PURIFICATION OF CYANIDE-DEGRADING NITRILASE FROM *Pseudomonas fluorescens* NCIMB 11764

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Thesis Prepared for the Degree of

MASTER OF SCIENCE

UNIVERSITY OF NORTH TEXAS

December 2010

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Chou, Chia-Ni. Purification of cyanide-degrading nitrilase from *Pseudomonas fluorescens* NCIMB 11764. Master of Science (Molecular Biology), December 2010, 44 pp., 5 tables, 7 figures, references, 44 titles.

Cyanide is a well known toxicant that arises in the environment from both biological and industrial sources. Bacteria have evolved novel coping mechanisms for cyanide and function as principal agents in the biosphere for cyanide recycling. Some bacteria exhibit the unusual ability of growing on cyanide as the sole nitrogen source. One such organism is *Pseudomonas fluorescens* NCIMB 11764 (Pf11764) which employs a novel oxidative mechanism for detoxifying and assimilating cyanide. A unique complex of enzymes referred to as cyanide oxygenase (CNO) is responsible for this ability converting cyanide to ammonia which is then assimilated. Because one component of the four member CNO complex was previously shown to act on cyanide independent of the other members, its characterization was sought as a means of gaining a better understanding of the overall catalytic mechanism of the complex. Preliminary studies suggested that the enzyme belonged to a subset of nitrilase enzymes known as cyanide dihydratases (CynD), however, a *cynD*-like gene in Pf11764 could not be detected by PCR. Instead, a separate nitrilase (Nit) linked to cyanide metabolism was detected. The corresponding *nit* gene was shown to be one of a conserved set of *nit* genes traced to a unique cluster in bacteria known as Nit1C. To determine whether the previously described CynD enzyme was instead Nit, efforts were undertaken to isolate the enzyme. This was pursued by cloning and expressing the

recombinant enzyme and by attempting to isolate the native enzyme. This thesis is concerned with the latter activity and describes the purification of a Nit-like cyanide-degrading nitrilase (NitCC) from Pf11764 to ~95% homogeneity. Purification was greatly facilitated by the discovery that fumaronitrile, as opposed to cyanide, was the preferred substrate for the enzyme (20 versus 1 U/mg protein, respectively). While cyanide was less effective as a substrate, the specificity for cyanide far outweighed that (10,000 fold) of the recombinant enzyme (NitPG) implying that the native NitCC protein purified in this work is different from that of the cloned recombinant. Further evidence of this was provided by molecular studies indicating that the two proteins differ in mass (34.5 and 38 kDa, respectively) and amino acid sequence. In summary, two different Nit enzymes are encoded by Pf11764. While the two share greater than 50% amino acid sequence identity, the results suggest that the native NitCC enzyme purified in this work functions better as a cyanide-degrading nitrilase and is one of four enzyme components comprising CNO required for Pf11764 cyanide assimilation.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my professor, Dr. Daniel Kunz, whose expertise, understanding, and patience, added considerably to my graduate experience. I am grateful for the opportunity to work in his laboratory and his encouragement, guidance, and vast help during the development of this study.

I would like to thank Dr. Barney Venables for generously investing his time and his assistance in mass spectrometry analysis. I would also like to thank my final committee member, Dr. Rebecca Dickstein, for helpful discussions and review of the manuscript. I owe a special thanks to follow students Pallab Ghosh and Trevor Burton for their advice, help, and friendship.

Finally, I thank my family and friends for the support and understanding they have provided me throughout my entire life.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

CNO	Cyanide oxygenase
CynD	Cyanide dihydratase
FMN	Fumaronitrile
h	Hour
KCN	Potassium cyanide
kDa	Kilodalton
min	Minute
MS	Mass spectrometry
Na-K	Sodium potassium buffer
Nit	Nitrilase
PAGE	Poly acrylamide gel electrophoresis
Pf	*Pseudomonas fluorescens*
SDS	Sodium dodecyl sulfate

CHAPTER I

INTRODUCTION

Cyanide is a potent biological inhibitor and toxic poison. It inhibits cytochrome oxidases and other metallo enzymes thus preventing respiration and other vital biological functions (Solomonson, 1981; Knowles, 1988). Cyanide arises in the environment from both biological and abiological sources. Abiological sources include wastes generated in the electroplating, coal processing and mining industries. It's occurrence in industrial fire smoke, automobile exhaust, and even cigarette smoke has also been reported (Homan, 1988; Agency for Toxic Substances and Disease Registry, 1993). Cyanide is also produced biologically as a natural product of metabolism by so-called cyanogenic organisms. Cyanogenic species include various plants, arthropods, bacteria and fungi (Conn, 1980; Castric, 1981; Knowles and Bunch, 1986; Poulton, 1988; Blumer and Haas, 2000; Zagrobelny, 2008). The classic cyanide poisoning from over consumption of various plant materials in the human diet (e.g., cassava roots, lima beans, and almonds) is well documented (Kojima et al., 1983; Frehner et al., 1990; Dicenta et al., 2002).

Chemically, cyanide exists either in its free form (CN^-/HCN) or complexed to various acceptors. It is the free form of cyanide that is most toxic because of its tendency to add as a nucleophile to various acceptor molecules. The most notorious of these are metal ions such as iron present as a prosthetic group in cytochrome oxidases. At pH 7.0 the free form exists predominantly as the protonated species (HCN), which is a weak acid (pKa 9.3). HCN is also quite volatile and boils at room

temperature. In its complexed form cyanide is generally much less toxic because the free form is unavailable. Cyanide readily forms complexes with various metals, particularly of the transition series, including Fe, Ni and Zn. It also adds readily to various organic acceptors, including for example, α-keto acids (Kunz et al., 1998).

The presence of cyanide in nature implies that their likely exist mechanisms that organisms have evolved to survive in its presence. It is well known, for example, that bacteria and plants adapt to cyanide by making alternative cytochrome oxidases that are less sensitive to inhibition (Bendall and Bonner, 1971; Grant and Hommersand, 1974; Rhoads, 1998). This permits respiration to occur at concentrations that otherwise would be inhibitory. Another way to overcome the toxic effects of cyanide is to modify it in some way chemically. A number of enzymes with this ability are known. In animals, for example, cyanide is detoxified by the enzyme rhodanese (thiosulfate cyanide sulfurtransferase, EC 2.8.1.1) (equation 1), which catalyzes its conversion to less toxic thiocyanate (equation 1)(Sorbo, 1953; Silver and Kelly, 1976; Ryan and Tilton, 1977). In contrast, the enzyme β-cyanoalanine synthetase is believed to be

$$S_2O_3^{2-} + HCN \xrightarrow{\text{Rhodanese}} \underset{\text{Thiocyanate}}{HSCN} + SO_3^{2-} \qquad (1)$$

the principal enzyme involved in cyanide detoxification in plants (Castric, 1981; Jenrich et al., 2007). It catalyzes the condensation of cyanide with cysteine as shown in equation 2.

$$HCN + HSCH_2-\underset{NH_2}{\overset{COOH}{\underset{|}{\overset{|}{C}}}}-H \xrightarrow{\beta\text{-Cyanoalanine Synthetase}} COOH-\underset{H}{\overset{NH_2}{\underset{|}{\overset{|}{C}}}}-CH_2CN + SO_3^{2-} \qquad (2)$$

