Alternative routes of enzymic cyanide metabolism in *Pseudomonas fluorescens* NCIMB 11764

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Cell-free extracts from *Pseudomonas fluorescens* NCIMB 11764 catalysed the degradation of cyanide into products that included CO₂, formic acid, formamide and ammonia. Cyanide-degrading activity (CDA) was localized to cytosolic cell fractions and was observed at substrate concentrations as high as 100 mM (2600 mg CN⁻ l⁻¹). At least two different CDAs could be distinguished by: (i) the determination of reaction product stoichiometries, (ii) requirements for NADH and oxygen, and (iii) kinetic analysis. The first activity produced CO₂ and NH₃ as reaction products, was dependent on oxygen and NADH for activity, and displayed an apparent *K_m* for cyanide of 1.2 mM. The second activity generated formic acid (and NH₃) plus formamide as reaction products, was oxygen independent, and had an apparent *K_m* of 12 mM for cyanide. The first enzymic activity was identified as cyanide oxygenase as previously described [Harris, R. E. & Knowles, C. J. (1983) *FEMS Microbiol Lett* 20, 337–341] whereas the second activity is believed to consist of two enzymes, a cyanide nitrilase (dihydratase) and hydratase (EC 4.2.1.66). In addition to these enzymes, cyanide-grown cells were also induced for formate dehydrogenase (EC 1.2.1.2) thereby providing a means of recycling NADH utilized by cyanide oxygenase. A mutant strain having lost the ability to grow on cyanide as a nitrogen source was isolated and shown to be defective in cyanide oxygenase, but not the cyanide nitrilase/hydratase enzymes. This finding together with results showing that the substrate affinity of cyanide oxygenase was tenfold greater than for the nitrilase/hydratase enzymes, indicates that it is this enzyme that is most important in cyanide assimilation. A cyanate-defective mutant was also isolated and shown to be unaffected in cyanide assimilation, indicating that the metabolism of these two compounds is physiologically distinct.

Keywords: *Pseudomonas fluorescens*, cyanide oxygenase, nitrilase/hydratase, formate dehydrogenase

INTRODUCTION

Pseudomonas fluorescens NCIMB 11764 is capable of growth on cyanide (CN⁻/HCN) as the sole nitrogen source (Harris & Knowles, 1983a). Growth requires that cyanide be enzymically converted to ammonia which, is then readily assimilated into cellular nitrogen. Harris & Knowles (1983b) first reported the conversion of cyanide into ammonia and CO₂ by cell-free extracts from this organism. These findings were confirmed in our lab-

oratory using intact cells, but formate and formamide were identified as additional reaction products (Kunz *et al.*, 1992). Furthermore, whereas formate and formamide accumulated under both aerobic and anaerobic conditions, CO₂ production was shown to be exclusively an oxygen-dependent process. The latter observation is consistent with earlier findings reported by Knowles and coworkers, who proposed that the enzyme responsible for cyanide oxidation in NCIMB 11764 was an oxygenase (Fig. 1) (Harris & Knowles, 1983b; Knowles & Bunch, 1986; Knowles, 1988). These investigators further proposed that cyanate was a metabolic intermediate, and subsequent studies showed that an enzyme resembling

Abbreviations: CDA, cyanide-degrading activity; HSS, high-speed supernatant; Tcn, tetracyanonickelate (II).

Fig. 1. Routes of cyanide assimilation in *P. fluorescens* NCIMB 11764.

cyanase (EC 4.3.99.1) could be detected in crude extracts of cyanate-grown NCIMB 11764 cells (Kunz & Nagappan, 1989). However, this enzyme was not present in cyanide-grown cells implying that cyanate and cyanase are not involved in cyanide assimilation (Dorr & Knowles, 1989; Nagappan, 1992). The identification of formate and formamide as additional reaction products suggested that in addition to a putative cyanide oxygenase, other enzymes capable of attacking cyanide were also elaborated by this organism. As illustrated in Fig. 1, these included a cyanide nitrilase (dihydratase) responsible for formate and ammonia production, and a cyanide hydratase yielding formamide. Formamide was eliminated as a possible precursor of formate since its further degradation by either intact cells or cell extracts was not observed (Kunz *et al.*, 1992). In this paper we report the recovery of cyanide-degrading activities (CDAs) in cell-free extracts consistent with the existence of these enzymes in NCIMB 11764. That separate enzymic reactions are involved is indicated by results showing that both the products of conversion and kinetics varied with the reaction conditions. In addition to the description of two different CDAs, evidence for the induction of formate dehydrogenase is also reported. Finally, the isolation of mutants defective in cyanide and cyanate utilization is described. Their properties establish the physiological role of cyanide oxygenase in cyanide assimilation and further exclude a role for cyanate as a metabolic intermediate.

