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Isolation and Characterization of a Cyanide Dihydratase from *Bacillus pumilus* C1

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A cyanide-degrading enzyme from *Bacillus pumilus* C1 has been purified and characterized. This enzyme consisted of three polypeptides of 45.6, 44.6, and 41.2 kDa; the molecular mass by gel filtration was 417 kDa. Electron microscopy revealed a multimeric, rod-shaped protein approximately 9 by 50 nm. Cyanide was rapidly degraded to formate and ammonia. Enzyme activity was optimal at 37°C and pH 7.8 to 8.0. Activity was enhanced by Sc^{3+}, Cr^{3+}, Fe^{3+}, and Tb^{3+}; enhancement was independent of metal ion concentration at concentrations above 5 μM. Reversible enhancement of enzymatic activity by azide was maximal at 4.5 mM azide and increased with time. No activity was recorded with the cyanide substrate analogs CNO^-, SCN^-, CH_3CN, and N_3^- and the possible degradation intermediate $HCONH_2$. Kinetic studies indicated a K_m of 2.56 ± 0.48 mM for cyanide and a V_{max} of 88.03 ± 4.67 mmol of cyanide per min/mg/liter. The K_m increased approximately twofold in the presence of 10 μM Cr^{3+} to 5.28 ± 0.38 mM for cyanide, and the V_{max} increased to 197.11 ± 8.51 mmol of cyanide per min/mg/liter. We propose naming this enzyme cyanide dihydratase.

The high cost of chemical detoxification of cyanide-containing industrial wastewater has generated interest in the biological detoxification of these wastes (18). Despite the well-known toxicity of cyanide to a number of cellular processes (30, 31, 34), cyanide occurs as a common metabolite in various plants, fungi, and microorganisms (3, 4, 14, 18, 19, 24, 26). Several biochemical pathways have been suggested for the degradation of cyanide in bacteria (18). *Bacillus pumilus* (29), *Pseudomonas fluorescens* NCIMB 11764 (13, 15), and *Pseudomonas paucimobilis* (36) have all been shown to convert cyanide to ammonia and carbon dioxide. In the case of *P. fluorescens* NCIMB 11764, this reaction may proceed by means of an NAD(P)H-dependent dioxygenase (6, 15). The cyanide hydratase (formamidehydrolyase [EC 4.2.1.66])-catalyzed hydration of cyanide to formamide has been reported to occur in fungi (9–11) as well as in *P. fluorescens* NCIMB 11764 (20). Additionally, *P. fluorescens* NCIMB 11764, as well as an unidentified pseudomonad isolated from coke plant-activated sludge (35), has been shown to degrade cyanide to formate and ammonia under both aerobic and anaerobic conditions. Little work has been carried out on the enzymes responsible for cyanide degradation, although a Cyanidase (Novo Nordisk A/S) catalyzing the hydrolysis of cyanide to formate and ammonia has been purified from a strain of *Alcaligenes xylosoxidans* subsp. *denitrificans* (17). In addition, the cyanide hydratase from the phytopathogenic fungus *Gloeocercospora sorghi* has been purified and characterized (32). The gene encoding the 45-kDa polypeptide subunits of this enzyme has been cloned and characterized (33).

We have previously described an efficient cyanide-degrading bacterium, *B. pumilus* C1, isolated from a cyanide wastewater dam (23). We wish to investigate the properties of the enzyme responsible for this activity in order to assess its commercial application to the detoxification of cyanide-containing wastewaters from the mining industry. In this paper we report the purification and characterization of this enzyme, which was found to be a cyanide dihydratase catalyzing the conversion of cyanide to formate and ammonia.

MATERIALS AND METHODS

Materials. DEAE-cellulose was obtained from Whatman, Maidstone, United Kingdom; Bio-Gel A-5m was obtained from Bio-Rad Laboratories, Hemel Hempstead, United Kingdom; and Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Formate dehydrogenase was from Sigma, St. Louis, Mo. Deionized water (filtered through a Millipore Milli-RO4 apparatus) was used throughout these studies.

Culture conditions. *B. pumilus* C1 was grown as described previously (23) with the exception that Luria broth was used as the culture medium because it supported vigorous growth of the organism without exogenous Mn^{2+}. Some batches of medium required the addition of 0.25 μM Mn^{2+} for maximal cyanide-degrading activity of the bacteria. Experiments to determine whether Fe^{3+} or Cr^{3+} could replace Mn^{2+} as the effector of cyanide-degrading activity were carried out by growing *B. pumilus* C1 in Oxoid nutrient broth as described previously (23) before determining the cyanide-degrading activity of the whole bacteria.

$^{54}Mn^{2+}$ (91.4 μCi) or $^{51}Cr^{3+}$ (87.7 μCi) was added to the culture medium for experiments investigating whether the cyanide dihydratase was a metalloenzyme containing these metal ions. The enzyme was purified as described below.

Enzyme assay. Enzymatic activity was determined in a volume of 0.12 ml in 10 mM Tris-HCl–50 mM NaCl (pH 8.0) in duplicate sealed tubes incubated for 1 h at 37°C; 1 U of activity was defined as the degradation of 1 μmol of cyanide per min. Residual cyanide was determined by the absorption of the picric acid complex at 520 nm (8). To determine the effects of various metal ions on the enzymatic activity, the enzyme was initially dialyzed against 10 mM Tris-HCl–50 mM NaCl–5 mM EDTA (one change) and then against 10 mM Tris-HCl–50 mM NaCl–0.1 mM EDTA. Each metal ion

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was then added to a final concentration of 200 μM free metal ion, and the cyanide-degrading activity was determined.

