhibited significantly lower specific activities for (*R*,*S*)-mandelonitrile than their parental enzymes (Table 2). The NitAn W163A variant (but not the NitNc W168A variant) also exhibited a decreased activity for (*R*,*S*)-2-phenylpropionitrile (Table 3). An examination of the amounts of wild-type and mutant enzymes by SDS–PAGE (not shown) suggested similar expression levels for all enzymes. Thus the differences in specific activities of the cells were probably not caused by differing expression levels, but by differing specific activities of

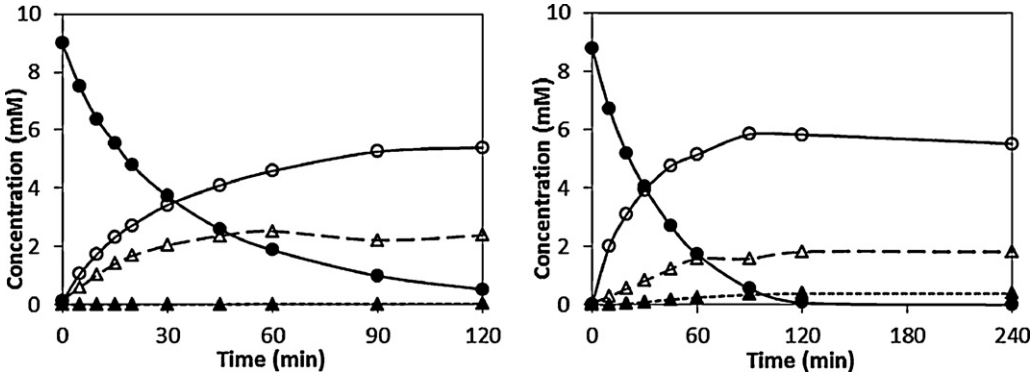

Fig. 2. Conversion of 10 mM acetophenone cyanohydrin at pH 4.5 by *E. coli* JM109 cells ($OD_{600\,nm}$ = 94) expressing NitAn (left) or NitNc (right). Acetophenone cyanohydrin (•), atrolactamide (▲), atrolactate (△), and acetophenone (○).