METHODS

Organisms and growth conditions. *P. fluorescens* NCIMB 11764 has been described in previous communications (Harris & Knowles, 1983a, b; Kunz *et al.*, 1992). Cells were grown in a minimal salts medium using the fed-batch cultivation procedure described by Kunz *et al.* (1992). In this procedure glucose (20 mM) is supplied as the carbon source and KCN is added at sub-toxic levels (2 additions per day to give 0.125 mM each). Cells were harvested by centrifugation after 3 d incubation and washed twice in 50 mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (Na-K) buffer, pH 7.0. Cells induced for CDA were obtained by collecting cells 3–5 h after the addition of KCN (0.1 mM) to a 24-h-old batch culture that had been grown on NH_4Cl (1 mM). Washed cell pellets were stored at -70°C until use.

Mutant strains of NCIMB 11764 that had lost the ability to grow on cyanide (JL102) or cyanate (ON101) as the sole nitrogen source were obtained by nitrosoguanidine mutagenesis and penicillin enrichment. The two mutants were obtained from separate mutagenesis performed as follows. A 50 ml overnight culture grown in minimal medium containing glucose (20 mM) as the carbon source and NH_4Cl (1 mM) as the nitrogen source (minimal glucose-ammonia) was harvested and washed twice

with Na-K phosphate buffer, pH 7.0. These cells were then resuspended in 50 ml 0.1 M sodium citrate buffer, pH 5.5, containing 100 μg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine ml^{-1} and incubated for 30 min at 30°C on a rotary shaker. Cells were removed by centrifugation and washed twice in the same buffer before being resuspended in 50 ml minimal glucose-ammonia medium and grown overnight. This culture was harvested and the cells washed twice in Na-K phosphate buffer, pH 7.0, before being inoculated into 50 ml minimal glucose supplied either with tetracyanonickelate (II) (Tcn; 1 mM) or with cyanate (KOCN; 1 mM) as the sole nitrogen source. Procedures for the growth of NCIMB 11764 on Tcn (Rollinson *et al.*, 1987; Silva-Avalos *et al.*, 1990) and KOCN (Kunz & Nagappan, 1989) have been described. After 12 h growth, mutant enrichment was achieved by adding penicillin G (10000 U ml^{-1}) and D-cycloserine (0.2 mg ml^{-1}) and allowing cultures to incubate for an additional 12 h, during which cell lysis occurred. At this time the cells were harvested and plated to minimal glucose agar plates containing NH_4Cl (1 mM). Cyanide- or cyanate-defective mutants were then detected after replicating colonies to plates containing either Tcn, KCN or KOCN as the sole nitrogen source. These substrates were added to minimal glucose agar plates at 1 mM, except for KCN, which was supplied as vapour as described by Harris & Knowles (1983a).

Preparation of cell extracts. Washed cells contained in Na-K phosphate buffer, pH 7.0 (1.0 g per 2 ml buffer), were broken in a French Press at 20000 p.s.i. (138 MPa). A small amount of deoxyribonuclease (20 μg ml^{-1}) was added to the resultant viscous liquid and the preparation was centrifuged at 30000 *g* for 30 min. The supernatant liquid was then subjected to ultracentrifugation at 150000 *g* for 90 min to produce high-speed supernatant (HSS; cytosolic) and pellet (membrane) fractions. The pellet was resuspended in the original volume of phosphate buffer and washed by an additional high-speed centrifugation before it and other fractions were assayed for cyanide conversion activity as described below. The protein content in cell-extract fractions was determined by the Lowry procedure. NAD^+ was removed from HSSs by incubation with 0.2 U NADase (NAD^+ glycohydrolase; EC 3.2.2.5) ml^{-1} for 1 h at 30°C .

Cyanide-degradation assay. The time-dependent disappearance of cyanide (CDA) was measured in sealed vials (15 ml Hypo-Vials crimp-sealed with Tuf-Bond Teflon-rubber laminated discs, Pierce Chemical, or 2 ml HPLC screw-cap auto-sampler vials sealed with silicon rubber/PTFE septa, Rainin Instrument) at 30°C by an adaptation of the procedure described previously for intact cells (Kunz *et al.*, 1992). The standard reaction mixture contained in 0.25 ml: cell extract (9–10 mg protein ml^{-1}), 2 mM KCN, 4 mM NADH (where indicated), and 50 mM Na-K buffer, pH 7.0. Reactions were initiated by the injection of KCN and 25 μl samples were withdrawn at intervals and diluted as necessary for the determination of cyanide by the method of Lambert *et al.* (1975). Separate reaction vials containing cell extract incubated in the absence of cyanide were also included as a reference for colorimetric determinations. Reaction mixtures were made anaerobic by flushing vials with oxygen-free N_2 for 5 min prior to injecting KCN.