To determine whether cyanide could be degraded to CO_2, 5 mM cyanide was incubated with the purified enzyme in the presence of 1 μCi of $K^{14}CN$ for 1 h at 37°C. These incubation conditions permitted complete degradation of cyanide as determined by the picric acid assay. The radioactivity present in triplicate 1-μl samples was measured with a Beckman LS 1701 liquid scintillation counter before and after the incubation as well as after the incubated samples had stood uncovered for 1 h at room temperature.

Freshly purified enzyme was used for all characterization experiments. For the determination of the effects of various concentrations of thiocyanate, formate, and acetate on enzymatic activity, the enzyme was incubated at 37°C for 1 h in the presence of 5 mM KCN together with increasing concentrations of the relevant test compounds. After incubation, the residual cyanide was determined as described above. The decrease in the A_{520} of each set of samples (relative to that of appropriate controls) was expressed as a percentage of the decrease in the A_{520} of the enzyme controls in each case. The effects of various azide concentrations were determined in a similar way, except that the samples were incubated for 16 h at 4°C in the presence of various concentrations of azide before cyanide-degrading activity was measured.

Protein purification. All purification steps were carried out at 4°C with 10 mM Tris-HCl–50 mM NaCl (pH 8) as the buffer. The cell extract from 800 ml of bacterial culture (23) was applied to a Whatman DE 52 cellulose column (1.6 by 23 cm) and eluted with a linear 250 to 360 mM NaCl gradient (245 ml of each). Fractions containing cyanide-degrading activity were pooled, concentrated by ultrafiltration (Amicon PM 10 membrane), and applied to a Bio-Gel A-5m agarose column (1.6 by 90 cm). The active fractions were again pooled and concentrated before being applied to a 12 to 30% sucrose gradient. After centrifugation for 16 h (SW40 Ti rotor, 35,000 rpm), the gradient was fractionated into 5-drop fractions and assayed for activity. Calibration of the gel filtration column was performed with proteins of known molecular weight (apoferritin, β-amylase, alcohol dehydrogenase, and bovine albumin). It was important to use the amount of bacterial culture stated for the column sizes described; the cell extract from 200 ml of culture eluted from the DE 52 column with a complete loss of enzymatic activity. We interpreted this loss of activity to be due to dilution, since that amount of activity should have been readily detected in the column eluate.

pH and temperature optima. Determination of the pH optimum was carried out by dilution of the purified enzyme into solutions of 600 mM Tris-HCl–50 mM NaCl buffered at pH values between 7.0 and 9.0. The resulting enzyme solutions were then mixed with unbuffered 2 mM KCN in a total volume of 0.12 ml. Shadow experiments, in which the components were mixed in the same proportions, showed that the pH was controlled at the expected value. Triplicate enzyme samples at each pH were incubated at 37°C for 1 h, after which the residual cyanide was determined as described above. The sample showing the greatest decrease in A_{520} (relative to that of duplicate controls at the same pH) was taken as having 100% activity, and the changes in A_{520} of all the other samples were expressed as a percentage of that of the most active sample.

Determination of the temperature optimum for enzymatic activity was carried out by incubating triplicate samples of purified enzyme, buffered at pH 8.0, for 10 min at the stated temperatures before adding KCN and assaying the enzymatic activity at the same temperature for a further 1 h. Residual cyanide was measured as described above. The sample showing the greatest decrease in A_{520} was taken as having 100% activity.

Other assays. Ammonia production was assayed by the A_{570} of the nitroprusside complex (7) in 100 mM phosphate–50 mM NaCl (pH 8) because Tris-HCl prevented color development. Cyanate was assayed by the A_{680} of the dicyanatodipyridine copper(II) complex (22). Thiocyanate was determined by the A_{490} of the ferric complex (1). Formamide was converted to hydroxamic acid and determined by the A_{570} of the resulting ferric complex (10, 16). Formate was determined by the reduction of NAD^+ with formate dehydrogenase (25).

PAGE. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed on 15% polyacrylamide gels according to the method of Laemmli (21). Proteins were stained with Page Blue G90 after electrophoresis. The molecular masses of the enzyme polypeptides were determined by comparison with the mobilities of standard proteins of known molecular mass.

Electron microscopy. A sample from the sucrose gradient was dialyzed against 10 mM Tris-HCl–50 mM NaCl to remove the sucrose. Lower-ionic-strength buffers could not be used because enzymatic activity was lost; thus, dialysis against 1 mM Tris-HCl–10 mM NaCl resulted in a loss of 80% of the enzymatic activity. After dialysis, the sample was concentrated by dialysis against dry polydextran (Sephadex G-200), applied to Formvar-carbon-coated grids, rinsed with distilled water, and stained with phosphotungstic acid (1%), pH 6.2. The prepared samples were examined with a Hitachi-600 electron microscope.

