Expression of the cyanide hydratase enzyme from Fusarium lateritium in Escherichia coli and identification of an essential cysteine residue

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Abstract

The filamentous fungus Fusarium lateritium is cyanide tolerant, due partly to the induction of the enzyme cyanide hydratase in the presence of cyanide. This enzyme catalyses the hydration of cyanide to formamide. The expression in Escherichia coli of a cDNA clone encoding cyanide hydratase is described. The cDNA cloned was expressed as a transcriptional fusion in the expression vector pKK233-2 and a high level of activity of cyanide hydratase was detected in E. coli. Site-directed mutagenesis of the cys-163 residue inactivated the enzyme.

Keywords: Cyanide hydratase; Nitrilase; Site-directed mutagenesis; Fusarium lateritium

1. Introduction

The enzyme formamide hydrolyase (EC 4.2.1.66), which is more commonly known as cyanide hydratase, is produced in Fusarium lateritium on exposure of the fungus to cyanide [1]. In maximally induced cells, cyanide hydratase represents approximately 25% of the soluble protein. The gene encoding cyanide hydratase in Fusarium lateritium has been cloned as both genomic and cDNA clones [1]. The predicted protein sequence of cyanide hydratase has strong identity (max. 34%) to nitrilases, which convert organic cyanide compounds to the corresponding carboxylic acids, but shows no identity with the more functionally related nitrile hydratases which hydrolyse organic cyanide compounds to the corresponding amides [1]. Here the expression of cyanide hydratase from Fusarium lateritium in E. coli is described. The cysteine residue, which has been shown to be essential for the activity of a number of nitrilases [2,3], is also essential for cyanide hydratase activity.

2. Materials and methods

The Fusarium lateritium strain used was as described previously [1]. The E. coli strain M15 (pREP4) obtained from Qiagen was used in the
expression studies. The expression vector pKK233-2 [5] was obtained from Pharmacia. The plasmid pMC311, containing the complete cyanide hydratase ORF, was described previously [1]. Standard methods for routine analysis and subcloning of DNA were used as described in Sambrook et al. [4]. Site-directed mutagenesis was carried out using the Promega ‘Altered sites’ mutagenesis system. The EcoRI insert of pMC311 was subcloned into the pSelect I vector (Promega) and mutagenesis was carried out according to the manufacturer’s instructions. The oligonucleotides used for mutagenesis were (i) 5’-GATAGCCATGGTGAAGGTTG-3’ for the generation of the NcoI site at the start of the cyanide hydratase ORF and (ii) 5’-CATGTCTCCAGGCCGTTGAGCTGACC-3’ for the conversion of cys163 to alanine. The mutated genes were then subcloned into pKK233-2 [5] for expression analysis. Methods for the determination of cyanide hydratase enzyme activity and for the analysis of crude cell extracts of E. coli by SDS-PAGE and Western blotting were as described previously [1].

3. Results and discussion

The cDNA clone pMC311 [1] contains the complete cyanide hydratase ORF together with 35 bp of 5’ untranslated sequence and 66 bp of 3’ untranslated sequence. To express cyanide hydratase in its native form in E. coli the sequence surrounding the chy1 translation initiation ATG was converted to an NcoI site by site-directed mutagenesis of the sequence 5’-TCATGG-3’ to 5’-CCATGG-3’. The complete ORF was then cloned as an NcoI–NcoI–HindIII fragment into the expression vector pKK233-2 [5]. Cloning into the NcoI site of pKK233-2 generates a transcriptional fusion of the chy1 gene to the strong trc promoter and positions the ORF at the correct distance from the ribosome binding site in this plasmid. The pKK233-2 derivative containing the chy1 ORF is called pDB711. Another product of the cloning experiment was pDB712 in which the NcoI fragment containing the 5’ region of the gene has inserted in the incorrect orientation for expression.

3.1. Expression of cyanide hydratase in E. coli

The trc promoter of pKK233-2 is inducible with IPTG. Crude cell extracts of E. coli strain M15(pRep4) and M15(pRep4) containing pDB711 or pKK233-2 were analysed by Western blotting for the production of the cyanide hydratase protein (Fig. 1). It can be seen that cells harbouring pDB711 produce significant quantities of protein which is of the same molecular mass and showing the same immunoreaction as the cyanide hydratase enzyme isolated from Fusarium lateritium itself. The control cells do not produce an immunoreactive protein of 43 kDa. Both IPTG induced and uninduced cultures of M15(pRep4)/pDB711 appear to produce the cyanide hydratase protein. This suggests that even in this strain which is overproducing the lac Iq gene on the plasmid.

