Discovery of a Novel Enzyme, Isonitrile Hydratase, Involved in Nitrogen-Carbon Triple Bond Cleavage*

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Isonitrile containing an N=C triple bond was degraded by microorganism sp. N19-2, which was isolated from soil through a 2-month acclimatization culture in the presence of this compound. The isonitrile-degrading microorganism was identified as Pseudomonas putida. The microbial degradation was found to proceed through an enzymatic reaction, the isonitrile being hydrated to the corresponding N-substituted formamide. The enzyme, named isonitrile hydratase, was purified and characterized. The native enzyme had a molecular mass of about 59 kDa and consisted of two identical subunits. The enzyme stoichiometrically catalyzed the hydration of cyclohexyl isocyanide (an isonitrile) to N-cyclohexylformamide, but no formation of other compounds was detected. The apparent $K_m$ value for cyclohexyl isocyanide was 16.2 mM. Although the enzyme acted on various isonitriles, no nitriles or amides were accepted as substrates.

Nitriles are very toxic and are generally organic compounds containing a C–N moiety that are not biodegradable. We have studied nitrile metabolism (1–3); we clarified the structures and functions of enzymes involved (i.e. nitrilase (4–7), nitrile hydratase (8–11), and amidase (12–14)) and their genes and regulatory mechanisms in metabolism.

On the other hand, no reports have appeared on an enzyme involved in the metabolism of isonitrile (more generally called isocyano-compounds, which is an isomer of nitrile containing an isocyanate group).

$$R - C = N$$
$$\text{Nitrile}$$

$$R' - N' = C'$$
$$\text{(Isonitrile)}$$

(Eq. 1)

Like nitrile, isonitrile is generally highly toxic, and some organisms produce isocyanide compounds that probably have a self-defense function (15, 16). For example, Penicillium notatum synthesizes an isocyanate metabolite, xanthocillin, that exhibits a wide spectrum of antibiotic activity (17). A marine sponge, Axinella cannabina, produces a sesquiterpenoid compound containing the isocyanate structure, axisotin-1 (18). 9-Isocyanopupukeanane, an unusual smelling substance lethal to fish and crustaceans, is found in the mucus of a nudibranch, Phyllidia varicosa, and its prey, a sponge, Hymeniacidon sp. (19). There have been many other reports about such naturally occurring isocyanides (for reviews, see Refs. 15 and 16). However, the details of their synthetic and degradative pathways remain unknown, and no enzyme involved in the metabolism of such isocyano-compounds has yet been reported. Among so far known enzymes, only nitrogenase has been reported to act on an isonitrile; it reduces methyl isocyanide to give dimethylamine (by four electrons transfer) or methane plus methylamine (by six electrons transfer) (20, 21). However, methyl isocyanide is just an alternative substrate for nitrogenase. The enzymes metabolizing isonitriles as its physiological substrate remain unknown.

We are interested in how C–N hydrolases evolved. Because isonitrile contains a nitrogen-carbon bond in its structure, an isonitrile-hydrating enzyme (which has not been discovered) would also belong to the category of C–N hydrolases. A search for such an enzyme and its functional analysis would probably provide us with new knowledge about C–N hydrolases, which might serve as clues for elucidating the pathways of their functional and structural evolution. In the present paper, we describe the isolation of an isonitrile-degrading microorganism, Pseudomonas putida N19-2, from soil and the purification and characterization of an enzyme named isonitrile hydratase that catalyzes the hydration of isonitrile to the corresponding N-substituted formamide.

EXPERIMENTAL PROCEDURES

Materials

Cyclohexyl isocyanide and N-cyclohexylformamide were purchased from Fluka Chemical Co. (Buchs, Switzerland) and Tokyo Kasei Kogyo Co. (Tokyo, Japan), respectively. DEAE-Sephacel and a low molecular weight standard kit were obtained from Amersham Pharmacia Biotech. TSK gel Butyl-Toyopearl 850M was purchased from Tosoh Co. (Tokyo, Japan). Cellulose GCL-2000 superfine was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Marker proteins for molecular mass determination by high performance liquid chromatography (HPLC) were purchased from Oriental Yeast Co. (Tokyo, Japan). All other chemicals used were from commercial sources and were of reagent grade.

