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We previously discovered N-substituted formamide defformylase (NfdA) in Arthrobacter pascens F164, which degrades N-substituted formamide (Fukatsu, H., Hashimoto, Y., Goda, M., Higashibata, H., and Kobayashi, M. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 13726–13731). In this study, we found an enzyme involved in the first step of isonitrile metabolism, isonitrile hydratase, that hydrates isonitrile to the corresponding N-substituted formamide. First, we investigated the optimum culture conditions for the production of isonitrile hydratase. The highest enzyme activity was obtained when A. pascens F164 was cultured in a nutrient medium containing N-benzylformamide. This Arthrobacter isonitrile hydratase was purified, characterized, and compared with Pseudomonas putida N19-2 isonitrile hydratase (InhA), which is the sole one reported at present. Arthrobacter isonitrile hydratase was found to have a molecular mass of about 530 kDa and to consist of 12 identical subunits. The apparent $K_m$ value for cyclohexyl isocyanide was 0.95 ± 0.05 mm. A. pascens F164 grew and exhibited the isonitrile hydratase and N-substituted formamide defformylase activities when cultured in a medium containing an isonitrile as the sole carbon and nitrogen sources. However, both enzyme activities were not observed on culture in a medium containing glycerol and (NH₄)₂SO₄ as the sole carbon and nitrogen sources, respectively. These findings suggested that the Arthrobacter enzyme is an inducible enzyme, possibly involved in assimilation and/or detoxification of isonitrile. Moreover, gene cloning of the Arthrobacter enzyme revealed no sequence similarity between this enzyme and InhA. Comparison of their properties and features demonstrated that the two enzymes are biochemically, immunologically, and structurally different from each other. Thus, we discovered a new isonitrile hydratase named InhB.

We have extensively studied the biological metabolism of toxic compounds containing a C=N moiety, such as nitriles (1–5) and isonitriles (6–9). Isonitrile (more generally called isocyanide) containing an isocyano group (−N=) is an isomer of nitrile. Like nitrile, isonitrile is generally highly toxic and produced in nature, probably for self-defense, by various organisms, including bacteria, fungi, marine sponges, etc. (10, 11). For example, xanthocillin was first isolated as an isocyano metabolite from Penicillium notatum, and this isonitrile exhibits a wide antibiotic activity spectrum (12). There have been many other reports about such naturally occurring isocyanides, but information is quite limited on the metabolism of isonitrile. Some metabolic isonitrile intermediates have been elucidated through incorporation experiments, and we initially reported two enzymes involved in isonitrile metabolism. First, we discovered isonitrile hydratase in Pseudomonas putida N19-2, which catalyzes the hydration of an isonitrile to the corresponding N-substituted formamide (R-NH-CHO) (6, 7). It has been approved as a new enzyme by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), EC 4.2.1.103. Next, we discovered N-substituted formamide defformylase in Arthrobacter pascens F164, which catalyzes the hydrolysis of a N-substituted formamide to the corresponding amine (R-NH₂) and formic acid (8). It has also been approved as a new enzyme by the NC-IUBMB, EC 3.5.1.91. These enzymes that are involved in the metabolism of isonitrile and N-substituted formamide, respectively. These enzymes have provided us with new knowledge about isonitrile metabolism; however, their biochemical properties (e.g. three-dimensional structure and reaction mechanism) have not been clarified in detail. Furthermore, it remains unknown why A. pascens F164 has an isonitrile-metabolizing enzyme.

In this study, we discovered a novel isonitrile hydratase in A. pascens F164. Comparative study of the two isonitrile hydratases, this Arthrobacter isonitrile hydratase and InhA, revealed differences in physicochemical properties and structural features between the strains. Arthrobacter isonitrile hydratase is completely different from InhA and thus is a new type of isonitrile hydratase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cyclohexyl isocyanide and N-cyclohexylformamide were purchased from Fluka Chemie GmbH (Buchs, Switzerland) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. TOYOPEARL Butyl-650 M was purchased from Tosoh Co., Ltd. (Tokyo, Japan). A low molecular weight standard kit was obtained from GE Healthcare. Thyroglobulin and molecular weight markers for molecular mass determination with an AKTA purifier (GE Healthcare) were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan), and Oriental Yeast Co., Ltd. (Tokyo, Japan), respectively. All other chemicals used were from commercial sources and were of reagent grade.