Fungi detoxify cyanide mainly by way of cyanide hydratase yielding formamide as a less toxic product (equation 3). It is unclear what role, if any, cyanide detoxifying

$$HCN + H_2O \xrightarrow{\text{Cyanide hydratase}} \underset{\text{Formamide}}{HCONH_2} \quad (3)$$

enzymes play in bacteria since mutants lacking related enzymes have not been described. An additional cyanide-modifiying enzyme described thus far only in bacteria is cyanide dihydratase (CynD) (also referred to as cyanide nitrilase or cyanidase). This enzyme is unique in that it catalyzes the one step hydrolysis of cyanide to less toxic formate and ammonia (equation 4). Although a physiological role in cyanide detoxification for this enzyme has been suggested, the rather high

$$HCN + H_2O \xrightarrow{\text{Cyanide dihydratase (\textbf{CynD})}} \underset{\text{Formic acid}}{HCO_2H} + NH_3 \quad (4)$$

concentrations of cyanide required (Km values ranging from ~2-7 mM have been reported) (Raybuck, 1992; Jandhyala et al., 2005) make it difficult to rationalize a primary role for this enzyme as one of detoxification. This is particularly so when one considers that most bacteria will not grow above cyanide concentrations of ~0.5 mM (Silva-Avalos et. al, 1990). Both cyanide hydratase and CynD belong to the large superfamily of enzymes known as nitrilases (E. C. 3.5.5.3) a characteristic feature of which is a conserved glutamate-lysine-cysteine catalytic triad (Pace and Brenner, 2001; O'Reilly and Turner, 2003; Martinkova and Kren, 2010). An additional feature is a tendency to form large oligomeric structures from component monomers averaging ~40 kDa in mass (Thuku et al., 2008). In our laboratory (Kunz et al., 2001, Fernandez et. al.,2004a, Fernandez and Kunz, 2005; Dolghih, 2004) it has been

shown that certain bacteria make a novel enzyme that detoxifies cyanide at concentrations as low as $\leq$ 5μM. This is far below the concentration reported for any other known cyanide detoxifying enzyme. The enzyme responsible has been described as cyanide oxygenase (CNO) because molecular oxygen is also required as a substrate. This mechanism is novel in biochemistry because nitriles, of which cyanide is the simplest example, are generally metabolized hydrolytically not oxygenatively.

The biochemical properties of CNO have thus far been described in one organism only namely, *Pseudomonas fluorescens* NCIMB 11764 (Pf11764). Pf11764 was originally isolated for its ability to use cyanide as the sole nitrogen source in Great Britain (Harris and Knowles, 1983a). The responsible enzyme was first proposed to be an oxygenase because the degradation of cyanide by Pf11764 cell-extracts was linked to the consumption of molecular oxygen and NADH (Harris and Knowles (1983b). A body of studies in our laboratory, including those demonstrating that isotopic oxygen-18 was incorporated when cyanide was degraded (Wang et al., 1996), confirmed the original proposal that an oxygenase-mediated mechanism was involved. Further work by Dr. Kunz and students which showed that mutants that failed to make CNO could no longer grow (Kunz et al., 1994; Fernandez et al., 2001) provided strong evidence that CNO was needed for growth on cyanide. Additional studies aimed at characterizing the CNO enzyme have shown it to be fairly complex. For example, rather than a single enzyme, studies have shown that CNO is comprised of several enzymes identified as: NADH oxidase (Nox), NADH peroxidase (Npx), an enzyme with properties similar to those of CynD enzymes, and carbonic anhydrase (CA)

(Fernandez and Kunz, 2005). A diagram depicting the four as components of CNO is illustrated in Fig 1. Evidence that all four are needed for enzyme activity comes from studies which showed that if any one of the four was not included in reactions no activity was observed (Fernandez and Kunz,2005). Ironically, CNO generates the same reaction products as those formed by CynD enzymes namely, formate and ammonia (equation 4), however, instead of using water to accomplish substrate cleavage, CNO utilizes molecular oxygen and NADH (equation 5). The ammonia

$$HCN + O_2 + 2NADH + H^+ \xrightarrow{\textbf{CNO}} HCO_2H + NH_3 + 2NAD^+ \quad (5)$$

$$HCO_2H + NAD^+ \xrightarrow{\textbf{FDH}} CO_2 + NAD^+ \quad (6)$$

thus formed is assimilated and helps to explain how cells acquire nutritional nitrogen from cyanide. The formate is further oxidized to carbon dioxide by an enzyme separate from CNO previously identified as formate dehydrogenase (FDH) (equation 6.)(Kunz et al, 2004). Together, the CNO and FDH enzymes mediate the complete oxidation of cyanide to carbon dioxide and ammonia recycling one NADH equivalent in the process.

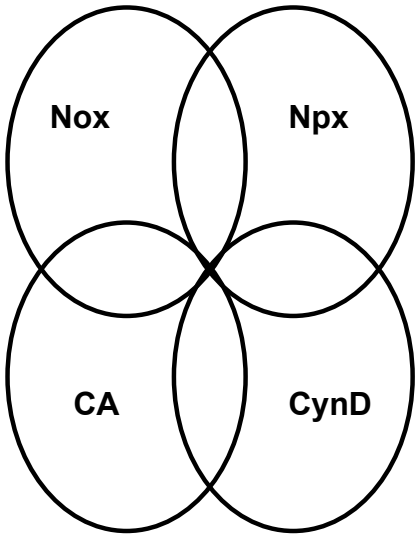


FIG 1. Hypothetical model of interacting proteins identified in previous studies as being required for cyanide oxygenase (CNO) enzyme activity form *Pseudomonas fluorescens* NCIMB 11764. Nox, NADH oxidase; Npx, NADH peroxidase; CynD, cyanide dihydratase; CA, carbonic anhydrase.

How the four enzymes of CNO accomplish cyanide conversion is unknown. A perplexing issue has been the discovery that one of its members, CynD, is capable on its own of converting cyanide to formate and ammonia. However, as demonstrated for other CynD enzymes, the concentration of cyanide required for optimal activity is much higher than that displayed by CNO (> 5 mM vs $\leq$ 5 μM, respectively). Because CynD is the only member of CNO for which there is evidence that cyanide is a substrate there is strong interest in characterizing this enzyme. Thus, acquiring information on this enzyme is believed to shed light on and how the four enzymes accomplish catalysis.

Other than a demonstrated ability to convert cyanide to formate and ammonia with the parallel appearance on SDS gels of a polypeptide with a mass of approximately 38kDa (Fernandez, 2005), no further molecular information on the identity of the CynD component has since been acquired. Working from the hypothesis that the enzyme was a likely CynD homolog, considerable effort was put forward in both Dr. Kunz's laboratory and that of his collaborator, Dr. Michael Benedik (Texas A&M University) to amplify and clone a CynD gene from Pf11764 but these efforts were unsuccessful. One PCR product annotated as a nitrilase enzyme (Nit) was obtained, but since the translated amino acid sequence shared only 31% amino acid sequence identity with CynD enzymes it was concluded that this most likely was not a CynD-like enzyme. However, recent studies in our laboratory have given cause to think that this Nit enzyme may be more important to cyanide metabolism than initially thought. The basis for this reasoning comes from the discovery of a gene in Pf11764 coding for a small protein (17.8 kDa) of unknown function (Hyp1) the expression of which was

found to increase when cells were exposed to cyanide (Ghosh, 2009). Moreover, an examination of bacterial genomes revealed that the *hyp1* gene is well conserved among bacteria, and almost invariably resides immediately adjacent to a gene coding for Nit enzymes that show high homology to the *nit* discovered in Pf11764. Indeed, the conserved *hyp1* and *nit* genes reside in a conserved cluster of seven genes referred to as Nit1C the physical arrangement of which is shown in Fig 2. This cluster was so named because a survey of bacterial genomes for nitrilase-encoding genes showed that a high number of such genes were located among similar genes. Because the clustering of genes in bacteria is frequently suggestive of a related metabolic function, it was thought that this might be the case for Nit1C but so far its function remains unknown. However, we hypothesize that these genes may have a physiological role to play in cyanide assimilation as a nutritional nitrogen source based on (i) findings that the expression of Hyp1 in Pf11764 is elevated in response to cyanide, (ii) in addition to *hyp1* the genome of Pf11764 also contains a gene for *nit*, and (iii) preliminary laboratory studies conducted in Dr. Kunz's laboratory having shown that several other known Nit1C-containing bacteria also exhibit the ability to grow on cyanide as the sole nitrogen source. Because the Nit enzyme is considered to be a key enzyme in allowing bacteria to grow on cyanide (and as already discussed is believed to be related to or one and the same as CynD), a coordinated effort to isolate and fully characterize this enzyme was undertaken. One part of this effort was to try and clone the gene and over-express the enzyme which Pallab Ghosh, a fellow graduate student in the laboratory concentrated on and succeeded at to some measure. This thesis concentrates on the native enzyme which was purified to

approximately 95% homogeneity. The properties of both enzymes are compared in the Results section leading to the conclusion that the two enzymes are not the same.