Isolation of Isonitrile-degrading Bacteria

Isonitrile-degrading bacteria were isolated from soil samples as follows. A spoonful of a soil sample was added to a test tube containing 10 ml of medium (pH 7.0) consisting of 10 g of glycerol, 0.5 g of K$_2$HPO$_4$, 0.5 g of KH$_2$PO$_4$, 0.5 g of MgSO$_4$·7H$_2$O, 0.005 g of FeSO$_4$·7H$_2$O, 1 ml of a vitamin mixture (0.4 g of thiamine hydrochloride, 0.2 g of riboflavin, 0.4 g of pyridoxine hydrochloride, 0.4 g of nicotinic acid, 10 mg of folinic acid, 0.4 g of calcium pantothenate, 2 mg of biotin, 2 g of inositol, and 0.2 g of p-aminobenzoic acid in 1 liter of distilled water), and cyclohexyl isocyanide at a final concentration of 0.01% (v/v) of tap water.

1 The abbreviations used are: HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis.

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A Novel Enzyme, Isonitrile Hydratase

Cultivation was performed with shaking at 28 °C for 1 week. Once a week, 1 ml of the culture was inoculated into 10 ml of fresh medium. After 3 weeks of cultivation, the concentration of cyclohexyl isocyanide was increased to 0.02% (v/v). After a month of further cultivation, the microorganisms were spread on agar plates and isolated.

**Assaying of the Isonitrile-degrading Abilities of the Isolated Strains**

Each of the isolated strains was inoculated into a test tube containing 10 ml of 2× YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl/liter of distilled water) supplemented with cyclohexyl isocyanide at a final concentration of 0.02% (v/v) and then incubated at 28 °C for 48 h with reciprocal shaking. Then the cells were harvested by centrifugation, washed twice with 0.01 M potassium phosphate buffer (pH 7.5), and suspended in 0.1 M potassium phosphate buffer (pH 7.5).

The isonitrile-degrading abilities of the isolated strains were assayed by means of the resting cell reaction. The reaction mixture (400 μl) was composed of 40 μmol of potassium phosphate buffer (pH 7.5), 4 μmol of cyclohexyl isocyanide, 20 μl of methanol, and an appropriate amount of cell suspension. Methanol was added to enhance the solubility of the substrate. The reaction was carried out at 20 °C for 30 min and stopped by placing the reaction mixture on ice water and then rapidly removing the cells by centrifugation at 0–4 °C. The residual amount of cyclohexyl isocyanide in the reaction mixture was determined by HPLC with a Shimadzu LC-6A system (Kyoto, Japan) equipped with a Cosmosil 5C18-AR-II column (reversed-phase; 4.6 by 150 mm: Nacalai Tesque, Kyoto, Japan) and a UV spectrophotometric detector (SPD-6A) of the original system. The following solvent system was used: 5 mM KH2PO4–H2PO4 buffer (pH 2.9), acetonitrile (1:1 v/v) at a flow rate of 1.0 ml/min.

**Identification of the Compound Produced from Cyclohexyl Isonitrile by P. putida N19-2**

The product in the reaction mixture with cells of *P. putida* (which was identified as described under "Results") was extracted with chloroform/methanol (2:1 v/v), concentrated, and then analyzed by gas chromatography–mass spectrometry (GC-MS) analysis. GC-MS was performed with a Shimadzu GCMS-QP5050 equipped with an FFISULBON HR-1 capillary column (0.25 × 50 m; Shinwa Chemical Industries, Ltd., Kyoto, Japan). The initial column temperature of 50 °C was raised at 5 °C/min to 120 °C. The injection and detector temperatures were 250 °C. The carrier gas was helium, at a flow rate of 28 ml/min.

**Culture Conditions for P. putida N19-2**

*P. putida* N19-2 was collected from an agar plate and then inoculated for the first subculture. The first subculture was carried out at 25 °C for 24 h with reciprocal shaking in a test tube containing 10 ml of 2× YT medium supplemented with cyclohexyl isocyanide at a final concentration of 0.02% (v/v). Then 1 ml of the first subculture was inoculated into a 500-ml shaking flask containing 90 ml of the second subculture medium (pH 7.0) consisting of 40 g of tryptone, 13.4 g of K2HPO4, 6.5 g of KH2PO4, 1 g of NaCl, 0.2 g of MgSO4·7H2O, 0.01 g of FeSO4·7H2O, 1 ml of a vitamin mixture, and cyclohexyl isocyanide at a final concentration of 0.02% (v/v). The second subculture was also performed at 28 °C for 24 h with reciprocal shaking. Then 5 ml of the culture was inoculated into a 2-liter shaking flask containing 500 ml of the same medium as used for the second subculture, followed by incubation at 28 °C with reciprocal shaking. After 24 h of incubation, the cells were harvested by continuous flow centrifugation at 18,000 × g at 4 °C and then washed twice with 0.01 M potassium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol.