**Enzyme Assay**—All the reactions were performed under linear conditions as to protein (−0.4 mg/ml) and time (10 min). Isonitrile hydratase activity was assayed in a reaction mixture...
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(200 μl) consisting of 100 mM potassium phosphate buffer (KPB) (pH 7.5), 40 mM cyclohexyl isocyanide, 10% 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 25 °C for 10 min and stopped by adding a 3-fold volume of cold methanol to the reaction mixture. The amount of N-cyclohexylformamide formed was determined by HPLC with a Shimadzu LC-6A system (Kyoto, Japan) equipped with a Cosmosil 5C18AR-II column (reversed-phase, 4.6 × 150 mm; Nacalai Tesque, Inc., Kyoto, Japan). The absorbance was measured at 210 nm. The following solvent system was used: 10 mM KH2PO4/H3PO4 buffer (pH 2.5), acetonitrile (2:1 v/v) at a flow rate of 1.0 ml/min.

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

The nitrilase activity was assayed under the same conditions as for the isonitrile hydratase assay system, using a nitrile instead of an isonitrile. Also, the reaction product was determined with a gas chromatograph (GC-14BPF; Shimadzu) equipped with a flame ionization detector and a glass column (3.2 mm × 2.1 m) packed with Gaskuropack 56 (80/100 mesh; GL-Science, Tokyo, Japan). One unit of nitrilase hydratase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol carboxylic acid/min from the corresponding nitrile under the above conditions.

The specific activity is expressed as units/mg of protein. Protein concentrations were determined by the Coomassie Brilliant Blue R-250 dye binding method of Bradford (13), using dye reagent supplied by Nacalai Tesque Inc.

Culture Conditions for A. pascens F164—A. pascens F164 was taken from a glycerol stock and then inoculated for the first subculture. The first subculture was carried out at 28 °C for 24 h with reciprocal shaking in a 500-ml shaking flask containing 100 ml of 2YT medium (14). Then 10 ml of the first subculture was inoculated into a 2-liter shaking flask containing 990 ml of NZCYM medium (14) containing NBFA3 at a final concentration of 0.05% (w/v). Unless otherwise stated, the above medium containing NBFA was used for the enzyme preparation. The second culture was also performed at 28 °C with reciprocal shaking. After 60 h of incubation, the cells were harvested by centrifugation at 10,400 × g at 4 °C and then washed twice with 10 mM KPB (pH 7.5).

The culture medium for investigating the role of the Arthrobacter isonitrile hydratase was composed of 0.4% (w/v) glycerol, 0.07% (w/v) (NH4)2SO4, 0.5% (w/v) K2HPO4, 0.5% (w/v) KH2PO4, 0.5% (w/v) MgSO4·7H2O, 0.005% (w/v) FeSO4·7H2O, and 0.1% (v/v) vitamin mixture (6) (pH 7.0). Unless otherwise stated, glycerol and (NH4)2SO4 were used as the sole carbon and nitrogen sources, respectively.

Purification of Isonitrile Hydratase from A. pascens F164—The washed cells from 2 liters of culture broth were suspended in 100 ml of 100 mM KPB (pH 7.5) and then disrupted by sonication at 200 watts for 10 min/10 ml with an Insonator model 201 M (Kubota, Tokyo, Japan). The cell debris was removed by centrifugation. The resulting supernatant solution was fractionated with ammonium sulfate (20–35% saturation) and then dissolved in 10 mM KPB (pH 7.5), containing 10% saturated ammonium sulfate. The dialyzed solution was applied to a first TOYOPEARL Butyl-650 M column (5.0 × 30 cm) equilibrated with 10 mM KPB (pH 7.5) containing 10% saturated ammonium sulfate. The enzyme was eluted by lowering the concentration of ammonium sulfate (10 to 0% saturation) in 1.5 liters of the same buffer. The active fractions were combined and precipitated with ammonium sulfate to give 40% saturation. The precipitate was collected by centrifugation and then dissolved in 10 mM KPB (pH 7.5) containing 10% saturated ammonium sulfate. The enzyme solution was put on a second TOYOPEARL Butyl-650 M column (5.0 × 30 cm) equilibrated with 10 mM KPB (pH 7.5) containing 10% saturated ammonium sulfate. The enzyme was eluted by lowering the concentration of ammonium sulfate (10 to 0% saturation) in 1.5 liters of the same buffer. The active fractions were combined and precipitated with ammonium sulfate to give 40% saturation. The precipitate was collected by centrifugation and then dissolved in 10 mM KPB (pH 7.5). The enzyme solution was dialyzed against 10 mM KPB (pH 7.5) and then centrifuged. The homogeneity of the purified protein was confirmed by SDS-PAGE.

