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The cyanide hydratase enzyme of *Fusarium lateritium* also has nitrilase activity

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Abstract

The filamentous fungus *Fusarium lateritium* produces cyanide hydratase when grown in the presence of cyanide. The cyanide hydratase protein produced at a high level in *Escherichia coli* shows a low but significant nitrilase activity with acetonitrile, propionitrile and benzonitrile. The nitrilase activity is sufficient for growth of the recombinant strain on acetonitrile, propionitrile or benzonitrile as the sole source of nitrogen. The recombinant enzyme shows highest nitrilase activity with benzonitrile. Site-directed mutagenesis of the *F. lateritium* cyanide hydratase gene indicates that mutations leading to a loss of cyanide hydratase activity also lead to a loss of nitrilase activity. This suggests that the active site for cyanide hydratase and nitrilase activity in the protein is the same. This is the first evidence of cyanide hydratase having nitrilase activity.

Keywords: Cyanide hydratase; Nitrilase; Nitrile; Site-directed mutagenesis

1. Introduction

The gene encoding the enzyme cyanide hydratase has been isolated and sequenced from *Fusarium lateritium*, *Fusarium solani*, *Gloeocercospora sorghi* and *Leptosphaeria maculans* and in all cases the predicted protein sequences show strong homology to all available nitrilase sequences [1–4] and to cyanide dihydratase [5]. This is interesting as cyanide hydratase converts HCN to the amide product formamide with the addition of one water molecule while nitrilases hydrolyse RCN substrates to the corresponding carboxylic acid with the addition of two water molecules. The enzyme cyanide dihydratase, which converts HCN to formate and ammonia, has been characterised from *Pseudomonas stutzeri* AK61 [5] and is more closely related to nitrilases than to cyanide hydratase with approximately 60% amino acid identity to the nitrilases of *Comamonas testosteroni* and *Bacillus* sp. OxB-1 [6,7] but only 35% identity to cyanide hydratase sequences. The nitrilases from *Pseudomonas* [8], *Rhodococcus* ATCC39484 [9] and *Fusarium oxysporum* [10] have been shown to produce a small amount of amide product ($<6\%$ of total product) while the nitrilase of *Rhodococcus rhodochrous* J1 can use benzamide as a substrate albeit at a very low rate (0.0002% of benzonitrile rate) [11]. These findings suggest an amide intermediate in the reaction mechanism of nitrilase as proposed by Kobayashi et al. [11] and shown in Fig. 2. In this paper it is shown that the cyanide hydratase of *F. lateritium* has nitrilase activity and site-directed mutagenesis indicates that mutations leading to loss of cyanide hydratase activity also lead to loss of nitrilase activity in the enzyme.

2. Materials and methods

2.1. Bacterial growth

The *F. lateritium* cyanide hydratase gene was cloned in pDB711 in XL1-blue and expressed as described previously [12]. Growth on nitriles as a sole nitrogen source was carried out in M9 minimal salts medium at 37°C

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Table 1
Primers used for site-directed mutagenesis using the the QuikChange™ kit (Stratagene)

Mutant name	Mutation	Primer sequence
SDM1	T12Q	a. 5′-AG GCC GCC GCT GTC **CAA** TCC GAG CCT GGA TG-3′
		b. 5′-CA TCC AGG CTC GGA **TTG** GAC AGC GGC GGC CT-3′
SDM2	T12P	a. 5′-AG GCC GCC GCT GTC **CCA** TCC GAG CCT GGA TG-3′
		b. 5′-CA TCC AGG CTC GGA **TGG** GAC AGC GGC GGC CT-3′
SDM3	S13A	a. 5′-GCC GCT GTC ACC **GCC** GAG CCT GGA TG-3′
		b. 5′-CA TCC AGG CTC **GGC** GGT GAC AGC GGC-3′
SDM4	K136R	a. 5′-CC ACT CAT GTT GAG **AGG** CTC GTT TAC GGT GA-3′
		b. 5′-TC ACC GTA AAC GAG **CCT** CTC AAC ATG AGT GG-3′
SDM5	F170L	a. 5′-C ATG AAC CCC **TTA** CTC AAG TCT CTC-3′
		b. 5′-GAG AGA CTT GAG **TAA** GGG GTT CAT G
SDM6	D275E	a. 5′-G AAA GAC TTC **GAA** GGT CTT CTA TTT-3′
		b. 5′-AAA TAG AAG ACC **TTC** GAA GTC TTT C-3′
SDM7	V281A	a. 5′-GT CTT CTA TTT **GCT** GAC ATC GAT CT-3′
		b. 5′-AG ATC GAT GTC **AGC** AAA TAG AAG AC-3′
SDM8	M302S	a. 5′-TT GCT GGC CAC TAC **AGT** CGC CCT GAT CTC ATT-3′
		b. 5′-AAT GAG ATC AGG GCG **ACT** GTA GTG GCC AGC AA-3′