FIG 2. Organization of conserved Nit1C cluster found in various (Podar et al., 2005): Hyp1, hypothetical protein; Nit, Nitrilase; SAM, radical S-adenosyl methionine superfamily member; GNAT, acetyltransferase; AIRs, AIR synthase; Hyp2, hypothetical protein; FP, predicted flavoprotein. Adapted from Podar et. al, 2005.

CHAPTER II

MATERIAL AND METHODS

Organism and Cultivation Conditions

Pseudomonas fluorescens NCIMB 11764 (Pf11764) was originally isolated in Great Britain in 1983 and deposited in the National Collection of Industrial and Marine Bacteria (NCIMB) (Torrey, Scotland) (Harris and Knowles, 1983a). It was acquired 24 years ago from NCIMB and has been maintained in the Kunz laboratory ever since.

The complete medium used for growing cells on agar plates was Lennox medium (LB) whose components have previously been described (Lennox, 1995; Fernandez, 2004a). The minimal medium used for growth of cells (referred to from here on forward as GAM), is shown in Table 1. Cultivation of cells for enzyme isolation were routinely cultivated in 2L Erlenmeyer flasks supplied GAM and inoculated (10% v/v) with a 48 hour-old starter culture grown on the same medium. After 24 h incubation at 30^{o}C on a gyratory shaker, cells where indicated were exposed to cyanide by supplying KCN at 0.1 mM. After 3 h of further incubation (total 27 h) the cells were harvested and stored at -80^{o}C until use. For cells not exposed to cyanide, cells were grown similarly in GAM and harvested after 27 h. For the bulk of the enzyme isolation experiments reported herein, cells were grown in large quantity (400 L fermenter) at the USDA Forest Products Laboratory (Madison, Wi) employing the same cyanide-exposure and harvesting-timed protocol. Cells were recovered by centrifugation at 10,000 x g (or continuously with a Sharples centrifuge) and washed

twice in Na_2HPO_4-KH_2PO_4 (Na-K) phosphate buffer pH 7.0 before freezing the wet cell pellet at -80^{o}C (approximately 0.9~1.0 gram wet weight/liter).

TABLE 1. Composition of glucose-ammonia minimal medium (GAM) for cultivation of *P. fluorescens* NCIMB 11764

Component	Concentration
KH_2PO_4 (pH 7.0)	67 mM
$MgSO_4 . 7H_2O$[a]	400 µg ml^{-1}
$FeSO_4 . 7H_2O$[a]	10 µg ml^{-1}
Glucose	10mM
NH_4Cl	1mM

[a] Added aseptically to sterilized KH_2PO_4 buffer (P1X) at a 200-fold dilution from a sterile stock solution (R-salts) prepared by mixing 400 ml of $MgSO_4 . 7H_2O$ with 100 ml of $FeSO_4 . 7H_2O$ to which was added 2 ml of concentrated HCl. Glucose and NH_4Cl were also added aseptically after autoclaving to sterile P1X. Adapted from Kunz et al., (1998).

Enzyme Assays

Nitrilase. Nitrilase (Nit) activity was assayed either with cyanide (CN, supplied as KCN) or the dinitrile, fumaronitrile (FMN), as substrates. For *cyanide-dependent Nit activity* (also referred to as cyanide dihydratase [CynD] activity) reaction mixtures contained 20 mM KCN in 50 mM Na-K phosphate buffer (pH 8.0) and protein (3 μg – 1.5 mg). Reactions were conducted in sealed vials incubated without shaking at 30°C, and at various times, samples were removed with a syringe and the amount of remaining cyanide (or ammonia formed) determined colorimetrically. In cases where it was preferred to have oxygen eliminated from reaction, vials were purged with argon before reactions were initiated. *Fumaronitrile-dependent Nit activity* was determined by following the formation of ammonia from FMN (10 mM). For each measured assay the change in substrate disappearance (or product formed) was corrected for changes observed in the absence of added protein by conducting simultaneous incubations under identical conditions but without added protein.

Cyanide oxygenase (CNO). CNO activity was assayed as described previously (Fernandez et al., 2004a; Fernandez and Kunz, 2005) by measuring the disappearance of cyanide (or formation of ammonia) in reaction mixtures (100 μl) that contained 50 mM Na-K phosphate buffer (pH 7.0), 100-200 μM KCN, 500 μM NADH and protein (1-10 mg/ml). Reactions were incubated at 30°C with shaking (200 rpm) and conducted in sealed vials to prevent cyanide escape.

Substrate Disappearance and Product Analysis

Cyanide was quantified colorimetrically by the Lambert method as described previously (Lambert et al., 1975; Kunz et al., 1992). For this purpose, 10µl of sample was added to a solution containing 700µl of oxidizing reagent (1 g succinimide and 0.1 g N-chlorosuccinimide in 100ml diH_2O) and 300µl of diH_2O. After mixing, 50µl of the above solution was added to

1.1 ml of water containing 50µl of oxidizing reagent, followed immediately by the addition of 50µl of barbituric acid reagent (60 mg barbituric acid in 0.3ml pyridine and 0.7ml diH_2O). Color

was allowed to develop for 15 minutes after which the optical density of the solution was read at 580 nm and cyanide quantified from a standard curve.

Ammonia was determined by modification of the indophenol method of Fawcett and Scott (1960). For this purpose, 5 µl of reaction mixture was added into 120 µl of diH_2O, followed by 250µl of sodium phenate (0.25 g phenate and 0.78 ml of 4N NaOH in 10ml diH_2O), 375µl of 0.01% of sodium nitroprusside, and 375µl of 0.02N sodium hypochlorite. Reactions were allowed to incubate for 30 minutes at room temperature and the optical density read 630nm. Ammonia was quantified from a standard curve.

Enzyme Purification

The basic protocol for enzyme purification is shown in Fig 3. Individual steps are discussed in order.

Preparation of cell extracts. Frozen cell pellets were suspended in Na-K phosphate buffer (pH 7.0 (2 ml/1 g cells) containing 1mM dithiothreitol, 1% glycerol and 50µg/ml DNase. Cells were broken at 4^{o}C in a French Press (20,000 psi) followed by low-speed centrifugation at 30,000 x g. The resulting supernatant was then subjected to ultracentrifugation at 150,000 x g for 90 minutes and the supernatant (HSS, high-speed supernatant) retained.

Anion exchange chromatography. A Source 30Q (GE healthcare) anion exchange column (bed volume 55 ml) was equilibrated with 20 mM Piperazine buffer (pH 10.0) (running buffer) for 3 column volumes (flow rate 3 ml per min) before applying approximately 200 mg of HSS protein (~ 13 ml HSS equilibrated with 2 equal volumes of running buffer). Non-binding proteins were removed by applying an additional 3 column volumes of running buffer before eluting bound proteins with a linear gradient of 0-0.5 M Na_2SO_4 applied over five column volumes. The 2nd ion-exchange step was performed similarly except that a smaller amount of protein (about 25 mg) was applied. Also, prior to initiating the 2nd round of Source30Q chromatography the column was reversed in orientation and cleaned successively with 2M NaCl (3 ml/min 30-60 min), 1 N NaOH (3 ml/min 1-2 hours) followed by 70% ethanol (1ml/min 1-2 hours). Following chromatography, proteins recovered in fractions (6 mL) were tested for Nit activity with FMN. For this purpose, 90µl of each fraction was placed in 2 ml sealed HPLC vial to which 10 mM fumaronitrile was added. Vials were incubated for 90 min to 2 h and analyzed for evidence of ammonia formation by visible inspection. Those fractions exhibiting activity were collected and desalted with Na-K buffer (3 times original volume) (50 mM, pH 7.0) containing 1 mM dithiothreitol, and 1% glycerol