Determination of cyanide reaction products and stoichiometries. The products of cyanide metabolism were quantified in reaction mixtures containing either 2 or 25 mM KCN supplied as the ^{12}C -, ^{13}C - or ^{14}C -isotopic species. Reactions were carried out in sealed vials as described above, or in serum-stoppered flasks when radioactive ^{14}C CO_2 production from cyanide was determined. In the latter case, the main compartment of a 25 ml

serum-stoppered flask fitted with a centre well contained 0.4 ml cell extract (9–10 mg protein ml⁻¹; HSS), NADH (where indicated) and 50 mM Na-K phosphate buffer, pH 7.0, in a total volume of 0.5 ml. The reaction was initiated by the injection of labelled (1–2.5 µCi; 37–92.5 kBq) and unlabelled KCN (2 or 25 mM), and when reactions were complete, as determined by simultaneous assays for cyanide, 0.3 ml 4 M NaOH was injected into the centre well. The flask was incubated for an additional 15 min to trap volatile ¹⁴CO₂ and the contents of the main compartment and centre well were fractionated with BaCl₂ as previously described (Fallon *et al.*, 1991; Kunz *et al.*, 1992). CO₂ was thus quantified as the sum of the radioactivity recovered in the centre well (volatile ¹⁴CO₂) and main compartments (non volatile H¹⁴CO₃). The identity of CO₂ as a reaction product was also verified by the detection of a 45 *m/e* species when the headspace from incubations conducted with K¹³CN as the substrate was analysed by mass spectrometry. This was accomplished by injecting 10 µl gas over a polydimethylsiloxane capillary column (Alltech Associates SE3-30M) coupled to a Hewlett-Packard 5992 GCMS equipped with a Teknivent Vector-One data acquisition system.

The quantification of formic acid and formamide in reaction mixtures supplied either isotopic or nonisotopic cyanide was determined following deproteinization of samples by treatment with 0.6 M ZnSO₄. For this purpose, 10 µl 6 M ZnSO₄ was added to 100 µl sample and protein removed by centrifugation in a microcentrifuge. Formic acid in the supernatant was then determined enzymically using commercial formate dehydrogenase as described previously (Kunz *et al.*, 1992). The identity of formic acid was further confirmed by GCMS. This was accomplished by injecting 10 µl of the reaction mixture onto an Alltech Associates AT1000 10 m megabore column (injector 110 °C, flow 6.5 ml min⁻¹ He, oven programmed at 40 °C for 2 min, then ramped to 110 °C at 10 °C min⁻¹) fitted to a mass spectrometer. The retention time was 9 min and masses of 46 (H¹²COOH) and 47 *m/e* (H¹³COOH) were detected, respectively, in incubations conducted with K¹²CN and K¹³CN. The presence of formamide in deproteinized samples was determined colorimetrically (Fry & Millar, 1972; Kunz *et al.*, 1992) and by chemical hydrolysis to formic acid, which was then determined enzymically. The latter procedure was used when the concentration of formamide was below that which could be detected colorimetrically (≤ 0.2 mM). Formamide hydrolysis was achieved by adding 10 µl 3 M H₂SO₄ to 90–100 µl deproteinized sample in a microcentrifuge tube and allowing the mixture to incubate at 95 °C for 2 h, after which the sample was neutralized with 5 M NaOH and assayed enzymically for formate.

Ammonia was determined colorimetrically using the indophenol method of Fawcett & Scott (1960). All spectrophotometric analyses were carried out on a Perkin-Elmer Lambda 6 spectrophotometer.

Oxidation of formic acid. The oxidation of formic acid to CO₂ was determined radioisotopically by fractionation of reaction mixtures with BaCl₂ as described earlier for ¹⁴CO₂ quantification from cyanide. Reactions were initiated by the injection of 1–2.5 µCi (37–92.5 kBq) sodium [¹⁴C]formate (0.5 mM), and NAD⁺, where supplied, was provided at 1 mM.

Formate dehydrogenase activity in cell extracts was measured spectrophotometrically as described by Höpner & Knappe (1974) and Kunz *et al.* (1992). Activity was measured anaerobically where indicated by flushing cuvettes with oxygen-free N₂ for 5 min prior to injecting HSS.