RESULTS

The cyanide-degrading enzyme was purified by column chromatography and sucrose gradient centrifugation (Fig. 1a to c) as described above. The cyanide-degrading activity was purified 42-fold by this procedure with an overall yield of 2.3%; the purification data are summarized in Table 1. The enzyme responsible for this activity had a molecular mass of approximately 417 kDa by gel filtration chromatography. Analysis of the purified protein by SDS-PAGE, however, indicated that the active enzyme consisted of three polypeptides of 41.2, 44.6, and 45.6 kDa (Fig. 1d) which copurified with the same ratio to one another throughout the purification procedure, suggesting that the enzyme was a multimeric complex. When the purification was carried out without stringent temperature control, SDS-PAGE of the purified enzyme revealed, in addition to the normal three bands, a further three faster-migrating bands of markedly lower intensity but with the same relative pattern of staining intensity. This reinforced evidence for the proposal that the holoenzyme consisted of multiples of a basic structure containing three polypeptide chains, rather than the possibility that the original bands arose from proteolysis of a larger monomeric protein.

Examination of the purified enzyme by electron microscopy (Fig. 2) revealed particles of heterogenous sizes and shapes. Tightly packed spiral structures with diameters of approximately 9 nm and lengths of approximately 50 nm could be readily observed on the phosphotungstic acid-stained grid (Fig. 2a). Many shorter, tightly packed spiral structures (Fig. 2b), as well as some smaller particles (Fig. 2b and c), were also visible. In addition, arrays of six of the