Electrophoresis—SDS-PAGE was performed in a 12.5% polyacrylamide slab gel according to Laemmli (15). The gel was stained with Coomassie Brilliant Blue R-250. The molecular mass of the enzyme subunit was determined from the relative mobilities of marker proteins, phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Molecular Mass Determination—The purified enzyme sample was applied to a Superose 6 HR 10/30 column (GE Healthcare), which was attached to an AKTA purifier (GE Healthcare), and then eluted with 50 mM KPB (pH 7.5) containing 0.15 M KCl 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 following standard proteins: thyroglobulin (660 kDa), 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 M HCl overnight and then exhaustively rinsed with distilled water before use. Prior to analysis, the enzyme was dialyzed against 10 mM KPB (pH 7.5). The dialysis had no effect on the enzyme activity (data not shown). The enzyme sample containing 1.6 mg of protein/ml was analyzed with an inductively coupled radiofrequency plasma spectrometer, Shimadzu ICPS-8100 (27.120 MHz; Kyoto, Japan). The metal contents of the enzyme sample were determined from the calibration curves for standard solutions.

Circular Dichroism Analysis—CD measurements were carried out with a Jasco spectropolarimeter, model J-720W (Japan Spectroscopic Co., Tokyo), equipped with a thermal incubation system at 20 °C with a 1-mm path length cell. CD spectra were

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3 The abbreviation used is: NBFA, N-benzylformamide.
obtained at the protein concentration of 0.2 mg/ml in the far-UV region (200–260 nm).

Substrate Specificity—The following isonitrile compounds were examined as to substrate specificity: cyclohexyl isocyanide, benzyl isocyanide, 1-pentyl isocyanide, tert-butyl isocyanide, methyl isocyanocetate, ethyl isocyanocetate, and isocyanomethyl phosphonic acid diethyl ether.

The levels of the reaction products were determined with the same HPLC system as used under “Enzyme Assay.” The following wavelengths were used for monitoring: 190 nm for N-tertbutyformamide, 192 nm for N-benzylformamide and N-1-pentylformamide, 195 nm for N-formylglycine methyl ester and N-formylglycine ethyl ester, 210 nm for N-cyclohexylformamide, and 230 nm for isocyanomethyl phosphonic acid diethyl ether, respectively.

The following nitrile compounds utilized as substrates by nitrilase that exhibited sequence similarity with Arthrobacter isonitrile hydratase (see “Results”) were examined as to substrate specificity: acetonitrile, propionitrile, acrylonitrile, nitrilase that exhibited sequence similarity with Arthrobacter isonitrile hydratase or InhA, respectively, and the partial NH₂-terminal amino acid sequence of the enzyme, respectively. A reaction mixture (50 μl) comprising 35 ng of genomic DNA, 200 pmol of each primer, and KOD plus DNA polymerase (TOYOBO, Osaka, Japan) was subjected to PCR (94 °C 30 s, 58 °C 30 s, and 72 °C 60 s; 30 cycles), and the amplified DNA fragment (about 300 bp) was gel-purified. The DNA fragment was then used as a probe for Southern hybridization and colony hybridization to clone the full-length Arthrobacter isonitrile hydratase gene.