Chemicals. KCN (97 %), KOCN (99 %), K¹³CN (99 atom %) and sodium [¹³C]formate (99 atom %) were purchased from Aldrich. K¹⁴CN (54 mCi mmol⁻¹; 1998 MBq mmol⁻¹) and Na [¹⁴C]formate (49 mCi mmol⁻¹; 1813 MBq mmol⁻¹) were ob-

tained from DuPont (NEN Research Products). Tcn was prepared as previously described (Silva-Avalos *et al.*, 1990). All other chemicals were from standard commercial sources.

RESULTS

Recovery of CDA in cell-free extracts

As a first step in identifying the enzymes responsible for cyanide degradation in NCIMB 11764 efforts were made to recover CDA in cell-free extracts. As previously reported for intact cells (Kunz *et al.*, 1992), cell extracts catalysed the time-dependent disappearance of cyanide at concentrations as high as 100 mM (2600 mg CN⁻ l⁻¹). Fractionation of crude extracts into cytosolic (150 000 *g*; HSSs) and membrane (pellet) fractions revealed that the bulk (90 %) of CDA was located in the cytosolic fraction. Fig. 2 shows the rate at which cyanide was consumed when supplied at low concentration (2 mM) under various reaction conditions. The fastest rate of cyanide consumption was observed when both oxygen and NADH were supplied, but activity in the absence of NADH or in reactions conducted anaerobically could still be detected. Fig. 3 shows the effect of protein concentration on CDA measured at a starting concentration of 10 mM KCN. Under both aerobic (in the presence of NADH) and anaerobic (in the absence of NADH) conditions activity was protein-concentration dependent. Non-enzyme controls indicated that < 1 % of cyanide disappeared due to loss of volatile HCN during incubation. Approximately 50 % of the initial activity measured at 2 mM cyanide (cyanide oxygenase) was lost after 3–4 d storage at 4 °C, but when extracts were stored at -20 °C greater than 90 % of the initial activity was retained after 1–2 months.

Kinetics of cyanide conversion

The kinetics of cyanide degradation were investigated under various reaction conditions including: aerobically

Fig. 2. Time-course of consumption of 2 mM cyanide by HSSs from *P. fluorescens* NCIMB 11764 measured under the following reaction conditions: ○, oxygen and NADH both present; □, oxygen present, NADH absent; ●, both oxygen and NADH absent. Reactions were conducted as described in Methods. Data represent the mean and mean deviations from two separate experiments.

Fig. 3. Cyanide-degrading activity from *P. fluorescens* NCIMB 11764 measured as a function of protein concentration. Activities were measured at 10 mM KCN under aerobic conditions (with 20 mM NADH; ○) or anaerobic (without NADH; ●) conditions. Data represent the mean and mean deviations from two separate experiments.

Fig. 4. Kinetics of cyanide degradation by HSSs from *P. fluorescens* NCIMB 11764. Rates of cyanide disappearance were measured in different sealed vials containing HSS (9–10 mg protein ml⁻¹) and KCN at concentrations ranging from 1–100 mM. NADH where supplied was provided at a twofold excess over KCN. Reaction conditions: ○, aerobic with NADH present; □, aerobic with NADH absent; ●, anaerobic (without NADH). Insert: Lineweaver-Burk plot of initial aerobic (with NADH) rates (○) after subtracting anaerobic (without NADH) rates (●). Data shown are representative data from three separate experiments.

with NADH present, aerobically with NADH absent, and under anaerobic conditions (without NADH). Fig. 4 shows that the initial rate of cyanide consumption increased as a function of substrate concentration under all three reaction conditions. The highest rate of substrate consumption was again observed when both oxygen and

NADH were supplied, with maximal activity recorded at 20 mM cyanide; above this the activity declined probably due to enzyme inhibition. A second activity functioning in the absence of oxygen (and NADH) was also apparent at cyanide concentrations as high as 100 mM. Rates of substrate consumption measured aerobically in the absence of NADH fell between those recorded under aerobic and anaerobic conditions, suggesting that this activity represented the contribution of at least two enzymes. Noteworthy, rates of substrate consumption under all three reaction conditions appeared to follow Michaelis-Menten kinetics; this despite the fact that more than a single activity appeared to be present in HSSs. In order to further differentiate between the various activities, rates of substrate consumption were further compared after initial rates recorded under anaerobic conditions (without NADH) were subtracted from those obtained under aerobic conditions (with NADH present). In this way enzymes contributing to the total CDA in HSSs could be distinguished on the basis of favoured reaction conditions. This allowed the apparent kinetic constants (denoting the use of non-purified enzyme preparations) for the different enzymes to be estimated without each enzyme having to be individually purified. The insert to Fig. 4 shows the results obtained when the corrected data were replotted in the Lineweaver-Burk form. Dramatically different K_m values for cyanide were observed. Under aerobic conditions the apparent K_m was estimated at 1.2 mM compared with 12 mM under anaerobic conditions. We concluded from these observations that one enzyme in HSSs had a low apparent K_m and was both oxygen- and NADH-dependent, and a second enzyme had a high apparent K_m and required neither oxygen or NADH. In contrast to the differences observed in apparent K_m , the apparent V_{max} values for the two were found to be essentially identical (125 $\mu\text{M min}^{-1}$; Fig. 4 insert).