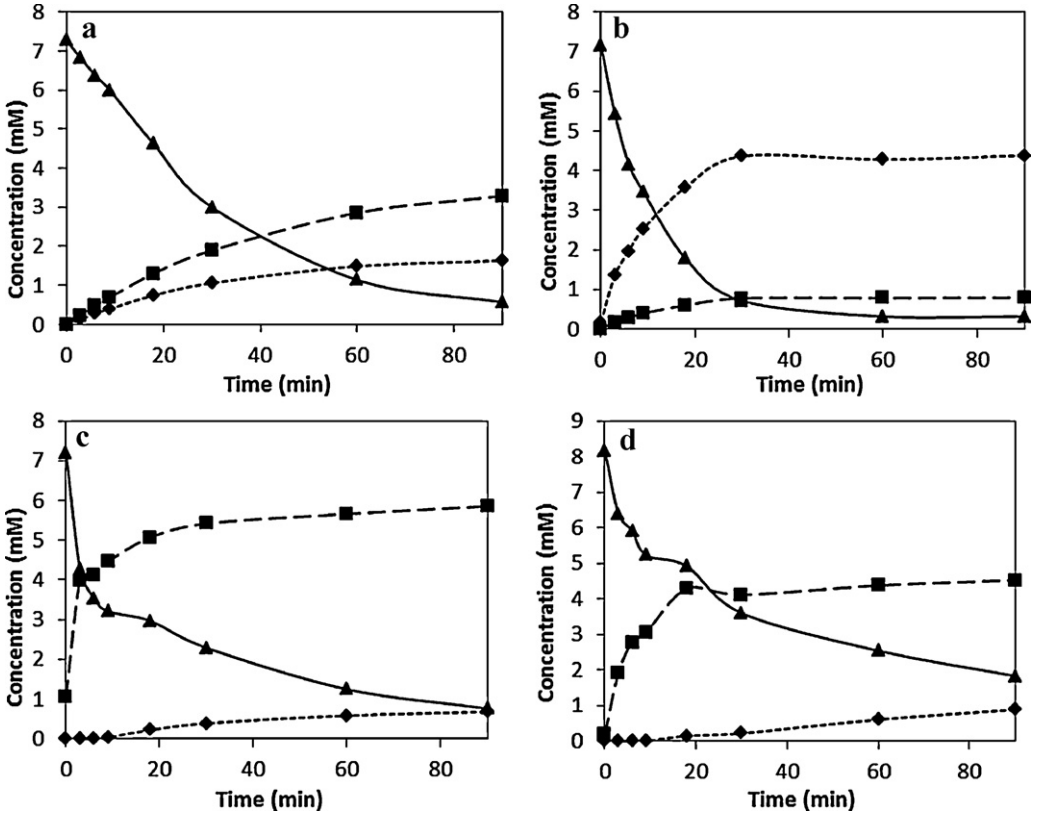

Fig. 3. Conversion of 10 mM (*R*,*S*)-mandelonitrile at pH 5.0 by *E. coli* JM109 cells expressing variants of NitAn (a and c) and NitNc (b and d). NitAn W163A ((a); OD_{600} = 5.8); NitNc W168A ((b); OD_{600} = 3.4); NitAn H165 N ((c); OD_{600} = 7.9); NitNc H170 N ((d) OD_{600} = 6.5). Mandelonitrile (▲), mandelic acid (■), and mandelamide (♦).

the enzymes. In the following experiments (Fig. 3), the amounts of cells producing enzyme variants were increased to obtain similar reaction rates to the wild-type enzymes (Fig. 1). A significant decrease in the specific activities of strains producing variants of the enzyme from *P. fluorescens* was also observed, while the amounts of recombinant enzymes produced were similar in all strains [20,24].

These replacements also caused significant changes in the acid/amide ratio of the reaction products (Table 2 and Fig. 3). The effect of this mutation was especially significant in NitNc variant W168A, which formed more than 85% mandelamide (compared to 40% with the parent enzyme). Furthermore in this experiment, the acid and the amide were formed simultaneously throughout the whole experiment and not in an ordered manner, as previously observed for the wild-type enzyme (Fig. 1b and d).

In both fungal nitrilases, the replacement of the tryptophan residues adjacent to the active cysteine with alanine residues exerted a negative effect on the (*R*)-selectivity observed during the hydrolysis of (*R*,*S*)-mandelonitrile (Table 2). Furthermore, the selectivity for (*S*)-mandelamide formation also decreased in the W168A variant of NitNc. In contrast, the W163A mutant of NitAn, whose wild type did not produce any mandelamide, exhibited a high (*S*)-selectivity for the formation of mandelamide, which accounted for 33% of the total product (Table 2).

Recently, the importance of the corresponding residue (A165) to catalytic performance (enantioselectivity, acid/amide ratio) was analyzed in the nitrilase from *P. fluorescens* EBC191. The enzyme variants with a bulky amino acid residue at this position (W, H, F, Y) produced less mandelamide than the wild-type enzyme or the variant with glycine at this position. The selectivity for (*R*)-mandelonitrile was higher with the former variants.

The NitNc W168A variant formed a higher percentage of mandelamide than any other tested nitrilase variant with a single amino

acid replacement [15,20]. These were variants of the *P. fluorescens* nitrilase with mutations in the amino acid residue directly adjacent to the catalytically active cysteine but toward the *N*-terminus (C163N and C163Q), which produced 43 and 51% mandelamide from (*R*,*S*)-mandelonitrile after complete conversion, respectively [15]. NitNc carried an asparagine-residue at this position (N166), and this may be one of the reasons for its pronounced tendency to synthesize large amounts of amides. In contrast NitAn, carrying an alanine at the respective position (A161), produced almost no amides. The corresponding enzyme variants of the nitrilase from *P. fluorescens* EBC191, which either carried an alanine or an asparagine residue at the respective position, also differed significantly in their degree of mandelamide formation (3 *vs.* 43%, [15]).