**Purification of Isonitrile Hydratase**

All purification steps were performed at 0–4 °C. Potassium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol was used throughout the purification steps. Centrifugation was carried out for 30 min at 18,000 × g.

**Step 1: Preparation of a Cell-free Extract—**Washed cells (40 g) from 12 liters of culture broth were suspended in 150 ml of 0.1 M Tris and then disrupted by sonication at 9 kHz for 30 min with an Insonator model 201M (Kubota, Tokyo, Japan). The cell debris was removed by centrifugation.

**Step 2: Ammonium Sulfate Fractionation—**The resulting supernatant solution was fractionated with ammonium sulfate (45–60% saturation), followed by dialysis against 10 mM buffer.

**Step 3: DEAE-Sephadex Column Chromatography—**The dialyzed solution was applied to a DEAE-Sephadex column (5 × 20 cm) equilibrated with 10 mM buffer. Protein was eluted from the column with 1 liter of the same buffer by increasing the concentration of KCl linearly from 0 to 1 M. The active fractions were pooled, and then ammonium sulfate was added to give 70% saturation. After centrifugation of the suspension, the precipitate was dissolved in 0.1 M buffer and then dialyzed against 10 mM buffer.

**Step 4: TSK Gel Butyl-Toyopearl 650M Column Chromatography—**The enzyme solution from step 3 was mixed with an equal amount of 50 mM buffer containing 40% saturated ammonium sulfate and then placed on a TSK gel Butyl-Toyopearl 650M column (2.6 × 2 cm) equilibrated with 50 mM buffer containing 20% saturated ammonium sulfate. The enzyme was eluted by lowering the concentration of ammonium sulfate (20 to 0% saturation) in 1 liter of the same buffer. The active fractions were combined and precipitated with ammonium sulfate to bring 70% saturation. The precipitate was collected by centrifugation, dissolved in 0.1 M buffer, and then dialyzed against 10 mM buffer.

**Step 5: Cellulofine GCL-2000Sf Column Chromatography—**The enzyme solution from step 4 was concentrated with a Centricron-10 microconcentrator (Amicon Inc., Beverly, MA) to 0.7 ml, and then placed on a Cellulofine GCL-2000Sf column (2.6 × 108 cm) equilibrated with 10 mM buffer. The rates of sample loading and column elution were kept at 15 ml/h. The active fractions were combined and then precipitated with ammonium sulfate at a final concentration of 70%. The precipitate was collected by centrifugation and then dissolved in 0.1 M buffer.

The enzyme solution was dialyzed against 10 mM buffer and then preserved in 10 mM buffer containing 50% (v/v) glycerol at −20 °C.

**Enzyme Assay**

Isonitrile hydratase activity was assayed in a reaction mixture (400 μl) consisting of 40 μmol of potassium phosphate buffer (pH 7.5), 4 μmol of cyclohexyl isocyanide, 20 μl of methanol, and an appropriate amount of enzyme. Methanol was added to enhance the solubility of the substrate. The enzyme activity was not inhibited even in the presence of 10% (v/v) methanol (data not shown). The reaction was carried out at 20 °C for 30 min and stopped by adding 400 μl of cold acetonitrile to the reaction mixture. The amount of N-cyclohexylformamide formed was determined by HPLC, which was performed with the same system used for the measurement of cyclohexyl isocyanide under "Assaying of the Isonitrile-degrading Abilities of the Isolated Strains," except that a UV spectrophotometric detector (SPD-6A) was used. The wavelength of 198 nm was used for the monitoring.

One unit of isonitrile hydratase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol N-cyclohexylformamide/min from cyclohexyl isocyanide under the above conditions.