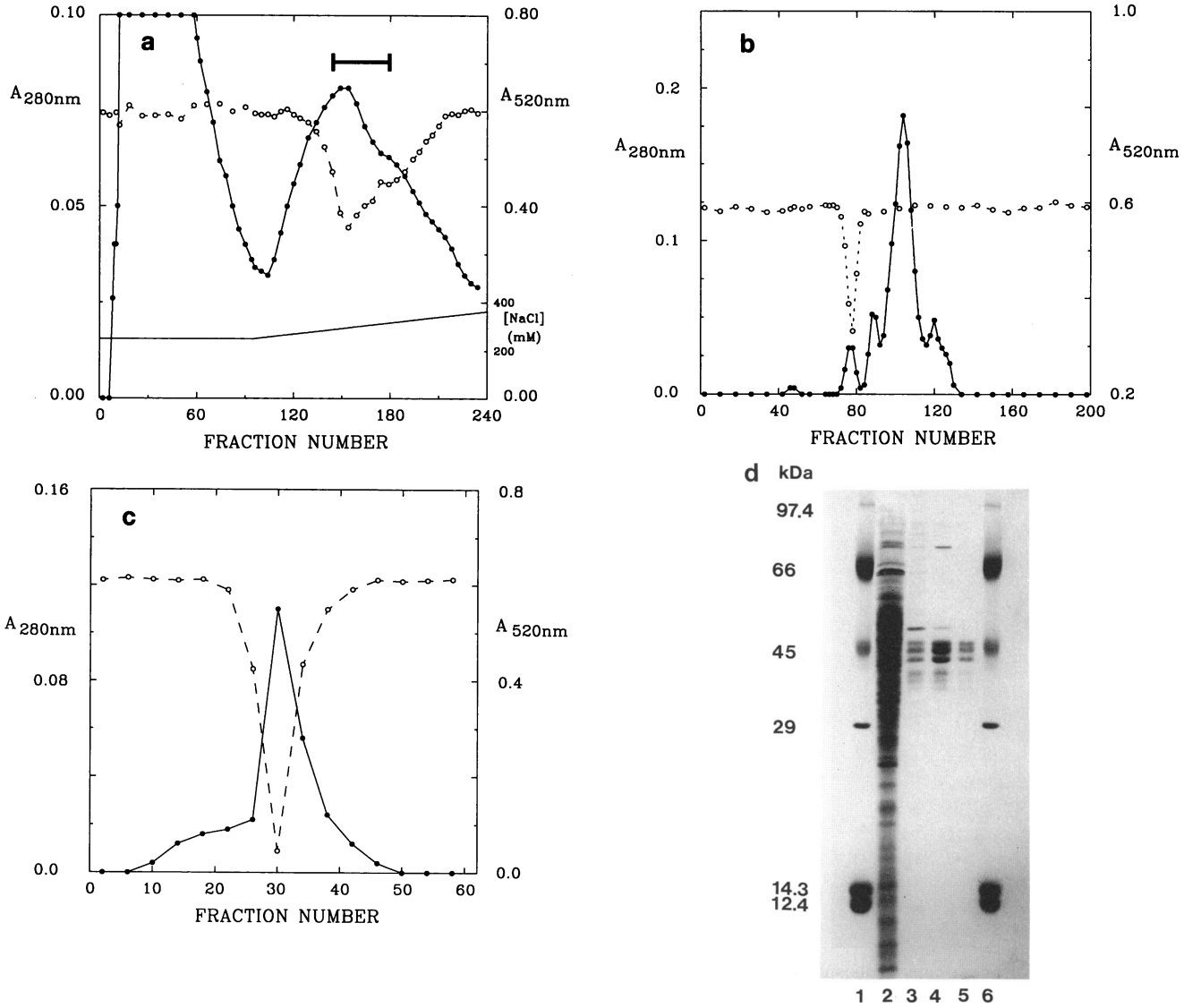

FIG. 1. (a) Whatman DE 52 chromatography (in 10 mM Tris-HCl, pH 8.0) of the *B. pumilus* C1 cell extract. The sample was loaded at 50 mM NaCl, the column was washed with 250 mM NaCl, and a 250 to 360 mM NaCl gradient was used to elute the enzymatic activity. Samples were assayed for protein content (●) and cyanide-degrading activity (○). Fractions 144 to 179 were pooled (bar), concentrated by ultrafiltration, and applied to a Bio-Gel A-5m agarose column. (b) Bio-Gel A-5m chromatography (in 10 mM Tris-HCl–50 mM NaCl, pH 8.0) of the pooled fractions from the Whatman DE 52 column shown in panel a. Samples were assayed for protein content (●) and cyanide-degrading activity (○). Fractions 63 to 76 were pooled and concentrated by ultrafiltration. Further purification was effected by sucrose gradient centrifugation. (c) Sucrose gradient centrifugation (12 to 30% in 10 mM Tris-HCl–50 mM NaCl, pH 8.0) of the pooled fractions from the Bio-Gel A-5m column shown in panel b. The gradient was fractionated into 5-drop fractions while the A_{280} was monitored (●). Every other fraction was assayed for cyanide-degrading activity (○). (d) SDS-PAGE of steps during the purification of cyanide dihydratase. Lanes: 1 and 6, molecular mass standards (phosphorylase *b*, bovine albumin, ovalbumin, carbonic anhydrase, lysosyme, and cytochrome *c*, in order of descending molecular mass); 2, cell extract; 3, DE 52 eluate; 4, Bio-Gel A-5m eluate; 5, peak fraction from the sucrose gradient.

smallest particles, in close association (Fig. 2b), were visible. These possibly arose by fragmentation of the spiral structure on application to the grid.

Although separation of the constituent polypeptide chains could be achieved by SDS-PAGE, we were unable to recover the individual chains by electroelution from the gel. C_{18} reverse-phase high-pressure liquid chromatography in 0.1% trifluoracetic acid was unsuccessful in separation of the protein into its constituent polypeptide chains, with all the bands coeluting at 60% acetonitrile. Accordingly, the entire

TABLE 1. Purification data

Fraction	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	225.0	272.9	1.2	100	1
DE 52 eluate	3.0	48.6	16.4	17.8	13.5
A-5m eluate	0.7	20.7	30.0	7.6	24.8
Sucrose gradient	0.12	6.3	50.9	2.3	41.9