Reaction products and stoichiometries

In order to identify the enzymes showing different cyanide-degrading kinetics the reaction products formed after incubating HSSs with cyanide at concentrations favouring one or the other activity were determined. Accordingly incubations were conducted at 2 and 25 mM KCN, which were considered optimal, respectively, for the apparent low and high K_m activities. Table 1 shows that greater than 90% of the cyanide carbon was recovered as CO₂, formic acid and formamide under all reaction conditions. In contrast, the accumulation of cyanide-nitrogen equivalents as formamide plus ammonia varied substantially (39–93 molar%). This is because ammonia was apparently further metabolized, particularly under prolonged incubation periods when cyanide was supplied at low concentration (2 mM). Therefore, the molar ratios of formamide:ammonia accumulated at 2 mM KCN under aerobic (with NADH present) and anaerobic (without NADH) conditions were only 10:31 and 32:7 (41 and 39% molar recovery), respectively. This compares with values of 56:37 and 53:40, respectively, for corresponding reactions conducted at 25 mM.

As previously also reported using intact cells (Kunz *et al.*,

Table 1. Products of cyanide metabolism by cell-free extracts of *P. fluorescens* NCIMB 11764

Experiments were conducted in sealed vials containing air or N₂ in the gas phase. Reaction mixtures contained HSS (9–10 mg protein ml⁻¹) prepared from cyanide-grown cells and NADH, where indicated, at twice the concentration of KCN. After ≥ 90% of the cyanide was consumed, reaction products were determined by the analytical methods described in Methods. Data shown are the means and mean deviations from at least two separate experiments.

Reaction conditions	KCN consumed (mM)	Incubation time (h)	Products formed (nmol per µmol KCN)			
			CO ₂	Formic acid	Formamide	Ammonia
Aerobic (with NADH)	2	0.25	871 ± 51	60 ± 8	102 ± 1	308 ± 2
	25	5	488 ± 40	65 ± 42	370 ± 28	562 ± 88
Anaerobic (without NADH)	2	3	86 ± 11	692 ± 89	324 ± 105	67 ± 46
	25	10	230 ± 42	418 ± 110	405 ± 87	489 ± 116

1992), the relative yield of carbon compounds recovered after HSSs were incubated with cyanide varied with the reaction conditions and concentration of cyanide supplied. For example, the molar ratios of CO₂:formic acid:formamide measured at 2 and 25 mM KCN under aerobic conditions were 87:6:10 and 49:6:37. These results indicated that the major carbon-derived product obtained when both oxygen and NADH were supplied was CO₂. It was therefore concluded that an enzyme similar to cyanide oxygenase as previously described (Harris & Knowles, 1983b) was responsible for CO₂ formation. Because of the rapid consumption of cyanide by the putative cyanide oxygenase (15 min for 2 mM), it was further concluded that it was this enzyme that displayed the lower apparent *K_m* for cyanide as indicated from kinetic studies. By comparison, the molar ratios of products formed at 2 and 25 mM KCN under anaerobic conditions were 9:69:32 and 23:42:40, respectively, indicating that while CO₂ was still made (for reasons discussed below), the major reaction products were formic acid and formamide. Detection of the latter chemical species is consistent with the reaction products expected for cyanide nitrilase and hydratase enzymes as earlier proposed (Kunz *et al.*, 1992). The detection of CO₂ under anaerobic conditions was a surprise until it was discovered that formate was subject to further oxidation as outlined below.