The positions changed are indicated in bold type.

with 2% glucose, nitrile at 0.5% (v/v) and 1 mM isopropyl thiogalactose (IPTG) for induction of enzyme activity.

2.2. Enzyme assays

Nitrilase activity was determined by the Nesslerisation method for ammonia determination as described previously [13]. Cyanide hydratase activity was determined using the formamide assay as described [1].

2.3. Molecular biological techniques

Standard DNA manipulation procedures were carried out as described in Sambrook et al. [14]. Site-directed mutagenesis was carried out using the QuikChange™ kit (Stratagene). The primers used are shown in Table 1. All mutants were sequenced to confirm the introduction of the correct mutation.

3. Results and discussion

3.1. Escherichia coli *expressing cyanide hydratase can grow on nitriles as nitrogen source*

The *F. lateritium* cyanide hydratase gene, *chy*, carried on the pKK233-2 derived plasmid pDB711, is expressed in *E. coli* XL1-blue at a very high level [12]. The maximum level seen in *E. coli* is five times higher than that seen in *F. lateritium*. Sequence analysis of a number of cyanide

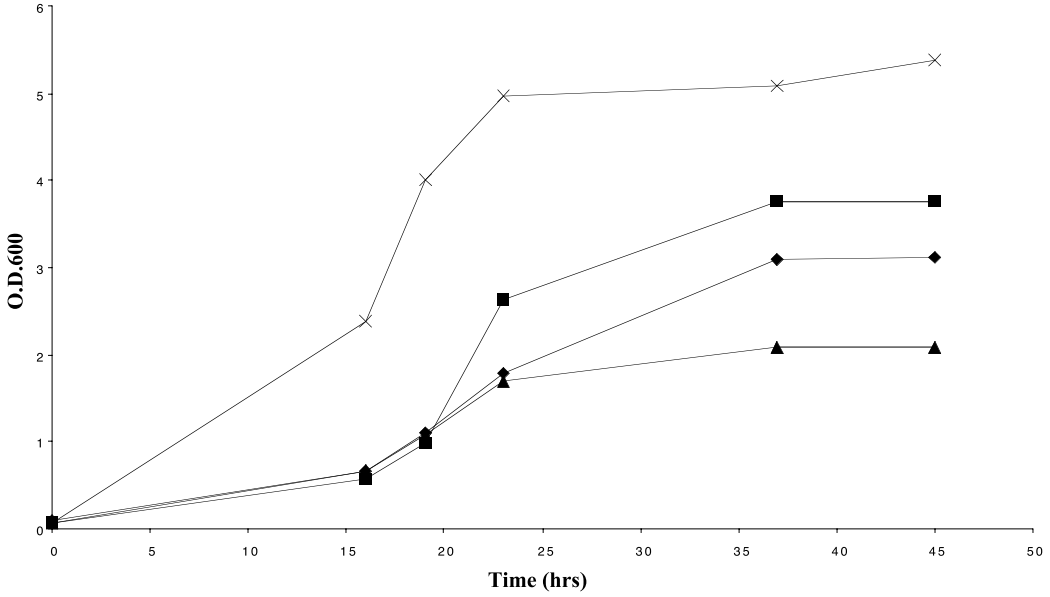

Fig. 1. Growth of *E. coli* XL1-blue/pDB711 expressing cyanide hydratase with NH_3Cl (×), acetonitrile (♦), propionitrile (■) and benzonitrile (▲) as sole nitrogen source. The untransformed *E. coli* strain showed no growth on nitriles as nitrogen source.