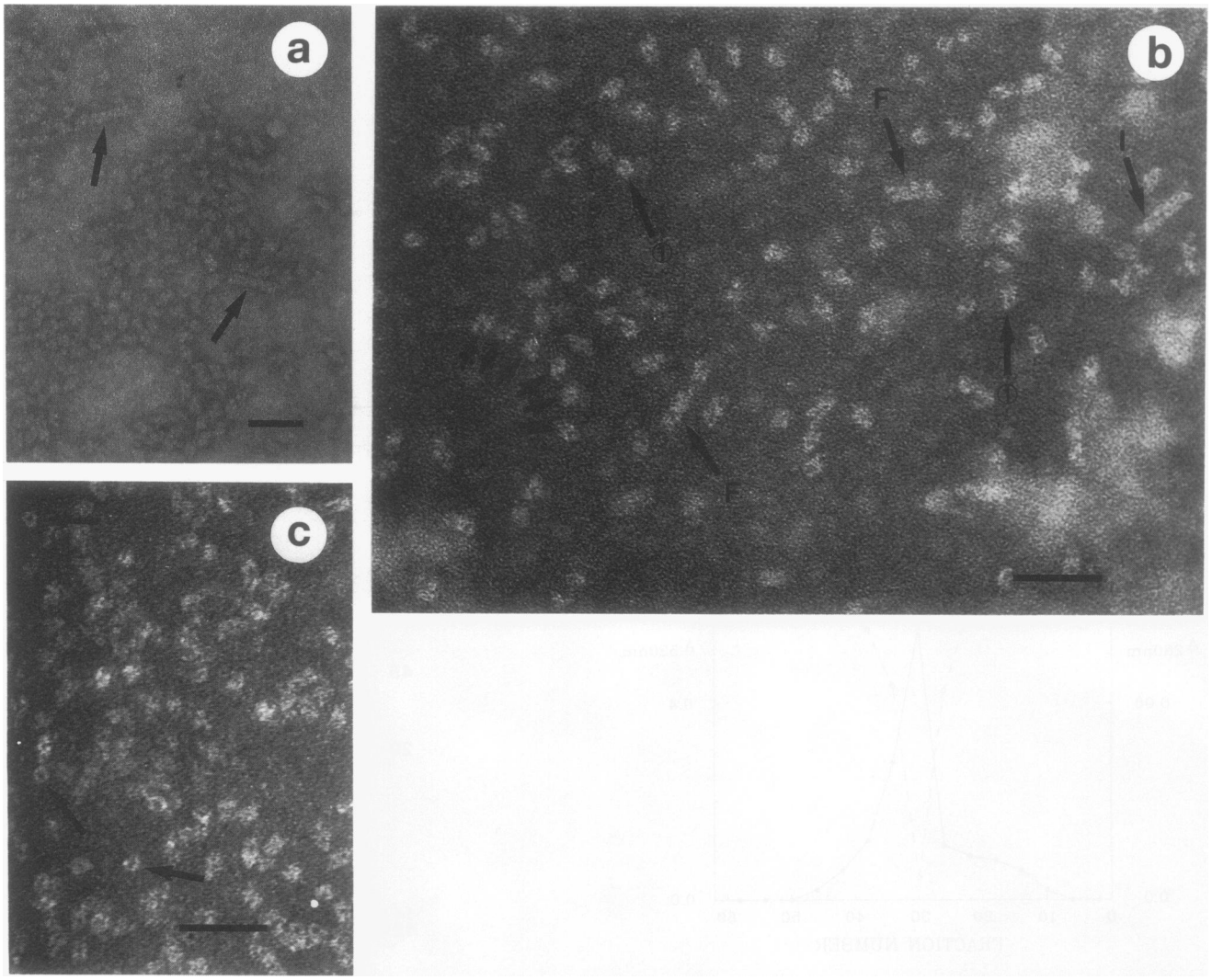

FIG. 2. Electron microscopy of phosphotungstate-stained cyanide dihydratase after sucrose gradient purification. Bars, 50 nm. (a) An area of the grid that contained several tightly packed spiral structures (arrows). Magnification, ×151,500. (b) An area of the grid that contained predominantly fragmented structures (F), although one intact tightly packed spiral structure (I) is visible. Monomers (→) and a hexameric array of monomeric particles are also visible. The latter structures may have arisen by fragmentation of the putative native tightly packed spiral structure; one such hexamer is indicated by unlabeled arrows. Magnification, ×244,000. (c) An area of the grid that contained predominantly monomeric structures. Three such monomers are indicated by arrows. Magnification, ×247,000.

protein was subjected to gas phase sequencing. Only one sequence was found: Thr-Ser-Ile-Tyr-Pro-Lys-Phe-X-Ala-Ala-X-Val-Gln. Possible reasons for the presence of only a single sequence are that two of the three polypeptide chains are blocked at the N terminus, that the three chains arose by gene duplication and have identical N termini, and that the three chains are transcribed from the same gene but are subjected to different posttranslational modifications.

Degradation of cyanide and related compounds. Since the ultimate sucrose gradient purification step increased the specific activity only 1.7-fold but resulted in the loss of 70% of the material, most kinetic analyses were carried out by using the A-5m column eluate. The purified cyanide-degrading enzyme was found to act as a nitrilase, converting cyanide to formate and ammonia according to the following equation: $HCN + 2H_2O \rightarrow HCOO^- + NH_4^+$. The production of formate (assayed by the formate dehydrogenase-catalyzed reduction of NAD^+) was equivalent to 83% of the decrease in the cyanide concentration. Although ammonia was present after cyanide degradation, the amount detected was not stoichiometric with the amount of formate produced, suggesting that some ammonia was stripped from solution. In order to demonstrate that cyanide was not converted to CO_2 plus NH_4^+, 5 mM cyanide was incubated with the purified enzyme in the presence of 1 μCi $K^{14}CN$. It was found that >95% of the radioactivity remained in solution after all the cyanide had been degraded, thereby eliminating CO_2 as a possible reaction product. Since the addition of only one molecule of water to cyanide would result in the formation of formamide, we investigated the relationship between this enzyme and formamide. We found that even small quantities of formamide could not be detected after cyanide degradation. Moreover, the purified enzyme was unable to degrade formamide, and the presence

TABLE 2. Effect of thiocyanate on cyanide dihydratase activity[a]

[Thiocyanate] (mM)	Activity (%)
0	100.0
2.5	114.7
5	113.1
10	110.4
20	104.0
40	99.2
80	92.4
160	62.5

[a] Cyanide dihydratase activity was determined by the relative degradation of 5 mM cyanide in 1 h at 37°C in the presence of the indicated concentrations of thiocyanate.

of formamide during the degradation of cyanide had no effect on the rate of cyanide utilization. It would therefore appear that if formamide is a reaction intermediate in the catalysis of cyanide to formate, it does not leave the active site of the enzyme. The enzyme was also unable to hydrolyze cyanate (CNO^-) and thiocyanate (SCN^-), nor did the enzyme show any ability to degrade azide (measured spectrophotometrically at 250 nm) or acetonitrile (as assayed by the generation of ammonia). We therefore propose naming the enzyme cyanide dihydratase to indicate its substrate specificity and mode of action. The effect of the presence of thiocyanate on cyanide degradation was investigated in order to determine whether this structurally similar compound was a competitive inhibitor. Surprisingly, up to 20 mM thiocyanate was found to slightly enhance the enzymatic activity; thiocyanate above this concentration was found to inhibit the enzyme (Table 2).

The initial velocity of cyanide degradation by cyanide dihydratase in 10 mM Tris-HCl–50 mM NaCl–1 mM EDTA (pH 8) at 37°C was investigated over the substrate concentration range 0.8 to 10.0 mM KCN. A Lineweaver-Burk plot (Fig. 3a) showed a linear response over this concentration range; the Michaelis constant (K_m) was 2.56 ± 0.48 mM, and the maximal velocity (V_{max}) was 88.03 ± 4.67 mmol/min/mg/liter.