Oxidation of formic acid by inducible formate dehydrogenase

The detection of CO₂ under various reaction conditions (Table 1) raised the possibility that in addition to its direct formation from cyanide, it could also arise from the oxidation of formate. To test this possibility, HSSs were incubated with [¹⁴C]formate and the production of ¹⁴CO₂ (and H¹⁴CO₃) was determined as described in Methods. Two separate experiments were conducted and the % molar yields of CO₂ are reported as the means and mean deviations. As much as 25 ± 2% of the formate supplied to HSSs was recovered as CO₂ after 2.5 h incubation; however, this was observed to increase to 88 ± 3% when reaction mixtures were supplied with NAD⁺. These

results suggested that formate oxidation was linked to an NAD⁺-dependent formate dehydrogenase present in HSSs. This was verified spectrophotometrically by observing the rapid formation of NADH when HSSs were added to reaction cuvettes containing both formate and NAD⁺. Activity was observed under both aerobic and anaerobic conditions, was protein-concentration dependent, and was inducible; the specific activity in HSSs from cyanide-grown cells was 14.0 ± 3.4 nmol NADH formed min⁻¹ (mg protein)⁻¹ compared with < 0.5 nmol min⁻¹ (mg protein)⁻¹ for cells grown on ammonia. The conversion of formate to CO₂ in 25% molar yield in incubations from which NAD⁺ was omitted was somewhat of a surprise. We hypothesized that this might be due to the presence of endogenous NAD⁺ in HSSs available for formate dehydrogenase activity. The removal of NAD⁺, therefore, should result in a decrease in the yield of CO₂, and in fact this was observed when HSSs were pretreated with NADase prior to incubation with [¹⁴C]formate. In this case the recovery of CO₂ from formate decreased to 4.8 ± 1.7%.

Effect of formate and NAD⁺ on cyanide oxygenase activity

The discovery of formate dehydrogenase in *P. fluorescens* NCIMB 11764 suggested that the NAD⁺-driven oxidation of formate might provide a means of generating the NADH utilized by cyanide oxygenase. To explore this possibility, the effect of NAD⁺ and formate on both cyanide oxygenase activity and the corresponding molar conversion yield of cyanide into CO₂ were tested, the rationale being that if these compounds were involved in NADH recycling an effect on both enzymic activity and production of CO₂ might be observed. Table 2 shows that when reactions were conducted with extracts that were pretreated with NADase, both cyanide oxygenase activity and the corresponding CO₂ yield were substantially lower than in comparable reactions in which untreated HSSs were employed. However, when reactions were supplemented with NAD⁺, both the CDA and CO₂ yield increased to 67 and 46% of maximal, and when both NAD⁺ and formate were supplied, these increased to 98

Table 2. Effect of formate and NAD⁺ on putative cyanide oxygenase activity in *P. fluorescens* NCIMB 11764

Reactions were conducted with 2 mM KCN in sealed vials with an air atmosphere. NADH, NAD⁺ and formate were supplied were provided at 4 mM. Data shown are the means and mean deviations from at least two separate experiments. Numbers in parentheses represent values relative to that obtained for reactions with NADH supplied.

Reaction conditions	Cyanide oxygenase [nmol min ⁻¹ (mg protein) ⁻¹]*	% Molar yield ¹⁴ CO ₂ from K ¹⁴ CN†
NADH	12.6 ± 1.4 (100)	87.1 ± 5.1 (100)
No NADH	2.6 ± 0.6 (21)	25.1 ± 2.8 (29)
NADase-treated HSS	1.2 ± 0.3 (10)	3.6 ± 0.8 (4)
NAD ⁺	8.5 ± 1.2 (67)	39.6 ± 0.6 (46)
Formate	2.4 ± 0.4 (19)	18.2 ± 2.4 (21)
NAD ⁺ + formate	12.4 ± 1.6 (98)	87.2 ± 1.3 (100)

* Calculated from initial rates of cyanide consumption by HSSs from cyanide-grown cells.

† Determined as the sum of volatile CO₂ and non-volatile HCO₃⁻ collected after ≥ 90% of the substrate was consumed.

and 100%, respectively. In contrast, the addition of formate alone had no significant effect on either enzymic activity or CO₂ production. A role for both formate and NAD⁺ in the recycling of NADH was thus inferred.