Table 2
Nitrilase activity of *F. lateritium* cyanide hydratase

Substrate	Activity	Relative activity (%)
Benzonitrile	0.059	0.033
Acetonitrile	0.024	0.013
Propionitrile	0.016	0.009
KCN	179.94	100

Nitrilase activity is given in μm NH_3 min^{-1} ml^{-1} per OD_{600} unit and cyanide hydratase activity in μm formamide min^{-1} ml^{-1} per OD_{600} unit. Relative nitrilase activity is given as % of cyanide hydratase activity.

hydratase proteins indicates strong homology to all the available nitrilase sequences. Previously it has been shown that the Cys163 residue of the *F. lateritium* cyanide hydratase, which is conserved in all the nitrilase related sequences, is essential for enzyme activity [12]. This residue is also essential for nitrilase activity.

In *F. lateritium* no nitrilase activity was detected under normal cyanide induced conditions but a low level of activity may not have been detected (P.D. Turner, unpublished). It was found that *E. coli* XL1-blue expressing cyanide hydratase from pDB711 is able to grow with acetonitrile, propionitrile or benzonitrile, but not with KCN, as the sole source of nitrogen (Fig. 1). Growth levels on acetonitrile and propionitrile are similar but less growth is found with benzonitrile. XL1-blue without cyanide hydratase activity does not grow with nitrile as the nitrogen source. No growth was detected on acetamide, propionamide or benzamide indicating that no amidase activity is present and that cyanide hydratase is acting as a nitrilase.

The nitrilase activity of XL1-blue expressing cyanide hydratase was determined, using the Nesslerisation method to determine the level of ammonia as described previously [13] with benzonitrile, propionitrile and acetonitrile as substrates. The results indicate that cyanide hydratase has low but significant nitrilase activity with benzonitrile the best substrate tested (Table 2). The mechanism proposed by Kobayashi et al. [11] as shown in Fig. 2 proposes a tetrahedral enzyme–hydroxylamine intermediate. In nitrilases this intermediate releases ammonia to yield an acyl–enzyme complex, which is then converted to the acid. This mechanism can also explain cyanide hydratase activity with the major route from the enzyme–hydroxylamine tetrahedral intermediate leading to the release of the amide when cyanide is the substrate. The minor nitrilase activity of cyanide hydratase is due to the enzyme–amide intermediate following the normal nitrilase route when nitriles are used as substrates.

3.2. Site-directed mutagenesis confirms nitrilase activity

Alignment of cyanide hydratase sequences with nitrilase and cyanide dihydratase sequences indicates that there are only 10 residues, which are highly conserved (i.e. at least in eight/nine sequences) in nitrilases and cyanide dihydratase but not in cyanide hydratase. An alignment of the relevant regions is shown in Fig. 3. Seven of these residues are conserved as a different residue in all the cyanide hydratase sequences available. To investigate the importance of these residues to cyanide hydratase activity site-directed mutagenesis has been used to change the cyanide hydratase sequence to that of the conserved nitrilase sequence at each of these seven positions as indicated in Fig. 3. The single mutants were then further mutated to generate various combinations of these changes as indicated in Table 3 with the ultimate aim of generating a mutant with all seven positions changed to the nitrilase sequence.

Analysis of the single mutants (SDM1–8) indicates that in cyanide hydratase F170 must form part of the active site as mutation of this nucleotide leads to normal protein level but no cyanide hydratase activity. This mutant has also lost nitrilase activity and cannot grow on nitriles as a nitrogen source. This confirms that cyanide hydratase has nitrilase activity and that the active site for the activities is