Requirement for Mn^{2+} and effects of other metal ions. *B. pumilus* C1 has previously been shown to require Mn^{2+} in the growth medium for the production of cyanide-degrading activity (23). Maximal enzymatic activity was found when greater than 0.2 μM Mn^{2+} was present; the absence of Mn^{2+} also resulted in poorer bacterial growth. To investigate whether cyanide dihydratase was a manganese-containing metalloenzyme, bacteria were grown in the presence of $^{54}Mn^{2+}$ and the enzyme was purified as described above. Despite the uptake of 97.7% (89.3 μCi) of the isotope by the bacteria, no radioactivity was found associated with the purified enzyme. Moreover, extensive dialysis against 10 mM Tris-HCl–50 mM NaCl–5 mM EDTA (pH 8) to remove any bound metal ions had no significant effect on enzymatic activity.

Since a wide variety of metal ions are known to interact with cyanide, forming both simple binary salts and multiligand complexes, we investigated the effects of some metal ions on the rate of cyanide degradation by cyanide dihydratase. Table 3 summarizes the results for the various metal ions tested. It was clear that the trivalent metal cations Sc^{3+}, Fe^{3+}, Cr^{3+}, and Tb^{3+} all enhanced the enzymatic activity approximately twofold, whereas Hg^{2+} and, to a lesser extent, Pb^{2+} and Ag^+ were inhibitory. Inhibition by Hg^{2+} suggested the presence of a sulfydryl moiety at or near the active site. Since 200 μM Cr^{3+} caused a significant enhancement of enzymatic activity, we investigated the relationship between this metal ion and cyanide dihydratase. It was found that maximum enhancement of enzymatic activity occurred when 5 μM Cr^{3+} was present in the assay (Fig. 4). Neither Cr^{3+} nor Fe^{3+} at 0.5 μM could replace Mn^{2+} as the effector of cyanide-degrading activity during overnight growth of *B. pumilus* C1. Despite the fact that growth of *B. pumilus* C1 in Luria broth containing $^{51}Cr^{3+}$ resulted in the uptake of 48% (42.1 μCi) of the radioactivity, purification of cyanide dihydratase resulted in no radioactivity being recovered with the purified enzyme. Moreover, the purified enzyme failed to bind $^{51}Cr^{3+}$ as determined by equilibrium dialysis. Analysis of the catalytic parameters for the enzyme in the presence of 10 μM Cr^{3+} (Fig. 3b) showed that the K_m for cyanide increased by a factor of 2.1 (to 5.28 ± 0.38 mM) and that the V_{max} increased by a factor of 2.2 (to 197.11 ± 8.51 mmol/min/mg/liter).

Characterization. Optimal enzymatic activity was found to

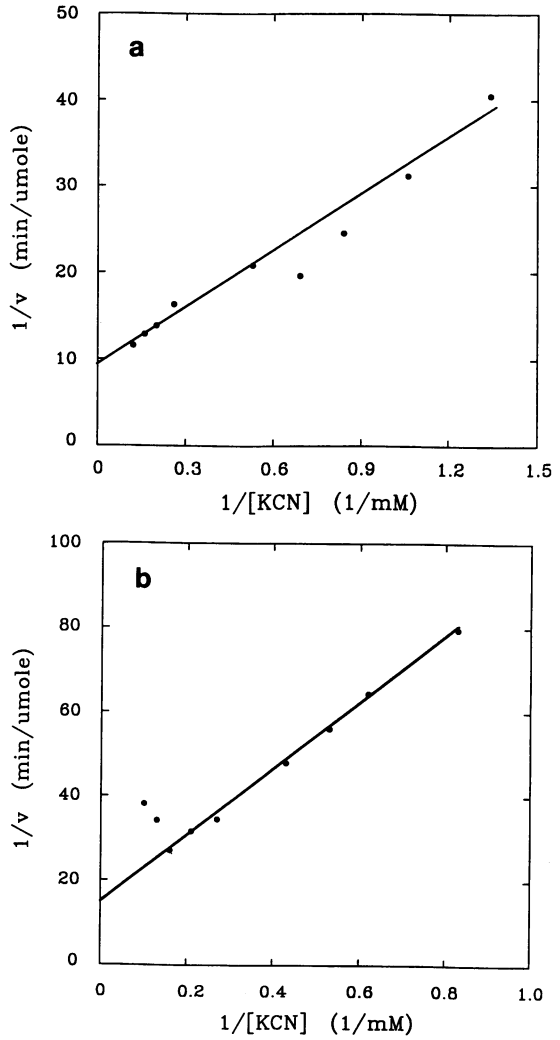

FIG. 3. Lineweaver-Burk plots of the reciprocals of initial velocity versus substrate concentration for cyanide dihydratase in the absence (a) and presence (b) of 10 μM Cr^{3+}. The data shown are the averages of two independent experiments with all measurements carried out in duplicate. A Bio-Gel A-5m eluate enzyme preparation was used for these determinations.