Isolation and characterization of cyanide metabolic mutants

Experiments were performed to determine whether one of the CDAs was more important physiologically for cyanide utilization. To accomplish this, a mutant strain

(JL102) that was no longer able to grow on cyanide was isolated and the effect on expression of cyanide oxygenase and cyanide nitrilase/hydratase activities was determined. Since the mutant was unable to grow, this necessitated testing for the induction of enzymic activity under non-growth conditions. For this purpose, cells were grown to stationary phase in a nitrogen-limiting medium and induced with cyanide as described in Methods. Under these conditions cyanide oxygenase could be detected in induced wild-type cells and, as shown in Table 3, the level of activity was actually higher than that detected in wild-type cells grown on cyanide. In contrast, the level of cyanide nitrilase/hydratase activities in induced cells of the wild-type was lower than in cultivated cells, suggesting that the regulation of synthesis of cyanide-degrading enzymes in NCIMB 11764 may be different. When HSSs from induced cells of the mutant strain JL102 were tested for cyanide oxygenase no activity was detected, but the level of cyanide nitrilase/hydratase enzymes was similar to that present in the wild-type. We concluded from these results that the inability of JL102 to grow on cyanide was related to its inability to produce cyanide oxygenase.

In order to determine the involvement of cyanate as a possible intermediate in cyanide metabolism as earlier proposed (Harris & Knowles, 1983b) a mutant strain (ON101) unable to grow on cyanate was isolated (Nagapan, 1992) and the effect on cyanide utilization determined. This mutant, while unable to grow on cyanate (phenotype designation Cnt⁻), could still utilize cyanide (Tcn or KCN, phenotype designation Cn⁺) as a nitrogen source. By comparison, strain JL102 having lost the ability to grow on cyanide (Cn⁻) was unaffected in cyanate utilization (Cnt⁺).

DISCUSSION

The recovery of putative cyanide oxygenase and cyanide nitrilase/hydratase enzymes in HSSs is consistent with an

Table 3. Synthesis of CDAs in wild-type and mutant strains of *P. fluorescens* NCIMB 11764

Data shown are the means and mean deviations from at least two separate experiments using HSSs prepared from cells cultivated under the following conditions.

Strain	Cultivation conditions*	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹ †	
		Cyanide oxygenase	Cyanide nitrilase/hydratase
Wild-type	Cyanide-grown	12.6 ± 1.4	11.8 ± 1.3
	Cyanide-induced	29.5 ± 2.5	1.7 ± 0.5
	Ammonia-grown	< 0.5	< 0.5
JL102	Cyanide-induced	< 0.5	1.3 ± 0.4
	Ammonia-grown	< 0.5	< 0.5

* Procedures for the growth and induction of cells are described in Methods.

† Measured as initial rates of cyanide disappearance. Cyanide oxygenase activity was measured aerobically (with NADH present) at 2 mM KCN and was uncorrected for cyanide nitrilase/hydratase activity measured anaerobically (without NADH) at 25 mM KCN.

Fig. 5. Pathways of cyanide transformation in *P. fluorescens* NCIMB 11764 showing the role of cyanide oxygenase (1), cyanide nitrilase (2) and formate dehydrogenase (3) in regenerating pyridine nucleotide cofactors.

earlier report from this laboratory indicating that cyanide is metabolized by more than a single mechanism in *P. fluorescens* NCIMB 11764 (Kunz *et al.*, 1992). Results showing that one of these, cyanide oxygenase, generated CO_2 (and NH_3) as reaction products (Table 1), and that activity was dependent both on oxygen and NADH, find analogy with previous reports in which initial cyanide degradation was proposed to be oxygenase-mediated (Harris & Knowles, 1983b). We have further independently confirmed the findings of Harris & Knowles (1983b) in which 1 mol oxygen and 1 mol NADH were reported to be consumed per mol cyanide degraded by this enzyme (C. S. Wang & D. A. Kunz, unpublished results). Unequivocal proof, however, that catalysis is oxygenase-mediated will require that the incorporation of ^{18}O into the product be demonstrated, but this may not be possible since CO_2 rapidly exchanges its oxygens with water (Pocker & Bjorkquist, 1977; Knowles, 1988). On the other hand, results showing that formate oxidation could be coupled to putative cyanide oxygenase activity (Table 2), apparently by recycling NADH (Fig. 5), further imply the involvement of an oxygenase-type mechanism.

The description of a combined activity containing both cyanide nitrilase/hydratase enzymes in cell-free extracts is consistent with previous work in which formic acid (and NH_3) and formamide were identified as additional cyanide conversion products (Kunz *et al.*, 1992). The increased production of these compounds relative to CO_2 (and NH_3) at higher substrate concentrations (≥ 10 mM) (Table 1) can now be better explained on the basis of differences in substrate affinity between cyanide oxygenase and the nitrilase/hydratase enzymes. Thus, cyanide oxygenase, with an estimated K_m of 1.2 mM, attacks cyanide more effectively at lower substrate concentration than does the combined activity of nitrilase/hydratase enzymes for which a combined apparent K_m of 12 mM was estimated (Fig. 4). Although the apparent K_m values of the two are different, the catalytic rates of cyanide turnover for each are the same. At present, we are unable to say for certain whether the CDA having cyanide nitrilase/hydratase activities consists of one or two enzymes. Data showing no inconsistencies in the apparent Michaelis-Menten kinetics of this activity (Fig. 4) suggest that only one enzyme is involved, but this does not agree with the observation that both formic acid (and NH_3) and