Southern hybridization was carried out by using an Alkphos Direct Labeling and Detection System with CDP-Star (GE Healthcare) according to the procedure recommended by the supplier. Colony hybridization was carried out as follows: recombinant colonies were transferred to a nylon membrane, lysed with denaturing buffer (0.5 M NaOH, 1.5 M NaCl) for 15 min, and then treated with neutralizing buffer (1 M Tris-HCl, 1.5 M NaCl (pH 7.5)) for 5 min and 2× SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate) for 15 min, successively. After DNA fixation by UV cross-linking, the membrane was washed in 2× SSC containing 0.1% SDS, and then hybridization was carried out with the same system as used for Southern hybridization. Nucleotides were sequenced by the dideoxy chain terminating method using an Applied Biosystems Prism 310 genetic analyzer.

RESULTS

Culture Conditions for A. pascens F164—We had already found that the isonitrile hydratase was produced in A. pascens F164 when NBFA was added to the culture medium (8). When benzyl isocyanide was used as the substrate for the cell-free extracts, we identified the following two metabolites in the reaction mixture: N-benzylformamide and benzylamine (data not shown). We could not determine the exact level of N-substituted formamide formation, because N-substituted formamide deformylase was also formed under the above culture conditions, which degraded the N-substituted formamide (formed from isonitrile by isonitrile hydratase) to the corresponding amine. Thus, we constructed a highly precise assay system for isonitrile hydratase activity. First, we attempted to find a suitable isonitrile that can be degraded by the Arthrobacter isonitrile hydratase whose resultant reaction product cannot be degraded by N-substituted formamide deformylase. In the previous study (8), we found that N-substituted formamide deformylase was not able to degrade N-cyclohexylformamide to the corresponding amine. Therefore, we changed the substrate from benzyl isocyanide to cyclohexyl isocyanide and detected the production of N-cyclohexylformamide but no production of cyclohexyl amine (data not shown). These results indicate the successful measurement of the actual isonitrile hydratase activity even in the presence of N-substituted formamide deformylase under the used assay conditions.

An oligonucleotide sense primer (23-mer, 256 variants, 5'-ATGGAYCAYCCNCCNTTYTTYGC-3') and an antisense primer (20-mer, 48 variants, 5'-ACCATDATRTRTTRT-CYTT-3') were synthesized based on the NH₂-terminal (MDHPKFKA) and internal amino acid (KDNIMV) sequences of the enzyme, respectively. A reaction mixture (50 μl) was analyzed by means of automated Edman degradation. To determine its internal sequences, the purified enzyme was digested with a lysyl endopeptidase (Wako Pure Chemical Industries Co., Ltd.) or an endoproteinase Asp-N (Takara Bio Laboratories, Inc., West Grove, PA) for 1 h. The immunoreactive proteins were detected with an ECL Western blotting detection kit (GE Healthcare).

Amino Acid Sequencing—The partial NH₂-terminal amino acid sequence of the purified Arthrobacter isonitrile hydratase was analyzed by means of automated Edman degradation. To determine its internal sequences, the purified enzyme was digested with a lysyl endopeptidase (Wako Pure Chemical Industries Co., Ltd.) or an endoproteinase Asp-N (Takara Bio Industries Co., Ltd.) or an endoproteinase Asp-N (Takara Bio Inc., Otsu, Japan), respectively. The peptide fragments were sequenced by automated Edman degradation.

Cloning and Nucleotide Sequencing of the Arthrobacter Isonitrile Hydratase Gene—Escherichia coli DH10B (Invitrogen) was used as the host for pUC plasmids (14). E. coli transformants were grown in 2YT medium (14). E. coli transformants were grown in 2YT medium (8).

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TABLE 1

| Purification of Arthrobacter isonitrile hydratase |
|-----------------------------------------------|
| Step                                      | Total protein | Total activity | Specific activity | Yield |
| Cell-free extract                        | 756           | 48.4 ± 0.1     | 0.0640 ± 0.0014  | 100   |
| (NH₄)₂SO₄ (0.20–0.35)                    | 163           | 38.3 ± 0.6     | 0.235 ± 0.016    | 79.1  |
| First Butyl-Toyopearl 650 M              | 35.2          | 29.1 ± 1.2     | 0.827 ± 0.039    | 60.1  |
| Second Butyl-Toyopearl 650 M             | 21.4          | 20.0 ± 0.3     | 0.935 ± 0.012    | 41.3  |