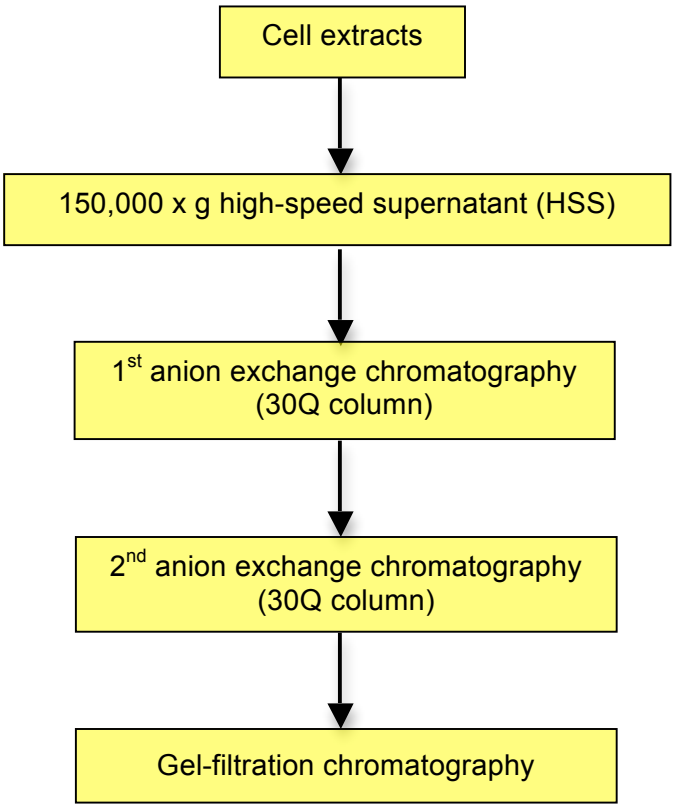


FIG 3. Steps used in isolation of cyanide-degrading nitrilase (Nit)

in Amicon Ultra 10K centrifugal filters (Millipore).

Gel filtration chromatography. Fractions exhibiting Nit activity with FMN following the second round of anion exchange chromatography were pooled and desalted prior to being loaded (approximately 5 mg) onto Superdex 200 column (bed volume 24 ml). The column was equilibrated in 20mM NaH_2PO_4-Na_2HPO_4 buffer (Na-Pi) (pH 7.0), and proteins eluted at a linear flow rate of 0.5 ml/min. Determination of the approximate molecular weight of eluting proteins was accomplished by comparing the elution volumes (times) to that of standard proteins (apoferritin, 443 kDa; alcohol dehydrogenase, 150 kDa; albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome C, 12.4 kDa) eluted under identical conditions. To those fractions exhibiting Nit activity, dithiothreitol (1 mM) and glycerol (1%) were added prior to concentration (~ 40X) in Amicon Ultra 10K centrifugal filters (Millipore). Concentrated proteins were stored at -80^{o}C.

Gel Electrophoresis

Discontinuous SDS-PAGE (12 or 16%) was carried out with a Mini-Protean (Bio-Rad) apparatus using either 12 or 16% acrylamide gels supported with a 5% stacking gel. A 12% gel (8.4 x 5.3 cm), was prepared by mixing (in order) 4 ml of 30% acrylamide/bis stock solution, 2.5 ml of 1.5 M Trizma buffer (pH 8.8), 100 μl of 10% SDS, 2.05 ml diH_2O, 50 μl of 10% ammonium persulfate, and 5μl TEMED (N,N,N,N-tetramethylenediamine). The gel was covered with isopropyl alcohol and the gel allowed to polymerize for 45 minutes before washing off the isopropyl alcohol and layering the separating gel with a 5% stacking gel. The stacking gel was prepared by

mixing 0.65 ml of acrylamide stock solution, 1.25 ml of 0.5 M Trizma (pH 6.8), 50 µl of 10% w/v SDS, 3.05 ml of ddH_2O, 25 µl of ammonium sulfate (10 % w/v) and 5 µl of TEMED. The gel was allowed to polymerize for 45 min and placed in an electrophoresis tank containing running buffer (25 mM Trizma, 192 mM glycine and 0.1% w/v SDS, pH 8.3). The samples were mixed with sample buffer (150mM dithiothreitol, 150mM Trizma pH 6.8, 21% glycerol, 6% lithium dodecyl sulfate, and 0.003% bromophenol blue) in the ratio of 2:1 and then were denatured by heating at 95 ^oC for 10 minutes. Samples were cooled, loaded onto the gel, and electrophoresis performed at 4 ^oC for 60 minutes at a constant voltage of 200 V. The gel was stained with Coomassie Blue staining solution (40% methanol, 10% glacial acetic acid, 0.1% Coomassie Brilliant Blue R-250 in diH_2O) for 30 minutes and destained with 40% methanol and 10% glacial acetic acid in diH_2O for 90 minutes. The gel was stored in 10% glacial acetic acid.

In-Gel Trypsin Digestion and Peptide Extraction

Protein bands from SDS-PAGE gels were excised and subjected to overnight trypsin digestion as described by Vergote et al., (2006). Protein bands were cut out (1 mm^2 gel slice) and placed into a microcentrifuge tube, and the gel contents washed 3 times with 0.5 ml water shaking the microcentrifuge tube continuously for 5 min. The contents were taken up in 0.5 ml 25 mM ammonium bicarbonate (ABC) and the microcentrifuge tube agitated for 10 minutes (2 times). In order to remove the blue Coomassie-stained, 0.2 ml of destaining solution (25 mM ABC in 50% acetonitrile

(ACN)) was added and agitated at least 20 minutes. The gel slice was washed with 0.2 ml of ABC in 50% ACN until the blue color of Coomassie color disappeared.

The gel slice was dried in speed vacuum for 15 to 20 minutes. After the gel slice was dry, the dried gel slice was treated with 15 to 25 µl of 33 mM dithiothreitol (DTT) in 25 mM ABC and incubated at 60^{o}C for 45 minutes. Then, the gel slice was reacted in dark at room temperature with 50 µl of 75 mM iodoacetic acid in 25 mM ABC for 50 minutes. The gel slice was washed and agitated with 0.1 ml of 25mM ABC for 15 minutes (2 times). The supernatant was discarded, and the gel slice was dried for 30 min in speed vacuum. The dried gel slice was treated with 10µl of 12.5 ng/µl trypsin in 10µl of 50mM ABC and incubated in ice about 45 min, and then incubated overnight (12~16 h) at 37^{o}C.

The supernatants from the trypsin-digested mixtures were collected in a new microcentrifuge tube. The gel slice was subjected to vortex for 10 min, sonicated for 15 min (65 rpm) and centrifuged for 5 min in 25µl of 25 mM ABC, 25µl of 50% ACN/ 5% formic acid (twice), 25µl of 50% ACN/ 50% isopropanol/ 2% formic acid, and 25µl of 95% ACN/ 5% formic acid. The supernatants were dried to the volume of about 15µl using speed vacuum, and stored at -80^{o}C.

Electro-Spray Tandem Mass Spectrometry (ESI-MS/MS)

The identity of proteins was verified by mass spectrometry on an Agilent 100 LC/MS (ESI-MS/MS) in collaboration with Dr. Berney Venables of the UNT Proteomics Laboratory. Peptides formed following trypsin digestion were separated by reverse-phase chromatography by injecting 8 µl of trypsinized sample onto a C_{18} capillary

column (0.3 mm x 150 mm). The mobile phase consisted of solvent A (H_2O/ 0.1% formic acid) and solvent B (acetonitrile/ 0.1 % formic acid) programmed to achieve a 0-100% gradient (solvent A to B) for 60 min at a flow rate of 4 µl/min. Separated peptides were introduced accordingly into the mass spectrometer with the most intense MS/MS peaks automatically selected after defining an intensity threshold. The mass spectral profile was then compared to those in available databases using the Mascot search tool from Matrix Science (www.matrixscience.com).