formamide accumulate, and the fact that formamide, as shown previously (Kunz *et al.*, 1992), is not further degraded. Thus, our working hypothesis is that there are two enzymes, a cyanide nitrilase (dihydratase) generating formic acid (and NH_3), and a cyanide hydratase (EC 4.2.1.66) giving formamide. Both enzymes would be expected to function in the absence of oxygen, which is consistent with our observations. The occurrence of cyanide nitrilase in NCIMB 11764 finds analogy with several reports of related enzymes in cyanide-degrading bacteria (White *et al.*, 1988; Ingvorsen *et al.*, 1991; Meyers *et al.*, 1993). These enzymes are believed to catalyse the direct hydrolysis of cyanide into formic acid and ammonia without the involvement of formamide as an intermediate. As such, they resemble true nitrilases (EC 3.5.5.1), but with a substrate range limited to cyanide. In contrast to cyanide nitrilases, cyanide hydratases have thus far been described only in phytopathogenic fungi (Fry & Millar, 1972; Fry & Evans, 1977; Fry & Myers, 1981), making the description of such an enzyme in NCIMB 11764 the first of its kind in bacteria. However, additional studies are needed before firm conclusions regarding the description of this and other enzymes elaborated by NCIMB 11764 can be made. Towards this end, efforts aimed at separating and purifying these enzymes are currently in progress.

While the involvement of formate dehydrogenase in cyanide metabolism has previously been proposed (Knowles, 1976; Knowles & Bunch, 1986), until now, direct evidence for this has not been provided. Its description thus adds a new dimension to cyanide metabolism by NCIMB 11764. As shown in Fig. 5, the production of CO_2 can now be visualized as arising by two separate routes; one from the oxidation of cyanide by cyanide oxygenase and the other from the oxidation of formic acid, the latter arising via cyanide nitrilase activity. Data showing that both formate and NAD^+ enhanced cyanide oxygenase activity (Table 2) suggest that formate dehydrogenase could be involved in the recycling of NADH as further depicted in Fig. 5. It appears this enzyme is similar to other NAD^+ -linked formate dehydrogenases described in bacteria (Quayle, 1966; Ferry, 1990), but the first of its kind reported to be induced by cyanide. We hypothesize that this enzyme may be important in preserving NADH under conditions of cyanide poisoning. For example, in the presence of cyanide (serving as a nitrogen source) the extent of NADH utilization generated from glucose (serving as a carbon and energy source) via respiratory chain enzymes would be expected to be inhibited by cyanide (Solomonson, 1981). Therefore, the consumption of NADH by a non-energy yielding reaction such as that catalysed by cyanide oxygenase would be expected to compromise energy yields and thus cell growth, but this may be overcome if the NADH recycling mechanism proposed for formate dehydrogenase operates. The inhibitory effect of cyanide on respiration is further thought to explain why growth of NCIMB 11764 at concentrations exceeding 0.25 mM is not observed (Silva-Avalos *et al.*, 1990).

In summary, growth of *P. fluorescens* NCIMB 11764 on

cyanide leads to the elaboration of several CDAs. Of these, cyanide oxygenase appears most important in initiating substrate attack at concentrations favourable for growth (≤ 0.25 mM). As a result cyanide is detoxified and ammonia is made available for assimilation by what are presumed to be established biochemical mechanisms (Tyler, 1978; Neidhardt *et al.*, 1990). The fundamental importance of cyanide oxygenase in cyanide utilization is further illustrated by results showing that this enzyme is not produced in a mutant strain no longer able to grow on cyanide (Table 3). While it could be argued that cyanide nitrilase on its own should be sufficient to support growth because it too generates ammonia, we hypothesize that its low substrate affinity precludes it from functioning exclusively for this purpose. Thus, in contrast to cyanide oxygenase the roles of cyanide nitrilase and cyanide hydratase in NCIMB 11764 remain to be further elucidated. In addition, the observation that a mutant defective in cyanate utilization can still grow on cyanide (phenotype Cn^+Cnr^-) finds analogy with earlier reported enzyme-induction studies (Dorr & Knowles, 1989; Nagappan, 1992) indicating that the enzymes involved in cyanide and cyanate metabolism are physiologically different.

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