**Protein was determined by the Coomassie Brilliant Blue G-250 dye binding method of Bradford (22), using dye reagent supplied by Bio-Rad. The specific activity is expressed as units/mg protein.**

**Electrophoresis**

SDS-polyacrylamide gel electrophoresis (PAGE) was performed in a 12.5% polyacrylamide slab gel according to Laemmli (23). The gel was stained with Coomassie Brilliant Blue R-250. The relative molecular mass of the enzyme subunit was determined from the relative mobilities of marker proteins, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

**Molecular Mass Determination**

The enzyme sample (20.4 μg) was subjected to HPLC on a TSK G25PW column (0.75 × 60 cm; Tosoh Co., Tokyo, Japan) and then eluted with 50 mM potassium phosphate buffer (pH 7.5) at a flow rate of 0.5 ml/min. The absorbance of the effluent was recorded at 280 nm. The molecular mass of the enzyme was calculated from the mobilities of the standard proteins, glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome c (12.4 kDa).

**Metal Analysis**

All glassware was soaked in 2 N HCl overnight and then exhaustively rinsed with distilled water before use. Prior to analysis, the enzyme was dialyzed against 10 mM potassium phosphate buffer (pH 7.5). The dialysis caused no effect on the enzyme activity (16.5 units/mg). The enzyme sample containing 0.9 mg protein/ml was analyzed with an inductively coupled radiofrequency plasma spectrophotometer, Shimadzu ICPS-8000 (27.120 MHz; Kyoto, Japan). The metal contents of
the enzyme sample were determined from the calibration curves for standard solutions.

**NH₂-terminal Sequence Analysis**

The purified enzyme sample (20.4 µg) was subjected to an Applied Biosystems model 476A protein sequencer (Foster City, CA), and the NH₂-terminal sequence was analyzed by automated Edman degradation.

**Substrate Specificity**

The following isonitriles, nitrile, and amide compounds were tested as to the substrate specificity: (a) isonitriles: cyclohexyl isocyanide, benzyl isocyanamide, methyl isocyanonate, ethyl isocyanonate, and isocyanomethyl phosphonic acid diethyl ether; (b) nitriles: propionitrile, n-butynitrile, isobutynitrile, n-valeronitrile, cyclohexyl cyanide, acrylonitrile, methacrylonitrile, crotononitrile, benzonitrile, benzyl cyanide, and 4-cyanopyridine; and (c) amides: propionamide, n-butynamide, isobutynamide, n-valeramide, cyclohexane carboxamide, acrylamide, methacrylamide, crotonamide, benzamide, phenylacetamide, and isonicotinamide.

The assaying of substrate specificity was carried out in a reaction mixture (400 µl) consisting of 40 µmol of potassium phosphate buffer (pH 7.5), 4 µmol of substrate, 20 µl of methanol, and an appropriate amount of enzyme. However, when propionitrile, n-butynitrile, isobutynitrile, n-valeronitrile, propionamide, n-butynamide, isobutynamide, and n-valeramide were used as substrates, their final concentrations were 100 mM. Methanol was added to enhance the solubility of the substrate. The reaction was carried out at 20 °C for 30 min (unless otherwise specified) and stopped by adding 400 µl of cold acetonitrile (when isonitriles were used as substrates) or 100 µl of 1 M HCl (when nitriles or amides were used as substrates) to the reaction mixture.

The levels of substrate consumption were determined with the same HPLC system as used under “Enzyme Assay.” The following wavelengths were used for monitoring: 194 nm for benzyl isocyanide, 195 nm for methyl isocyanonate and ethyl isocyanonate, 198 nm for cyclohexane carboxamide, 200 nm for methacrylonitrile, 205 nm for crotononitrile and benzyl cyanide, 220 nm for acrylonitrile, and 230 nm for the other nitriles. The isocyanomethyl phosphonic acid diethyl ether, propionitrile, n-butynitrile, isobutynitrile, n-valeronitrile, and cyclohexyl cyanide were measured by HPLC with a refractive index detector (RID-6A) instead of a UV spectrophotometric detector (SPD-6A).

**RESULTS**

**Isolation and Identification of P. putida N19-2**—After about 2 months from the start of the study, using the acclimatization culture method as described under “Experimental Procedures,” we finally isolated one microorganism, sp. N19-2, that is able to degrade cyclohexyl isocyanide.