TABLE 3. Effect of 200 μM concentrations of various metal ions on cyanide dihydratase activity[a]

Metal ion	Activity (%)
None	100
Sc^{3+}	190
Fe^{3+}	190
Cr^{3+}	177
Tb^{3+}	174
Co^{2+}	87
Cd^{2+}	66
Hg^{2+}	7
Pb^{2+}	23
Ag^{+}	54

[a] Cyanide dihydratase activity was determined by the relative degradation of 5 mM cyanide in 1 h at 37°C in the presence of the metal ions indicated. The following tested metal ions had no effect: Mg^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, and Mn^{2+}.

occur between pH 7.8 and 8.0 (Fig. 5a); increasing the pH above 8 had a dramatic effect on the activity, with no activity being exhibited above pH 8.4. The presence of the trivalent metal ion Cr^{3+} or Tb^{3+} at a concentration of 200 μM, however, protected the enzyme against the inhibitory effect of increasing the pH. Thus, the enzyme displayed identical activity in the presence of these metal ions at pH 8.0 and 8.6. The optimum temperature for cyanide degradation was found to be 37°C (Fig. 5b), with the activity rapidly lost at temperatures above 40°C. The enzyme was most stable at around 22°C, a temperature considerably lower than that optimal for activity. Enzymatic activity was reduced to 50% by 0.5 M urea and was completely inhibited by SDS concentrations above 0.01%, but it was unaffected by NaCl concentrations up to 3 M.

Addition of 0.02% sodium azide as a bacteriostat to solutions containing cyanide dihydratase was found to increase the specific activity of the enzyme. This enhancement increased gradually over a period of hours and was concentration dependent (Table 4), with maximal stimulation observed with 4.5 mM azide; removal of the azide by dialysis restored the specific activity to its original level, and readdition of azide once again increased the specific activity (Table 5). High concentrations of azide (150 mM and above) inhibited the enzyme (Table 4). Formate and acetate in the millimolar concentration range were also found to stimulate

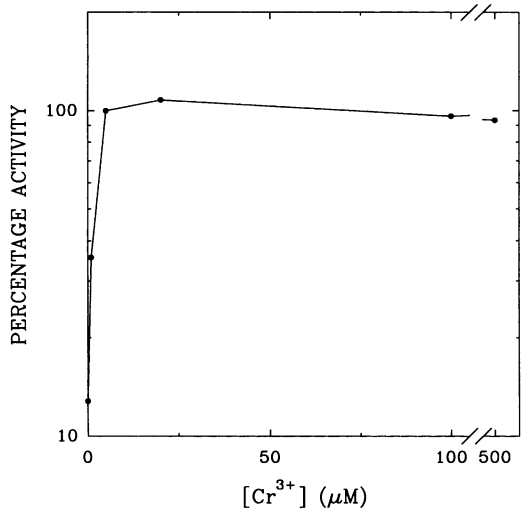

FIG. 4. Plot of cyanide dihydratase activity as a function of $[Cr^{3+}]$. A Bio-Gel A-5m eluate enzyme preparation was used for this determination.

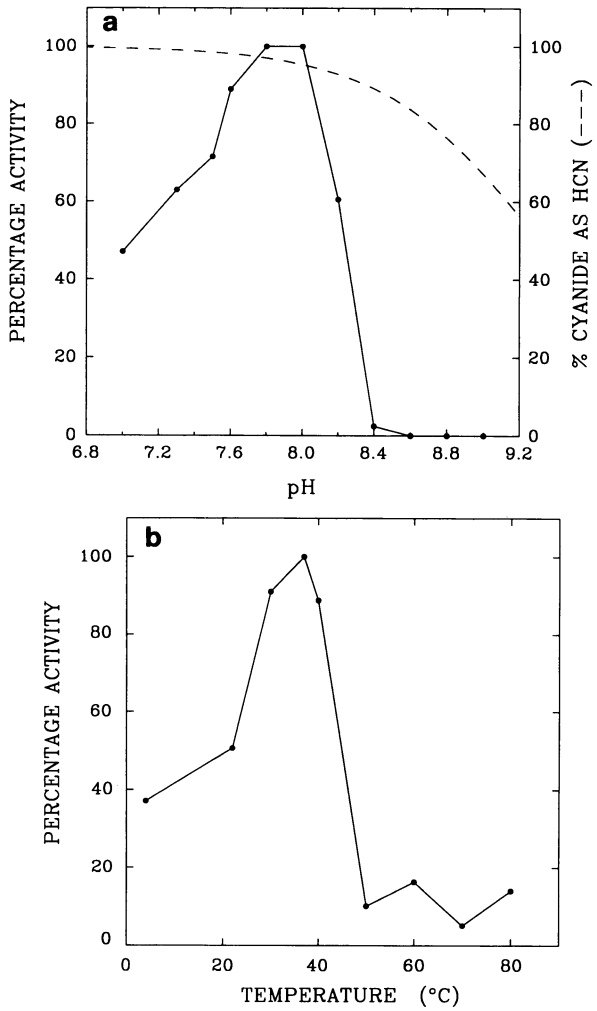

FIG. 5. Plot of cyanide dihydratase activity as a function of pH (a) and temperature (b). The dashed line in panel a represents the calculated ionization curve for $HCN \leftrightarrow CN^- + H^+$ as a function of the pH. A Bio-Gel A-5m eluate enzyme preparation was used for these determinations.

TABLE 4. Effect of azide on cyanide dihydratase activity[a]

[Azide] mM	Activity (%)
0	100
0.015	89.7
0.15	117.9
0.45	155.6
1.5	259.0
4.5	360.7
15	338.5
45	142.7
150	25.6
1,500	0

[a] Azide at the indicated concentrations was added to duplicate samples which were incubated at 4°C for 16 h before determination of the cyanide dihydratase activity at 37°C.