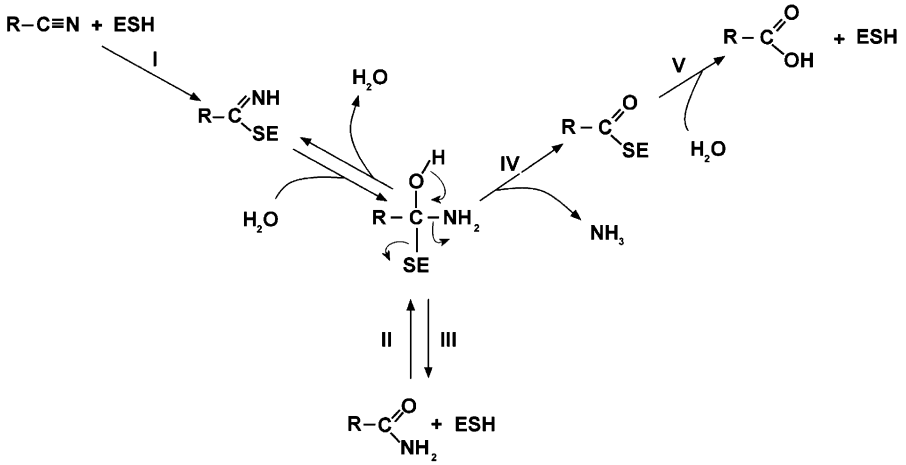

Fig. 2. Proposed mechanism for nitrilase/cyanide hydratase activity adapted from Kobayashi et al. [11]. With nitrilase route IV/V is the preferred route while with cyanide hydratase route III is the preferred route.

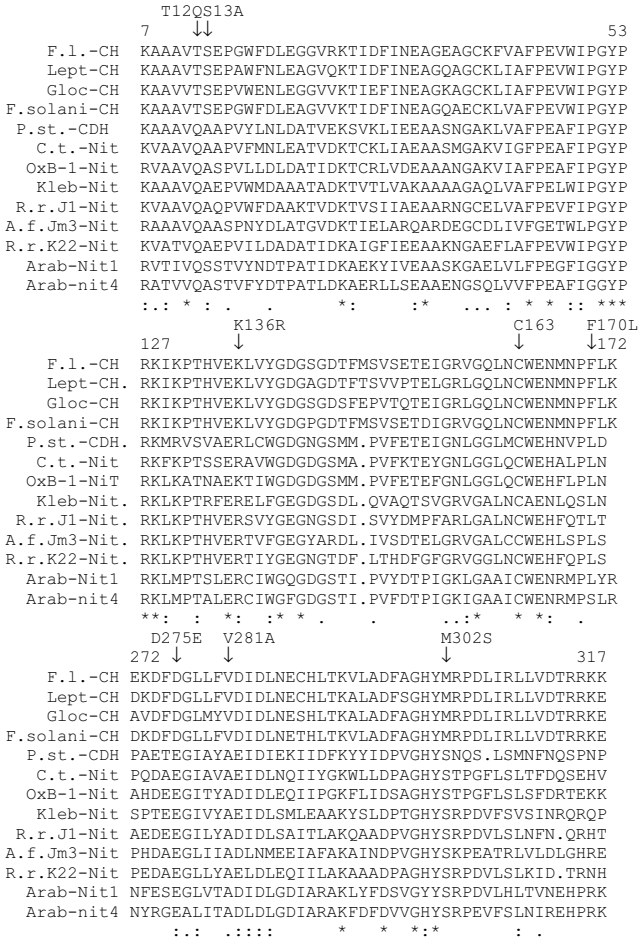

Fig. 3. Clustal W alignment of cyanide hydratase, cyanide dihydratase and nitrilase sequences. The sequences are: (1) cyanide hydratases: F.l.-CH, *F. lateritium* (P32963) [1], Lept-CH, *L. maculans* (Q9P8V3) [3], Gloc-CH, *G. sorghi* (P32964) [2], F.solani-CH, *F. solani* (Q96UG7) [4]; (2) cyanide dihydratase: P.st.-CDH, *P. stutzeri* AK61 (Q52445) [5]; and (3) nitrilases: C.t.-Nit, *C. testosteroni* (Q59329) [6], OxB-1-Nit, *Bacillus* sp. OxB-1 (P82605) [7], Kleb-Nit, *Klebsiella pneumoniae* subsp. *ozaenae* (P10045) [15], R.r. J1-Nit, *R. rhodochrous* J1 (Q03217) [16], A.f. JM3-Nit, *Alcaligenes faecalis* JM3 (P20960) [17], R.r. K22-Nit, *R. rhodochrous* K22 (Q02068) [18], Arab-Nit1, *Arabidopsis thaliana* Nit1 (P32961) [19] and Arab-Nit4, *A. thaliana* Nit4 (P46011) [20]. The residues that are conserved in nitrilases but conserved differently in cyanide hydratase are indicated with the changes made by site-directed mutagenesis. EMBL accession numbers are shown in brackets.

the same. Mutations at positions T12, S13 and D275 appear to affect protein folding as cyanide hydratase activity is very variable in these mutants from one culture to another although no dramatic effects on protein level were seen with these mutants, as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Interestingly, a mutant was generated by polymerase chain reaction (PCR) which has T12 mutated to proline (SDM2). This mutant has lost all activity and no cyanide hydratase protein is detectable by SDS–PAGE. This confirms the importance of T12 in protein folding and stability in cyanide hydratase and again confirms the nitrilase activity of cyanide hydratase. All the single mutations generated have some effect on cyanide hydratase activity confirming that all of these residues are important to activity.