Then we examined the isonitrile-degrading ability of the strain N19-2. Cyclohexyl isocyanide does not exhibit strong UV absorption, and it is difficult to measure the residual amount of it in a resting cell reaction mixture with a HPLC system equipped with a UV spectrophotometric detector (which is generally used for HPLC). Therefore, we developed a method for the measurement of cyclohexyl isocyanide involving a refractive index detector instead of a UV spectrophotometric detector. Because the compound was spontaneously decomposed at low pH, the reaction was stopped by the addition of an organic solvent (cold acetonitrile) and not a mineral acid (such as low pH, the reaction was stopped by the addition of an organic solvent (cold acetonitrile) instead of a UV spectrophotometric detector (RID-6A)).

As the result of the resting cell reaction, strain N19-2 degraded cyclohexyl isocyanide and produced an unknown compound concomitantly with the isonitrile degradation. The strain showed an activity level of 0.32 µmol isonitrile degradation/min/mg of dry cells.

Morphologically, strain N19-2 is a Gram-negative short rod, nonendospore forming, and motile. Its physiological characteristics are as follows: oxidase, positive; catalase, positive; oxidative/fermentative dissimilation of glucose, oxidative; reduction of nitrate and indole, negative; acid production from glucose and maltose, negative; decomposition of arginine, positive; de-

composition of urea, asesculin, gelatin, tyrosine, and casein, negative; β-galactosidase, negative; growth on sole carbon sources, positive with glucose, mannose, gluconate, caprate, malate, citrate, glycine, phenylalanine, benzylamine, and betaine and negative with arabinose, mannitol, N-acetylglucosamine, maltose, adipate, and inositol; cytochrome oxidase, positive; residual nitrate, positive; and production of pigment in King’s B broth, negative. The GC content of the genomic DNA of this strain was determined as 62.4% with a DNA-GC kit (Yamasu Shoyou Co., Choshi, Japan) according to the method developed by three groups independently (24–26). Based on these characteristics, strain N19-2 was identified as P. putida.

Identification of the Reaction Product—The unknown compound produced from cyclohexyl isocyanide through the cell reaction of P. putida N19-2 was analyzed by GC-MS, as described under “Experimental Procedures.” As a result, it was found that both the retention time (10.4 min under the experimental conditions) and the MS spectrum (Fig. 1A) of the reaction product agreed with those of authentic N-cyclohexylformamidine (Fig. 1B). Furthermore, their retention times on HPLC chromatography were the same (data not shown). No other compounds (which may be produced from cyclohexyl isocyanide), such as cyclohexylamine, cyclohexanol, cyclohexanone, formate, and ammonia, exhibited similarity to the reaction product on HPLC or GC-MS analysis (data not shown). Thus, the reaction product was identified as N-cyclohexylformamidine.

The amounts of residual cyclohexyl isocyanide and formed N-cyclohexylformamidine in the reaction mixture were determined to be 1.24 and 0.75 µmol, respectively, when the initial amount of cyclohexyl isocyanide as the substrate was 2.00 µmol. No other compounds were detected in the reaction mixture on HPLC or GC-MS analysis. The results demonstrated that N-cyclohexylformamidine was formed stoichiometrically with the consumption of cyclohexyl isocyanide. When the cells of P. putida N19-2 were heat-treated by boiling for 5 min before the resting cell reaction, no formation of N-cyclohexylformamidine was observed. This finding demonstrated that this conversion of cyclohexyl isocyanide to N-cyclohexylformamidine is not a chemical but an enzymatic reaction.

These findings indicated that P. putida N19-2 contains an enzyme that catalyzes the hydration of isonitrile to the corresponding N-substituted formamidine. Therefore, we named this enzyme “isonitrile hydratase.”

**Purification of Isonitrile Hydratase**—We found that the isonitrile hydratase activity of P. putida N19-2 was observed only when cyclohexyl isocyanide or another isonitrile was added to the culture medium. The highest activity was obtained by the addition of cyclohexyl isocyanide. Therefore, the purification of the enzyme was carried out from an extract of the cells cultured in the presence of cyclohexyl isocyanide. We

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2 M. Goda, Y. Hashimoto, S. Shimizu, and M. Kobayashi, unpublished results.
observed on SDS-PAGE that the crude extract contained a protein that was not synthesized without the addition of the isonitrile to the culture medium (Fig. 2, lane C). The ratio of the protein band in active fractions became greater as the purification procedure proceeded, demonstrating that it was isonitrile hydratase (data not shown).