CHAPTER III

RESULTS

Induction of Cyanide-Degrading Nitrilase

As shown in Table 1, cells of Pf11764 exposed to cyanide under minimal nutrient conditions induced two types of cyanide-degrading enzyme activities, one oxygen-dependent (aerobic) and the other oxygen-independent (anaerobic). The aerobic activity, as previously established (Kunz et al., 1994, Kunz et al., 2001), is conferred by CNO while the oxygen-independent (anaerobic) activity, was hypothesized to arise from Nit. Because the *hyp1* gene was shown in our laboratory to be upregulated by cyanide (Ghosh, 2009) it was hypothesized that the same would be true for Nit given the thought that the two reside next to each other in a probable Nit1C operon. However, to better evaluate the level of expression of Nit an improved method over assaying enzyme activity with cyanide as a substrate was needed given that the activity exhibited by cell extracts towards cyanide was quite low (~0.005 U/mg, see Table 2). An alternative substrate was therefore sought to better assay the enzyme. The literature was examined for compounds that might serve this purpose by searching for reports specifically describing the substrate requirements of Nit1C-derived Nit enzymes. Only one such report, that being of the characterization of a Nit enzyme from the blue-green bacterium *Synechocystis* sp. (strain PCC6803), was found (Heinemann et. al., 2003). Although it was not known at the time that this was a Nit1C enzyme, verification that the enzyme is truly Nit1C origin was confirmed by consulting the now available *Synechocystis* genome (Heinemann et. al., 2003) (locus s110784) (merR nitrilase)(http//ncbi.nlm.nih.gov). The best substrate found was the

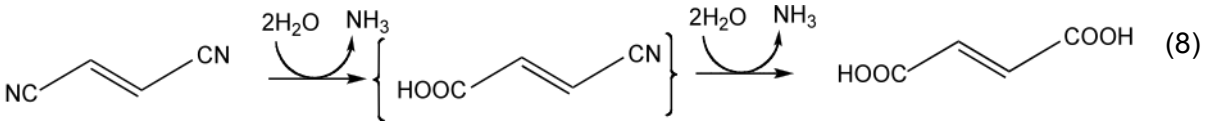
TABLE 2. Induction of cyanide-degrading enzyme activities in *P. fluorescens* NCIMB 11764

Cyanide exposure during cultivation	Specific Activity (nmol min^{-1} mg^{-1}) measured with cyanide:	
	Aerobically[a]	Anaerobically[b]
Yes	8 ± 4	5 ± 2
No	≤ 0.5	≤ 0.5

[a]Formally representing CNO measured by following the disappearance of cyanide (and/or appearance of ammonia) as described in the Materials and Methods.

[b]Assays conducted either anaerobically or aerobically by measuring cyanide (20 mM) disappearance (or ammonia formed) in the absence of added NADH.

dinitrile, fumaronitrile (1,2-dicyanoethene). For over-expressed enzyme was reported to have a specific activity of 2 U/mg. This is 100-times greater than that reported for a model substrate (benzonitrile) (0.02 U/mg). We tested cell extracts of cyanide-exposed cells of Pf11764 for nitrilase (Nit) activity towards fumaronitrile (FMN) and that indeed, cell extracts catalyzed its conversion to ammonia in a time-dependent manner (Fig. 4). Moreover, the activity for cells that had been exposed to cyanide during cultivation was significantly greater than that of non-exposed cells (0.5 and 0.02 U/mg, respectively) thus implying that expression of Nit, as earlier demonstrated for Hyp1 (Ghosh, 2009), was also induced by cyanide (as expected if genes for the two exist in an operon). The results in Fig 4 further show that in reaction mixtures supplied 10 mM FMN approximately 10 mM ammonia accumulated indicating that fumaronitrile is converted stoichiometrically to ammonia (and fumaric acid) as depicted in equation 8.



(8)

Purification of Cyanide-Degrading Nitrilase

Having discovered that FMN is a much better substrate for Nit than cyanide (KCN) we proceeded to purify the enzyme from cell extracts; the greater sensitivity of enzyme detection representing a big improvement over previous attempts made in our laboratory for isolating the enzyme. The approach to isolation of Nit employed three steps: two rounds of anion-exchange chromatography at high pH (10) followed by gel filtration chromatography (see Materials and Methods and Fig. 3). This approach was

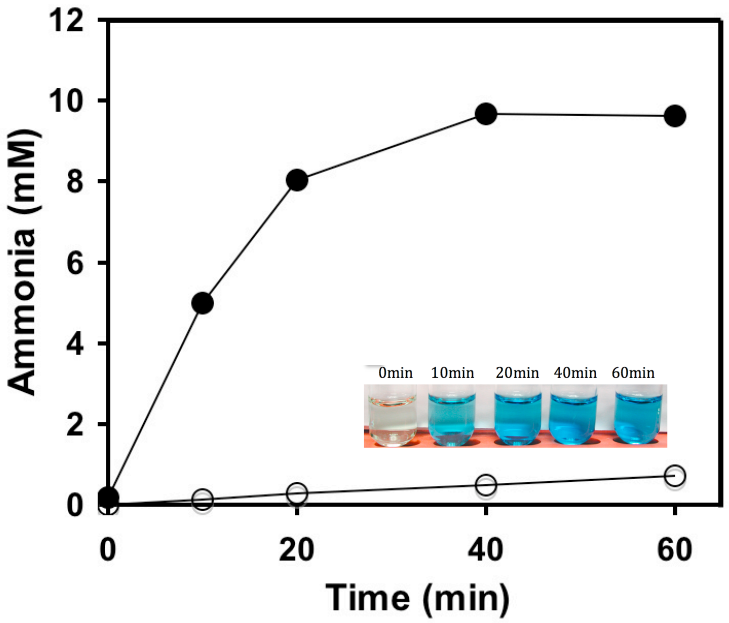


FIG 4. Time-course of fumaronitrile conversion to ammonia by cell extracts prepared from cyanide-exposed (●) and unexposed (○) cells of Pf11764. Inset: Color formation of ammonia for samples taken at the times indicated. Reaction mixtures contained, 10 mM fumaronitrile in Na-K phosphate buffer (pH 7) and 1 mg/ml protein.

taken because earlier work (Fernandez et al., 2004a) had shown that the enzymes comprising CNO (of which putative Nit is a component) could be recovered under these conditions. Fig 5A shows the protein elution pattern and recovery of Nit activity following the second round of anion exchange chromatography. Nit activity, as shown, was concentrated in fractions eluting between 0.3 and 0.4 M Na_2SO_4. These fractions were pooled, desalted by passage through 10,000 molecular weight cutoff (mwco) ultramembrane centrifugation tubes, and the concentrated protein was applied to a 24 ml capacity Superdex-200 column. The pattern of elution from this column is shown in Fig 5B and shows that activity was recovered in individual fractions calibrated to contain proteins in the approximate 110 kDa range. At each step of the purification process the relative Nit activity towards fumaronitrile versus cyanide as the substrate was measured and proteins recovered in various fractions analyzed by SDS-PAGE. The three-step protocol to enzyme isolation was performed on three separate occasions for which the overall results are summarized in Table 3. The results show that the enrichment in activity for assays conducted with FMN were paralleled by a simultaneous enrichment in activity towards cyanide suggesting that a single enzyme was responsible for the activity observed with both substrates. The final purified sample exhibited 18 times greater activity with FMN than for cyanide. In contrast, the overall purification achieved for the cyanide degrading activity was almost five times that achieved when FMN served as the assay substrate. Fig 6 shows the gel profile pattern for proteins present in a single fraction recovered after gel filtration chromatography. Visible were four major protein bands of 42, 40, 39, and 38 kDa. Similar migrating bands was also detected in fractions obtained following the 1st and

2nd rounds of anion-exchange chromatography steps, but overall, fewer proteins bands were visible on gels while progressing from one step to the next as expected. In order to determine which protein(s) was responsible for the observed FMN and cyanide degrading activities, individual bands from the gel were excised and subjected to mass spectrometry. These experiments were conducted in collaboration with Dr. Barney Venables of the UNT Protein Proteomics Laboratory. Table 5 shows a summary of the results in which it can be seen that only the 38 kDa species returned a match for a protein annotated as a nitrilase (Nit). Thus, it was concluded that a single Nit protein from Pf11764 was responsible for the observed activity towards both FMN and cyanide as substrates.