![Fig. 1. The left hand panel shows an SDS-PAGE gel of crude cell extracts of E. coli strain M15(pRep4) containing in lanes: 1, no plasmid; 2, pKK233-2; 3, pDB711 uninduced; 4, pDB711 induced; 5, pDB811 uninduced; 6, pDB811 induced. The right panel shows a Western blot with anti-cyanide hydratase antiserum of the same samples in lanes 2–7 and molecular mass standards in lane 1.](image-url)
pREP4 [6], the amount of repressor is still insufficient to fully repress expression from the trc promoter. It has also been observed that derepression of strong IPTG inducible promoters such as trc can take place in LB medium due to the presence of residual amounts of inducer in the medium [7].

The native cyanide hydratase isolated from Fusarium lateritium appears to aggregate [1] with the largest active species having a molecular mass of 1217 kDa and the minimum active form being a heptamer. Native PAGE analysis of the protein produced in E. coli indicates that the protein aggregates in an identical manner (data not shown).

### 3.2. Cyanide hydratase activity in E. coli

The activity of cyanide hydratase in E. coli was tested over a 4-h period following induction with IPTG, of exponentially growing cells. Cyanide hydratase in uninduced culture reached a maximum activity during this period of 330 μmol min⁻¹ ml⁻¹ while in the induced culture the maximum activity seen at the same time was 800 μmol min⁻¹ ml⁻¹ (representing a specific activity of 571 μmol min⁻¹ (mg dry weight)⁻¹). The level of activity seen in the induced culture is approximately 5.5 times greater than the maximum level seen in Fusarium cultures [1]. This somewhat extraordinary result can be explained in a number of ways. Firstly it may be the result of diffusional limitation in F. lateritium. The effect of densely packing enzyme molecules in large cells could be to prevent access of substrate to enzyme molecules in the cell interior as conversion to product would occur en route. Reducing the cell diameter from 10 μm to 1 μm might allow access of HCN to enzyme molecules that would otherwise not be exposed to substrate, thereby increasing activity. Secondly it may be that the enzyme is more stable in E. coli. This is supported by the observation that in E. coli, unlike F. lateritium, crude cell extracts did not show significant breakdown (as judged by native PAGE) of the very high molecular mass material typical of cyanide hydratase. Such instability might result in underestimation of the specific activity of purified enzyme and explain why the specific activity in E. coli is approximately half that previously seen for purified enzyme.

### 3.3. Identification of an essential cysteine residue

As previously discussed, the protein sequence of cyanide hydratase is remarkably similar to the sequences of a number of nitrilases. It has been shown for the nitrilases from Alcaligenes faecalis JM3 and Rhodococcus rhodochrous J1 that the cysteines at positions 163 and 165 respectively are required for enzyme activity [2,3]. This cysteine residue, which is at position 163 in cyanide hydratase, is conserved in all the nitrilases sequenced to date and in the two cyanide hydratases for which sequence is available. The importance of Cys-163 to the activity of the Fusarium lateritium cyanide hydratase was tested using site-directed mutagenesis to mutate the Cys-163 codon to an Ala codon, a change which should minimise conformational changes caused by the substitution. The change from cysteine to alanine required a two base change as indicated below.

574- GGT CAG CTC AAC TGC TGG GAG AAC ATG -600
154- gly gln leu asn cys trp glu asn met -167

574- GGT CAG CTC AAC GCC TGG GAG AAC ATG -600
154- gly gln leu asn ala trp glu asn met -167

The mutated cyanide hydratase gene was expressed in E. coli as a transcriptional fusion in pKK233-2 in precisely the same manner as the wild-type gene. The plasmid containing the mutated gene was designated pDB811. Expression of the cyanide hydratase protein in E. coli strain M15(rep 4) containing pDB811 was assessed by Western blotting and the results are shown in Fig. 1 (lanes 6 and 7). As with the wild-type gene, both IPTG induced and uninduced cells are producing immunoreactive material of the same molecular mass as cyanide hydratase. Cyanide hydratase enzyme activity in cells containing pDB811 was tested over a 4 h period following IPTG induction of exponentially growing cells. No enzyme activity was detected, indicating that as in nitrilases, a cysteine residue is essential for activity in cyanide hydratase. A number of models of nitrilase action have been proposed [8–10] and all of these propose nucleophilic attack by an SH (or possibly an OH group [10]) of the.
enzyme on the CN group of the nitrile as the initial step in the reaction. Nitrilases are defined as enzymes which convert nitriles to the corresponding carboxylic acid and ammonia with no amide intermediate (reviewed in [11]). However, a mechanism based on a thiol linked imine intermediate could in principle yield either the amide or the acid as product. The ratio between amide and acid was shown in practice to be pH dependent in the mercaptoethanol catalysed hydrolysis of N-benzyl-3-cyanopyridinium bromide [8]. Furthermore, ricinine nitrilase was observed to produce both acid and amide on a 91:9 ratio [10]. It may be postulated that cyanide hydratase exhibits the same chemical mechanism but possesses an active site that favours release of amide and not acid. This offers the possibility of exerting control over the substrate specificity of this biotechnologically important class of enzymes.

References