Through the purification steps described under "Experimental Procedures," the enzyme was purified 38.2-fold with a yield of 16.7% (Table I). The purified enzyme gave only one band on SDS-PAGE (Fig. 3), corresponding to a molecular mass of 29 kDa. Further evidence of the purity of the enzyme preparation was provided by the results of HPLC on a TSK G-3000SW column, which gave a single symmetrical protein peak. The molecular mass of the native enzyme was determined to be 59 kDa by gel permeation HPLC. Thus, the enzyme probably consists of two identical subunits.

Qualitative analysis of the following metals in the enzyme solution was performed with an inductively coupled radiofrequency plasma spectrophotometer: beryllium, boron, magnesium, aluminum, silicon, phosphorus, sulfur, calcium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, selenium, strontium, zirconium, molybdenum, palladium, silver, cadmium, tin, antimony, barium, tantalum, tungsten, platinum, gold, mercury, lead, lanthanum, and cerium. However, none of these 35 metals was detected within the limits of the assay (20 ng/ml). In this respect, the isonitrile hydratase is quite different from the nitrile hydratases of *Pseudomonas* (27, 28) and *Brevibacterium* (29) species, which contain tightly bound iron (1, 30), and the nitrile hydratase of *Rhodococcus rhodochrous* J1, which contains cobalt as a prosthetic group (1, 11, 31).

The absorption spectrum of the purified enzyme in 0.01 M potassium phosphate buffer (pH 7.5) showed the maximum absorbance at 278 nm. No other peak absorption or shoulder was observed, suggesting that no co-factor would be bound to the enzyme. The NH2-terminal sequence was determined as ALQIGFLLFP. It exhibited no homology with the amino acid sequences of the reported proteins.

**Stoichiometry**—The stoichiometry of isonitrile consumption and N-substituted formamide formation during the hydration of isonitriles was examined in a reaction mixture consisting of 40 μmol of potassium phosphate buffer (pH 7.5), 4 μmol of cyclohexyl isocyanide, 25 μl of methanol, and 0.42 nmol of the enzyme in a final volume of 400 μl. The reaction was carried out at 20 °C in an airtight tube. After 30 min of incubation, the amounts of residual cyclohexyl isocyanide and N-cyclohexylformamide were determined. The N-cyclohexylformamide formed and the cyclohexyl isocyanide remaining were 1.96 and 1.98 μmol, respectively. No formation of other compounds was noted. The results indicated that N-cyclohexylformamide was formed stoichiometrically with the consumption of cyclohexyl isocyanide.

**Stability of the Enzyme**—The stability of the enzyme was examined at various temperatures. After the enzyme had been preincubated for 30 min in 0.01 M potassium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol, an aliquot of each enzyme solution was taken, and then the enzyme activity was assayed under the standard conditions. It exhibited the following activities: 55 °C, 19%; 50 °C, 33%; 45 °C, 53%; 40 °C, 69%; 35 °C, 78%; 30 °C, 84%; 20 °C, 89%; and 10 °C, 97%.

**Effects of pH and Temperature**—The effects of pH and temperature on the enzyme activity were examined. The enzyme exhibited maximum activity at pH 6.0–6.5, as shown in Fig. 4A. The optimal temperature was 35 °C, and the enzyme activity was rapidly lost above 40 °C (Fig. 4B).

The substrate is very stable under 60 °C. As to the effect of pH, it is partly decomposed to N-cyclohexylformamide by spontaneous and chemical (nonenzymatic) reaction in the acidic conditions under pH 5.9. Thus, the enzyme activities under pH 5.0 shown in Fig. 4A were calculated from the modified amount of product; we subtracted the amount of N-cyclohexylformamide formed in control (the reaction mixture without the enzyme) from that of each sample before the calculation. The substrate is stable over pH 5.5.