The W168A variant of NitNc produced (*S*)-2-phenylpropionic acid and (*S*)-2-phenylpropionamide (in a molar ratio of 3:1) with high e.e.-values (98 and 90%, respectively, at 35% substrate conversion; Table 3). Thus for the formation of 2-phenylpropionic acid, an inversion from *R*-selectivity to *S*-selectivity was observed, as in the corresponding variants of NitAn (W163A; Table 3) and the nitrilase from *A. faecalis* (W164A) [16]. The NitNc W168A variant was the only enzyme tested here to produce amide from 2-phenylpropionitrile (31% of the total product formed). This was more than was found for any of the *P. fluorescens* nitrilase variants previously described (the highest was 16.6% in the A165R variant, the others were below 5%; [20]).

3.5. Site-directed mutagenesis of the histidine residue in the highly conserved sequence motif CWEH in fungal nitrilases

The CWEH motif is conserved among most nitrilases. A slight modification of the sequence (a CWEN motif) is found at the corresponding positions in the wild-type nitrilases from *Arabidopsis thaliana*, in cyanide hydratases [28,29] and in *A. niger* K10 [30]. The AtNit1 and AtNit4 isoenzymes from *A. thaliana* [32,33] and nitrilase from *A. niger* [30] are well-known for the production of larger amounts of amides. Furthermore, formamide is the sole product of cyanide hydratases. Therefore, the H165N and H170N variants were constructed from NitAn and NitNc, respectively.

Surprisingly, an increase in amide formation was only observed for the H165N variant of NitAn (Table 2 and Fig. 3c). In contrast, the analogous variant (H170N) of NitNc produced less mandelamide than the parent enzyme (Table 2 and Fig. 3d).

4. Conclusions

The aim of this work was to examine the potential of two recently discovered fungal arylacetonitrilases for the production of mandelic, 2-phenylpropionic and atrolactic acid, and to compare the catalytic properties of these enzymes and their variants with those of bacterial nitrilases.

NitAn and NitNc resembled most of the bacterial nitrilases discovered previously [29], as far as their high selectivity for the formation of (*R*)-mandelic acid is concerned. In contrast to these bacterial enzymes, however, NitNc produced high amounts of (*S*)-mandelamide (40% of total product), which is more than the nitrilase from *P. fluorescens* EBC191 [11]. The formation of (*R*)-mandelic acid and (*S*)-mandelamide occurred largely consecutively. The amide-forming capacity was further enhanced in the W168A variant of this enzyme, the amide comprising 85% of its total product. However, the enantiomeric excess of both products decreased. 2-Phenylpropionitrile was transformed by fungal enzymes with a lower specific activity than mandelonitrile and with a moderate to high *R*-selectivity. Production of the corresponding amide (in *S*-configuration) was only achieved with the aforementioned NitNc variant. Both enzymes also transformed the bulky acetophenone cyanohydrin into atrolactate (NitAn) or a mixture of atrolactate and atrolactamide (NitNc).

New fungal nitrilases have significant potential in the synthesis of enantioenriched α-substituted carboxylic acids or their amides from racemic substrates. They may be also suitable for the stereoretentive hydrolysis or hydration of enantiopure cyanohydrins prepared with oxynitrilases, as they are highly active at pH 4.5.

Certain amino acid residues in the proximity of the active site have similar effects on enantioselectivity and amide production in distantly related arylacetonitrilases (fungal enzymes and the nitrilase from *P. fluorescens* or *A. faecalis* [20]). In principle, this knowledge enables the creation of nitrilases with the desired properties (enantioselectivity, amide production) and may also be utilized in predicting the catalytic properties of putative nitrilases, whose sequences are available from databases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2012.01.005.

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