All of the double mutants have less cyanide hydratase activity than the individual single mutants, although, except for SDM7.3 and SDM4.3, this is probably the additive effect of the individual mutations and does not reflect any interaction between the residues. Of the double mutants constructed, the double mutation at positions K136 and S13 (SDM4.3) has the most significant effect with cyanide hydratase activity reduced to 3.8% of normal and very little cyanide hydratase protein seen on SDS–PAGE. This mutant cannot grow on nitriles as a nitrogen source. The serine-13 residue appears to be particularly important in protein folding as all mutants containing mutations at this position show decreased cyanide hydratase protein stability. The S13A mutation when combined with either the V281A or K136R mutations (SDM7.3 and SDM4.3, Table 2) leads to a very significant decrease in protein stability.

The importance of S13 is further supported by the finding that a mutant with changes at residues K136, V281 and M302 (SDM7.4.8) shows normal protein levels as does the mutant with an additional change at T12 (SDM1.7.4.8) but a mutant with changes at K136, V281, M302 and S13 (SDM3.7.4.8) has very little if any cyanide hydratase protein and no cyanide hydratase or nitrilase activity. This mutant was further mutated at position

Table 3
Characteristics of site-directed mutants

Mutant	Mutation	% Protein level[a]	Relative activity (%)[b]
SDM1	T12Q	100	52–90
SDM2	T12P	0	0
SDM3	S13A	100	57–81
SDM4	K136R	100	48
SDM5	F170L	100	0
SDM6	D275E	100	71–100
SDM7	V281A	100	92
SDM8	M302S	100	78
SDM7.4		100	45
SDM7.8		100	65
SDM7.3		40–60	28
SDM4.8		100	33
SDM4.3		20–40	3.8
SDM3.8		100	59
SDM7.4.8		100	30
SDM1.7.4.8		100	30
SDM3.7.4.8		0–20	0
SDM3.1.7.4.8		40–60	11
SDM6.3.1.7.4.8		80–100	22
SDM5.6.3.1.7.4.8		60–80	0

Mutants with multiple mutations are named according to the combination of mutations.

[a]Protein level is relative to normal levels seen in XL1-blue/pDB711 under IPTG induced conditions as judged by SDS–PAGE.

[b]Cyanide hydratase activity was measured as μm formamide min^{-1} ml^{-1} per OD_{600} unit and is expressed relative to expression from pDB711 in XL1-blue.

T12 (SDM1.3.7.4.8) and position D275 (SDM6.1.3.7.4.8) leading to restoration of some cyanide hydratase and nitrilase activity (11 and 22% respectively) and protein level is near normal in SDM6.1.3.7.4.8. The final addition of the change at F170 generates a protein sequence with all the changes at the target residues (SDM5.6.1.3.7.4.8) and leads to a mutant with reasonable protein levels but no cyanide hydratase or nitrilase activity. This again confirms the importance of the F170 residue for cyanide hydratase and nitrilase activity in the *F. lateritium* cyanide hydratase protein.

This work has shown that cyanide hydratase has a small but significant level of nitrilase activity. The active sites for cyanide hydratase and nitrilase activity appear to be same as any mutation leading to loss of cyanide hydratase activity leads to loss of nitrilase activity. The change of the phenylalanine residue at position 170 in *F. lateritium* to the nitrilase conserved amino acid, leucine, leads to complete loss of activity but to normal protein levels. This indicates that this residue forms part of the active site of cyanide hydratase and suggests a critical interaction between this residue and other cyanide hydratase specific conserved residues. The importance of the leucine residue at this position in nitrilases has not been analysed but may not be as essential as in one nitrilase, the plant nitrilase AtNIT4, this residue is not conserved [20].

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