TABLE 5. Effect of addition, removal, and readdition of azide on cyanide dihydratase activity

Treatment	Sp act (μmol/min/mg of protein)	
	Control (no N_3^-)	Sample (5 mM N_3^-)
None	28.25	60.93
Dialysis	23.06	24.27
Readdition of azide to sample	26.74	54.16

cyanide dihydratase activity (Table 6). The reason for the enhancement by these compounds is unknown.

DISCUSSION

We have described the purification and characterization of a cyanide dihydratase from the gram-positive bacterium *B. pumilus* C1 that converts cyanide to formate and ammonia. Interestingly, this enzyme and Cyanidase from the gram-negative organism *A. xylosoxidans* subsp. *denitrificans* (17) have some physicochemical properties in common. Both enzymes had molecular masses of above 300,000 Da, had similar pH optima around pH 8.0, and retained activity at very high ionic strength (2). Both enzymes displayed nitrilase activity exclusively against cyanide with a K_m of around 3 mM, converting cyanide to formate and ammonia in a single-step reaction. The two enzymes, however, are clearly different proteins in that the *B. pumilus* C1 dihydratase consists of three polypeptide chains whereas Cyanidase contains only two. Moreover, the N-terminal sequences bear no resemblance to one another, and the enzymes have different temperature optima (37°C for the *B. pumilus* C1 enzyme and 26°C for Cyanidase). This may account for the seemingly substantially higher V_{max} (88.03 ± 4.67 mmol/min/mg/liter for the *B. pumilus* C1 enzyme and 0.4 ± 0.01 mmol of cyanide per liter/min/g for the Cyanidase biocatalyst) (unless the Cyanidase/support ratio was very low). Other microbial cyanide-degrading enzymes that have been described include the formamide hydrolyases from *Stemphylium loti* (10) and *G. sorghi* (32). These were also reported to be large proteins with molecular masses of (at least) 600,000 and 300,000 Da, respectively, and displayed K_ms for cyanide of 25 and 12 mM, respectively. In contrast to these large cyanide-degrading enzymes, two small enzymes of 17,000 and 40,000 Da in *Fusarium solani* were reported (27, 28). Cyanide was degraded to ammonia, indicating that these were not formamide hydrolyases.

One possible interpretation of the electron microscopy data is that the enzyme responsible for cyanide degradation is a hexamer of a monomeric particle, the hexamer being readily dissociated. Evidence in favor of this hypothesis is that the enzyme displayed an unusual sensitivity to low-ionic-strength solutions and to dilution, with the catalytic activity being suddenly lost. If this interpretation is correct, the molecular mass of the active enzyme determined by gel filtration may be an overestimate, since the molecular mass determined by exclusion chromatography assumes that the protein is globular rather than rod shaped. An alternative interpretation of the electron microscopy data is that the rod-shaped structures visible in the electron microscope field were formed by association of the smallest particle and that the active enzyme is actually a smaller multimer than that indicated by the rod-shaped structures. In this regard it has been suggested that the tendency of the subunits of some nitrilases (EC 3.5.5.1) to associate into very large aggregates of several hundred kilodaltons may have led to an overestimate of their molecular sizes (12). Although our data preclude distinguishing between these interpretations, we would have expected the association of a basic structure to have led to a range of active species of increasing molecular masses which should have appeared as several active protein peaks during gel filtration chromatography and sucrose gradient centrifugation.

TABLE 6. Enhancement of cyanide dihydratase activity by formate or acetate

Formate or acetate concn (mM)	Activity (%) with:	
	Formate	Acetate
0	100	100
5	86.4	124.1
10	97.5	125.9
40	155.6	127.1
160	261.1	177.1
320	358.6	231.3

The enhancement of the enzymatic activity of the *B. pumilus* C1 enzyme by a variety of trivalent metal ions could possibly be due to these ions acting as cyanide carriers in order to present the substrate to the catalytic site in a more favorable manner. Chromium(III), however, which was also found to enhance enzymatic activity, is known to be relatively inert with respect to ligand-exchange reactions in solution (5). It is therefore unlikely that cyanide could replace H_2O in $[Cr(H_2O)_6]^{3+}$ during the course of a normal enzyme activity assay. Alternatively, enhancement of enzymatic activity could be due to changes in protein structure brought about by the presence of these ions. This latter explanation is more likely, as the presence of trivalent metal ions was found to increase the K_m for cyanide by a factor of two as well as to extend the pH range of enzymatic activity. Additionally, in the presence of Cr^{3+} and Tb^{3+}, the enzyme was found to function at pH 8.6 (at which no activity was recorded in the absence of trivalent metal ions) with approximately the same activity as at pH 8.0, the pH optimum. Trivalent metal ions either may maintain a favorable active-site conformation by electrostatic interactions with negatively charged residues or may influence the stability of the holoenzyme, since numerous incomplete structures were visible on the electron micrographs. This putative interaction was not caused by tight binding of the metal ions to the protein, since we were unable to demonstrate $^{51}Cr^{3+}$ associated with cyanide dihydratase purified from bacteria grown in the presence of this isotope. At pH 8.6, however, lysine residues are partially deprotonated, and it is possible that in their role in salt bridges they are being assisted or replaced by the trivalent metal ions.

In conclusion, the high specific activity of cyanide dihydratase from *B. pumilus* C1, its stability, and its ease of purification make it a strong candidate for the development of an industrial process for the biological detoxification of cyanide.

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