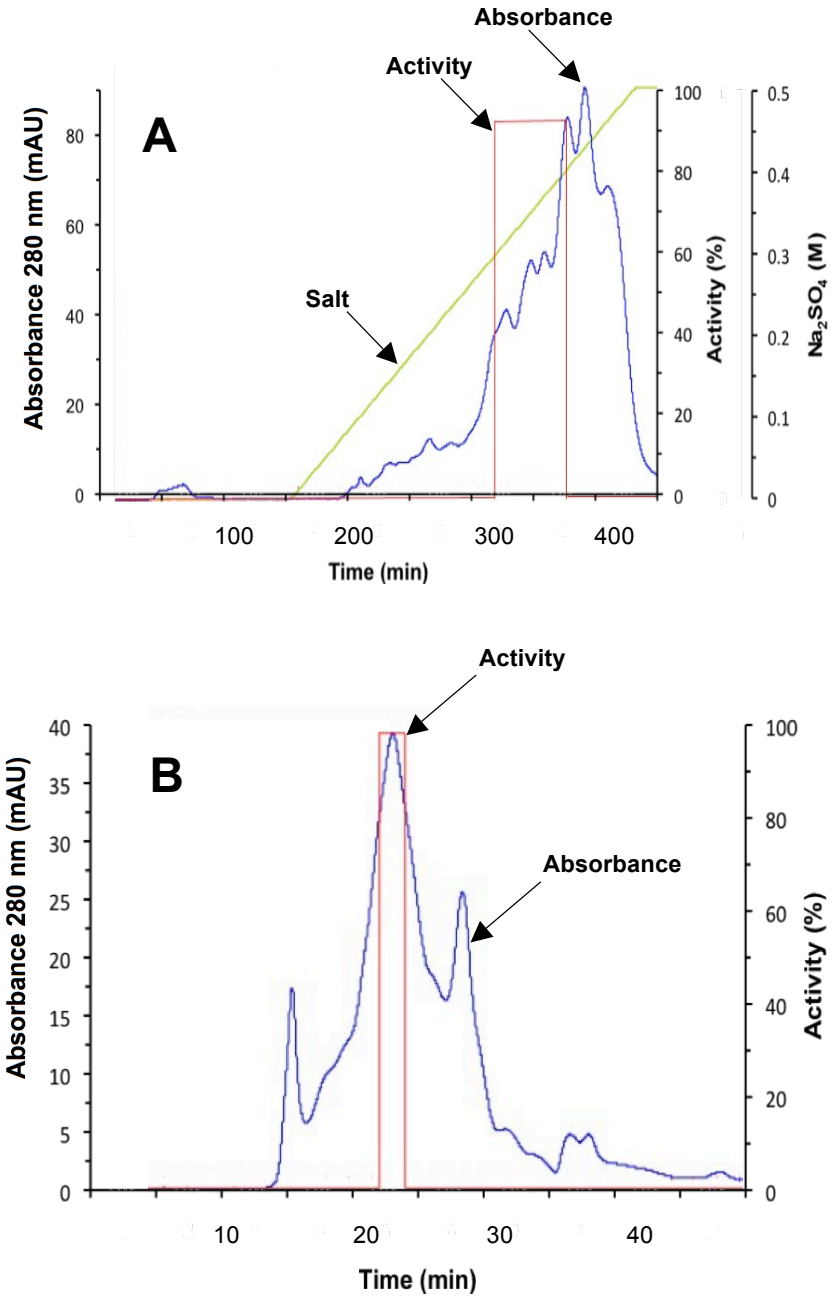


FIG 5. Elution of proteins and Nit activity present in cell extracts of *P. fluorescens* NCIMB 11764 following anion-exchange (A) and gel-filtration chromatography (B). In A and B respectively, 29 and 2.8 mg protein was applied. Proteins in (B) were eluted with 20 mM Na_2HPO_4, pH 8.0, at a flow rate of 0.5 ml/min.

TABLE 3. Copurification of fumaronitrile- and cyanide-degrading nitrilase (Nit) activities from *P. fluorescens* NCIMB 11764 induced with cyanide

Purification step	Protein (mg)	Activity (U)[a] with:		Purification fold		Yield (%)	
		Fumaronitrile	Cyanide	Fumaronitrile	Cyanide	Fumaronitrile	Cyanide
Cell extract	291.6 ± 17	0.46 ± 0.06	0.0053 ± 0.0015	1	1	100	100
1st 30Q Sepharose	22.2 ± 6	1.68 ± 0.12	0.026 ± 0.009	3.6	4.9	27	37.3
2nd 30Q Sepharose	5 ± 1.1	3.1 ± 0.98	0.094 ± 0.017	6.7	17.7	11.5	30.4
Superdex-200	0.035 ± 0.008	18.08 ± 1	1 ± 0.32	39.3	188.6	0.47	2.26

[a]1 unit (U) is defined as 1 μmol $min^{-1}mg^{-1}$ protein. Determined at 30°C by measuring the rate of ammonia formed or for cyanide its rate of disappearance in reaction mixtures supplied with 10 and 20 mM fumaronitrile and cyanide, respectively.

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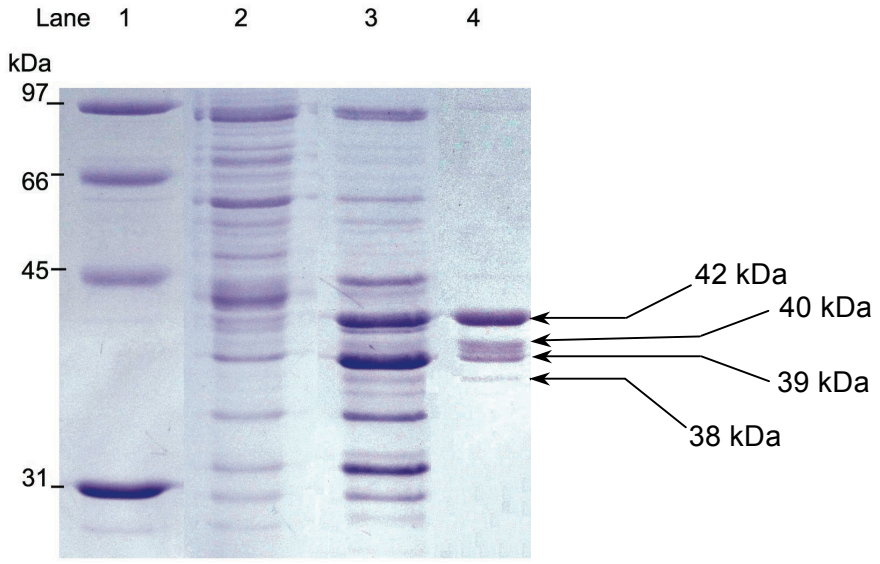

FIG 6. SDS-PAGE analysis of Pf11764 proteins recovered in fractions exhibiting Nit activity. Lane 1, molecular weight standards: phosphorylase B, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31kDa (5 μg); lane 2, 1st ion-exchange column (10 μg); lane 3, 2nd ion-exchange column (10 μg); lane 4, gel filtration column.