**Substrate Specificity**—The ability of the enzyme to catalyze the hydration or hydrolysis of various isonitriles, nitriles, and amides was examined. Table II shows that all of the tested isonitriles were active as substrates for the isonitrile hy-
Inhibitor Effects on the Enzyme Activity

| Inhibitor                  | Relative activity |
|----------------------------|-------------------|
| None                       | 100               |
| LiCl, NaCl, MgCl₂, CaCl₂   | 95–100            |
| BaCl₂, MnCl₂, PbCl₂, FeCl₃ |                   |
| and AlCl₃                  |                   |

**Discussion**

Isonitriles are unique in that they form the only class of organic compounds that contain a stable, formally mono-coordinated carbon (15). The isocyno group exhibits a dual nucleophilic/electrophilic character, which is often exploited for synthetic applications: e.g. nucleophilic addition reactions, multi-component condensation reactions, in the synthesis of peptides, in coordination chemistry, organometallic reactions, and carbohydrate chemistry (15).

On the other hand, there have been many reports of naturally occurring isonitriles (15, 16). To our knowledge, however, no enzyme involved in the metabolism of isonitriles has been found. Among the known enzymes, only nitrogenase is known to act on an isonitrile; it converts methyl isocyanide to the corresponding amine (20, 21). But isonitriles are not physiological substrates of the enzyme. Although it is possible that isonitrile-metabolizing organisms produce and utilize nitrogenase to degrade isonitriles, it remains unclear whether the isonitrile is degraded by nitrogenase or by an isonitrile hydratase (a novel enzyme).

**Table II**

**Substrate Specificity of Isonitrile Hydratase**

The reaction was carried out at 20 °C in the standard reaction mixture except that various isonitriles were used as substrates in place of cyclohexyl isocyanide. The enzyme activities towards the substrates other than cyclohexyl isocyanide were assayed as the consumption of the substrate, whereas the activity towards cyclohexyl isocyanide was determined as the formation of the product, as described under "Enzyme Assay." The synthesis of N-cyclohexylformamide, corresponding to 16.5 μmol/min/mg of protein, was taken as 100%.

| Substrate                  | Relative activity |
|----------------------------|-------------------|
| Cyclohexyl isocyanide      | 100               |
| Benzyl isocyanide          | 224               |
| Methyl isocyanocacetate    | 0.688             |
| Ethyl isocyanocacetate     | 0.105             |
| Isocyanomethyl phosphonic acid diethyl ether | 2.94 |

**Table III**

**Effects of Various Compounds on the Activity of Isonitrile Hydratase**

Each compound was added to the standard reaction mixture without the substrate, and then assaying of the enzyme was performed by adding the substrate. The final concentrations of the tested compounds were 1 mM.

| Inhibitor                  | Relative activity |
|----------------------------|-------------------|
| None                       | 100               |
| LiCl, NaCl, MgCl₂, CaCl₂   | 95–100            |
| BaCl₂, MnCl₂, PbCl₂, FeCl₃ |                   |
| and AlCl₃                  |                   |

**Summary**

The enzyme consists of two identical subunits and contains no metal. Isonitrile hydratase is different from nitrilase (which catalyzes the direct hydrolysis of nitrile to the corresponding carboxylic acid and ammonia; Refs. 32 and 33) and nitrile degrading bacteria (15, 16). In this work, we isolated an isonitrile-degrading bacterium, which has been identified as _P. putida_, from soil and purified isonitrile hydratase from the cells. This enzyme has been found to act only on isonitriles, leading to the formation of the corresponding N-substituted formamides. The reaction catalyzed by this enzyme is quite distinct from that of nitrogenase; the former is hydration, and the latter is reduction.

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N19-2 seems to be detoxification. We intended to isolate *Pseudomonas* to elucidate the reaction mechanism of isonitrile hydratase. Further studies are required to clarify the reaction mechanism of isonitrile hydratase.

The main physiological role of isonitrile hydratase for *P. putida* N19-2 seems to be detoxification. We intended to isolate an isonitrile-assimilating microorganism through acclimatization culture utilizing cyclohexyl isocyanide as a nitrogen source in the culture medium. However, the resting cells of *P. putida* N19-2 released almost all of the consumed cyclohexyl isocyanide metabolic pathways. For example, it has clearly been demonstrated that 2-isocyanopupukeanane is transformed to xanthocillin, although the origin of the carbon atoms of the isocyano groups remains a mystery, there has been one report suggesting that the synthetic and metabolic pathways for naturally occurring isonitriles. Moreover, it would also provide us with novel knowledge about C–N hydrolases and lead to a better understanding of their evolutionary pathways.

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