TABLE 4. Identity matches for proteins recovered after gel-filtration chromatography from Pf11764 by electrospray ionization mass spectrometry (ESI-MS/MS)

Bands[a]	***Gene Bank No***	***Protein Match in MASCOT MS/MS ion search***	***MASCOT Ion Score***	***No. of peptide matches***	***% coverage***	***Nominal mass (Mr), Da***	***Calculate pI value***
1	gi|77461686	delta-aminolevulinic acid dehydratase [*Pseudomonas fluorescens* Pf0-1]	244	7	17%	38897	5.22
2	gi|70728667	cysteine synthase [*Pseudomonas fluorescens* Pf-5]	82	3	4%	39886	5.87
3	gi|77459140	aldo/keto reductase [*Pseudomonas fluorescens* Pf0-1]	228	8	21%	37345	6.27
4	*gi|186473966*	Nitrilase *[Burkholderia phymatum STM815]*	105	2	7%	38027	5.56

[a] See figure 6

Comparative Analysis of Recombinant and Native Isolated Pf11764 Nit Enzymes

Pallab Ghosh and Dr. Jung-Huyn Lee of our laboratory succeeded in cloning Nit using Pf11764 DNA sequence arrived at by gene-walking both up and downstream of the identified Nit region (unpublished results). The *nit* content of the plasmid construct (pE101D::nit_{PG}) was verified by DNA sequencing and the translated amino acid sequence showed more than 50% identity to other Nit1C-derived Nit proteins providing strong evidence that the correct enzyme had been cloned (data not shown). However, in this case the predicted mass of the enzyme was 34.5 kDa which is somewhat below that determined for the enzyme isolated from the wild-type as described here. Limited expression studies for the recombinant protein showed that indeed, a protein of the correct mass was produced (as determined by gel electrophoresis and confirmed by mass spectrometry) (data not shown). Thus, a question then arose as to whether or not the cloned (34.5 kDa) recombinant (Nit_{PG}) and native (38 kDa) enzymes (Nit_{CC}) were different. To further compare them, the amino acid sequence for the Nit protein returned from a Mascot MS-ion search of matching peptides with the Pf11764 enzyme (e.g., gi|186473966 from *B. phymatum* STM815) was aligned with the sequence predicted for the recombinant. These data are shown in Fig 7. Included also in this comparison is the sequence of one additional Nit enzyme (gi|91784392) from the related bacterium, *B. xenovorans* LB400. Aside from the obvious differences between Nit_{PG} and Nit_{CC} as far as total amino acid content is concerned, additional differences may be noted. First, with regards to the highlighted regions (referencing peptides returned from the Mascot search), It may be noted that for the more carboxyl oriented peptide a 100% match for the two sequences

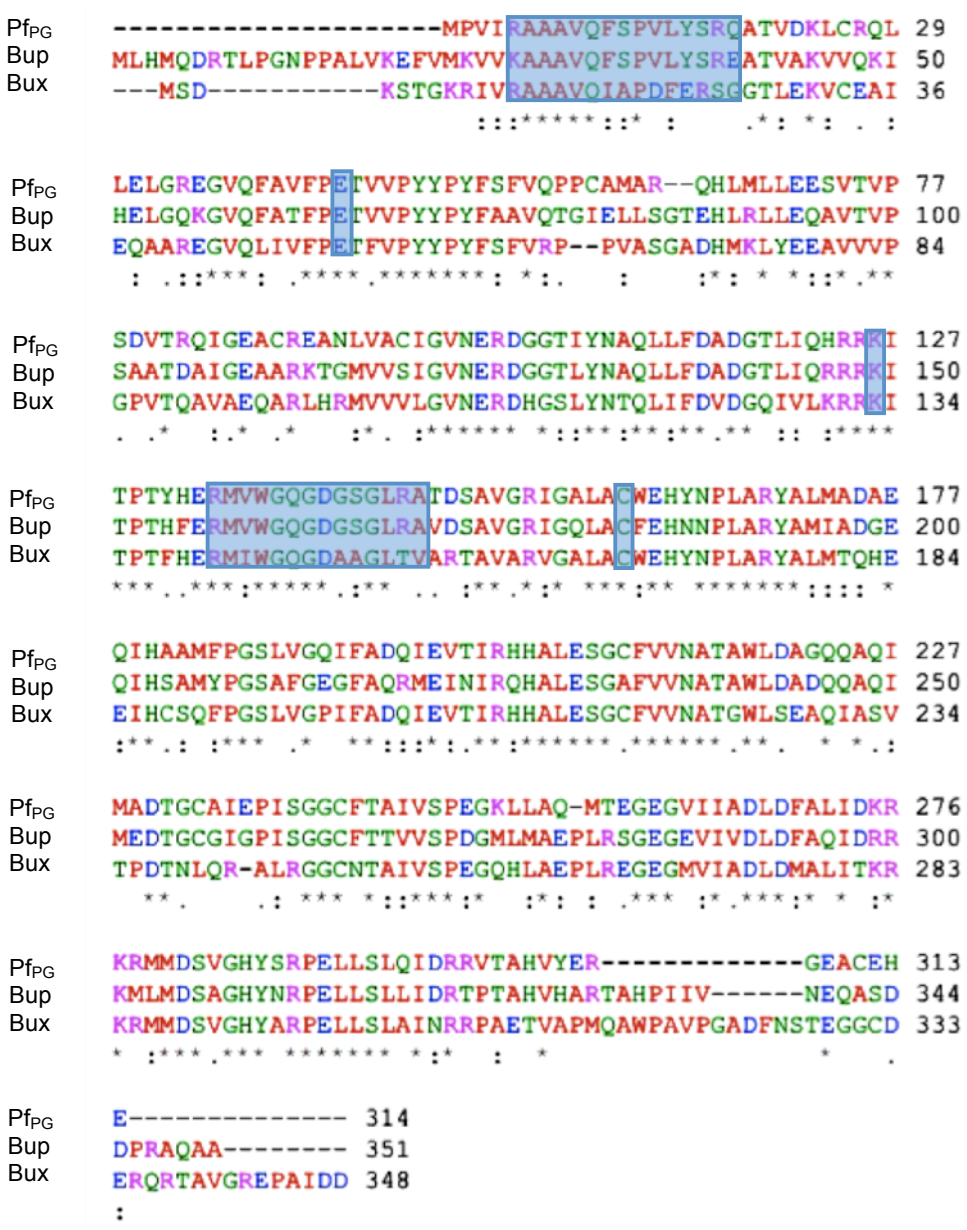

FIG 7. Alignment of amino acid sequences for the recombinant Nit_{PG} protein from *P. fluorescens* Pf11764 (Pf_{PG}), the MASCOT returned peptide match with the Pf11764 Nit_{CC} protein derived from *Burkholderia phymatum* STM815 (Bup), and Nit from *Burkholderia xenovorans* LB400 (Bux) using the ClustalW program available at ExPASy – Tools (www.ebi.ac.uk/Tools/clustalw2/index.html). Highlighted blocks of amino acids are those returned from a peptide search of amino acids matching those present in Pf11764 Nit_{CC}. Consensus amino acids representing the conserved catalytic triad are also shown highlighted. Identical residues are noted by asterisk (*), similar ones with a colon (:), and less similar by a dot (.). Non-conservative charges are left blank below the alignment.

exists. In contrast, for the more amino-terminal peptide there is a two amino-acid mismatch (one on each end, see also Table 5). There are additional differences that can be noted only one of which will attention be drawn to here. This includes the occurrence of a phenylalanine residue (F) next to the conserved catalytic triad-cysteine (C) for the Bup protein as opposed to tryptophan (W) in the case of Nit_{PG} and BxLB400. Thus, despite the relative high homology of all three proteins, differences can be discerned which could be important as far as biochemical function is concerned. This is important because both Nit_{PG} and Nit_{Bux} originate from known Nit1C cluster genes while the Nit_{Bup} enzyme does not as is evident from an examination of the Bup genome.

To further probe the question whether or not the Nit_{PG} recombinant and the isolated Nit_{CC} proteins were different the relative activities of the two towards FMN and cyanide were compared. As already noted, the native Nit_{CC} enzyme exhibited about a 18-fold higher activity with FMN than it did for cyanide. In contrast, studies performed with the recombinant enzyme by P. Ghosh revealed about a 10,000-fold higher activity with FMN compared with cyanide (Table 5). Consequently, these results gave further reason to think that the two enzymes were not the same. The comparative properties of the two are further summarized in Table 5.

TABLE 5. Comparative properties of native and recombinant Pf11764 Nit enzymes

Source of Nit	Molecular weight (Da)	Activity (U/mg) with:		Amino acid sequences for defined peptide regions[c]	
		Fumaronitrile	Cyanide	Region 1	Region 2
Wild-type (Nit_{CC})	38,051[a]	18	1	KAAAVQFSPVLYSRE	RMVMGQGDGSGLRA
Recombinant (Nit_{PG})	34,569[b]	500	0.05	RAAAVQFSPVLYSRQ	RMVMGQGDGSGLRA

[a]Determined by SDS-PAGE and match with protein returned from peptide search

[b]Predicted from translated gene sequence and confirmed by SDS-PAGE

[c]See also Figure 7. Variant amino acids are highlighted

CHAPTER IV

DISCUSSION

This project was initiated for the purpose of identifying the enzyme from *P. fluorescens* NCIMB 11764 shown previously to be capable of degrading cyanide independently but also capable of joining with several other enzymes to produce a novel enzyme complex with high cyanide scavenging ability (Fernandez and Kunz, 2005). The latter complex known as CNO has important physiological implications because of its ability to serve as an efficient cyanide detoxification system (micromolar range) and also because it is known to be required for utilization of cyanide as the sole nitrogen source. Previous studies suggested that the enzyme component of CNO capable of attacking cyanide on its own (but only at much higher concentrations then when combined with other CNO partners) was a likely cyanide dihydratase (CynD). These enzymes represent a small subset of enzymes grouped in branch 1 of the large superfamily of enzymes classified as nitrilases (E.C. 3.5.53) that act on cyanide specifically, catalyzing its direct hydrolysis to ammonia and formic acid. Besides CynD enzymes, branch 1 includes many enzymes that catalyze a similar conversion of higher nitriles referred to in general, as nitrilases (Nit) (Pace and Brenner, 2001). Extensive efforts in our laboratory to verify that the enzyme in question was indeed related to other CynD enzymes from bacteria were unsuccessful. Instead, while the enzyme was shown to fall into the same branch of the superfamily as CynD's (branch 1) it was not sufficiently homologous to warrant its being considered an ortholog thereof. Nonetheless, for reasons already discussed there was reason to think that

the Pf11764 Nit enzyme could be involved in cyanide metabolism, and therefore, efforts to isolate and characterize the enzyme were put forward.

In the final analysis, two Nit proteins have now been successfully isolated from Pf11764. One of these is a recombinant enzyme (Nit_{PG}) arrived at by cloning the corresponding gene and the other is the native enzyme (Nit_{CC}) whose purification from Pf11764 is described here. One possible outcome of the research undertaken is that the same Nit enzyme as already cloned (Nit_{PG}) might have been expected to be recovered in attempts to isolate Nit from the native organism, however, this turned out not to be the case. Instead differences between the two in terms of molecular mass, predicted amino acid sequence and substrate specificity were found suggesting that the two are different enzymes. This is not completely surprising when one considers that duplicate genes (paralogs) in organisms encoding different enzyme isoforms are fairly common among genomes. A question then arises what is the genetic origin of the Nit_{CC} enzyme since it has already been established that Nit_{PG} originates from a Nit1C cluster (based on extensive gene-walking experiments performed surrounding the *nit* region)? One possibility is that there exists in Pf11764 a second Nit1C cluster from which Nit_{CC} originates. That two separate Nit1C clusters might exist in one organism is certainly a possibility and indeed exists for some bacteria as an examination of their genomes reveals (e.g., *Gluconoacetobacter diazotrophicus* PA1 5). At a minimum, results showing that cyanide induced Nit enzyme activity and that this parallels the apparent induction also of a Nit1C-encoded Hyp1 protein (Ghosh, 2009), provides strong evidence of an effect of cyanide on Nit1C expression (regardless of whether there exist one or two clusters). On the other hand, there is

nothing to exclude the possibility that the Nit_{CC} product is encoded by a gene completely separate from that residing among Nit1C genes but whose expression also responds to cyanide as an environmental signal. If the Nit_{Bup} match with the Pf11764Nit_{CC} enzyme for peptides detected by MS is any indication, then it might be deduced that Nit_{CC} is not of Nit1C origin because Nit_{Bup} is not Nit1C encoded.

A question that arises is, why was the native counterpart of Nit_{PG} not recovered during enzyme purification if, as expected, significant levels of the enzyme could also be assumed to be present? First, it is possible that the purification protocol employed was not selective for the Nit_{PG} enzyme. This seems somewhat unlikely because no evidence for the recovery of Nit activity in fractions other than those containing proteins eluting within a narrow range was observed. Second, it is possible that the Nit_{PG} protein was present but somehow was degraded perhaps by protease action. The detection of smaller polypeptides in fractions subjected to multiple rounds of gel filtration chromatography which was occasionally observed (data not shown) could be taken as evidence that protein degradation occurs. Similarly, the identification of proteins on gels matching those of known chaperones (e.g. groEL) (data not shown) gives reason to think that the presence of such proteins might be explained on the basis that they are needed stabilize Nit or prevent it from being degraded. Finally, another possibility is that despite the expected induction of Nit_{PG} by cyanide, it is, in fact, not induced but instead, it is the Nit_{CC} protein that is the major Nit isoform made.

Is there reason to think that the recovered Nit_{CC} enzyme is more closely related to what was earlier proposed to be a CynD-like enzyme? The answer to this question depends somewhat on being able to combine the purified enzyme with other

components necessary for CNO and determine whether the reconstituted system supports CNO activity. However, this will require that all other components are re-isolated since quantities needed to perform such a reconstitution experiment are not currently available. The higher activity exhibited towards cyanide by the Nit_{CC} enzyme compared to Nit_{PG} gives reason to believe that of the two, it is the stronger candidate for a match with what was earlier published as a possible CynD (Fernandez and Kunz, 2005). Indeed, the similarity in specific activities (~ 0.026 U/mg when assayed with cyanide) of the two recovered at the same stage of purification (e.g., 1st round anion-exchange) gives further reason to think they are the same.

A curious observation that deserves comment is the rather dramatic increase in enrichment achieved for cyanide serving as the assay substrate opposed to FMN. As shown in Table 3, for cyanide the enrichment achieved was approximately 188 fold compared to 39-fold when FMN was employed as the assay substrate. The reasons for this are not understood, but repeated observations have shown that as proteins in Pf11764 are resolved the observed Nit activity appears to increase over what might otherwise be expected based on the rather low activity present in crude extracts. Why this should be the case is not known but one possible explanation is that the Nit protein is unavailable for enzyme action or inactive when there are many other proteins present (for example, in crude extracts). As already mentioned, it was not uncommon to detect chaperone proteins co-purifying with Nit_{CC}. While this may stabilize the enzyme it may at the same time restrict its availability to the substrate (e.g., cyanide). The large oligomeric structures that nitrilases are known to take (Thuku et al., 2008) also implies that rather complex interactions may occur which

could influence enzyme activity. Whether these types of interactions if proven serve any biological role remains to be determined. However, that they are not outside the realm of possibilities is supported by findings that the known mammalian Nit1 counterpart (and the fused NitFhit protein in *Drosophila melanogaster* and *Caenorhabditis elegans*) has tumor suppressor capability (Pace et al., 2000). In this case, one of the proposed Nit1 roles is that of a stabilizing agent for cell growth regulators (Semba et al., 2006). Another reason to suspect that Nit_{CC} has a tendency to bind other proteins comes from the fact that it was initially discovered to be one of four components of CNO (Fernandez and Kunz, 2005). This gave rise to a proposed model for CNO (see Fig. 1, Introduction) of a novel assembly of enzymes responsible for cyanide conversion. The high affinity for cyanide displayed by the combination of CNO enzymes compared with the apparent low affinity for putative Nit_{CC} (e.g., 5 μM versus 5 mM) (Nit_{CC} being assumed here to be the same as CynD for which the apparent Km for cyanide was earlier estimated at 5 mM [Fernandez et al., 2004b] implies that not only does the substrate affinity change when the component proteins interact, but curiously, so does the mechanism – from hydrolytic to oxygenolytic in chemistry. To explain how these rather dramatic physical and chemical changes may be taking place will require that each CNO component be separately characterized. Towards that objective, this project has provided new insights on the Nit component.

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