Using this cell-free extracts assay method, we investigated the optimum culture conditions for Arthrobacter isonitrile hydratase production in this strain. Because we used a minimal medium containing glycerol and N-benzylformamide as the sole carbon and nitrogen sources, respectively, the bacterial growth rate was slow, and the total and specific activities in the cells were low (0.00137 ± 0.00002 units/ml of culture and 0.011 ± 0.0002 units/mg, respectively). At first, we examined the effects of various nutrient media containing NBFA and found that NZCYM medium was the most suitable for the preparation of cells with high enzyme activity (0.00517 ± 0.00097 units/ml of culture and 0.016 ± 0.003 units/mg). Next, we investigated the effects of the following compounds added to the NZCYM medium on the enzyme formation: two isonitriles (i.e. benzyl isocyanide and cyclohexyl isocyanide), NBFA, N-benzylacetamide, and benzylamine. Isonitrile was added to a final concentration of 0.05% (v/v) to the medium and each of the others was added to a final concentration of 0.05% (w/v) to the medium. Benzylamine and N-benzylacetamide did not induce isonitrile hydratase activity (0.00045 ± 0.00005 units/mg and 0.00048 ± 0.00008 units/mg, respectively). Furthermore, we did not obtain a large amount of cells from the culture in the medium containing benzyl isocyanide or cyclohexyl isocyanide at a high concentration (0.05%). However, the highest isonitrile hydratase activity was obtained in the presence of NBFA in the culture medium (0.016 ± 0.0003 units/mg). As for examination of cultivation time, cultivation was carried out in the above medium at 28 °C with shaking. The highest specific activity was observed in the cells grown for 60 h.

We established the optimum conditions for the production of isonitrile hydratase by A. pascens F164 (0.0156 ± 0.00005 units/ml of culture and 0.029 ± 0.0001 units/mg); the enzyme activity in the culture broth reached ~11.4 and 2.6 times higher than that under the initial conditions (0.00137 ± 0.00002 units/ml of culture and 0.011 ± 0.0002 units/mg).

**Purification of Arthrobacter Isonitrile Hydratase**—Purification of the enzyme was carried out from an extract of cells cultured in the presence of NBFA. Through the purification steps described under “Experimental Procedures,” the enzyme was purified 14.6-fold with a yield of 41.3% (Table 1). The purified enzyme gave only one band on SDS-PAGE (Fig. 1), which corresponded to a molecular mass of 43 kDa. Further evidence of the purity of the enzyme preparation was provided by the results of gel filtration on a Superose 6 HR 10/30 column, which gave a single symmetrical protein peak (Fig. 2). The molecular mass of the native enzyme was determined to be 530 kDa by gel filtration chromatography.

Qualitative analysis of the following metals in the enzyme solution was performed with an inductively-coupled radiofrequency plasma spectrometer: lithium, boron, magnesium, silicon, sulfur, calcium, titanium, chromium, iron, nickel, zinc, germanium, selenium, strontium, zirconium, molybdenum, rhodium, silver, indium, antimony, barium, cerium, neodymium, europium, terbium, holmium, thulium, lutetium, tantalum, rhenium, iridium, gold, thallium, bismuth, beryllium, sodium, aluminum, phosphorus, potassium, scandium, vanadium, manganese, cobalt, copper, gallium, arsenic, rubidium, yttrium, niobium, ruthenium, palladium, cadmium, tin, tellurium, lanthanum, praseodymium, samarium, gadolinium, dysprosium, erbium, ytterbium, hafnium, tungsten, osmium, platinum, mercury, and lead. However, none of these 67 metals was detected within the limits of the assay.

The absorption spectrum of the purified enzyme in 10 mM KPB (pH 7.5) showed maximum absorbance at 280 nm; however, no other peak absorption or shoulder was observed (Fig. 3). These findings suggested that no cofactor would bind to the
enzyme. The CD spectrum of *Arthrobacter* isonitrile hydratase was examined (Fig. 4).

**Stoichiometry**—The stoichiometry of isonitrile consumption and *N*-substituted formamide formation during enzyme reaction was examined in a reaction mixture consisting of 100 mM KPB (pH 7.5), 5 mM benzyl isocyanide, and 0.02 mg/ml enzyme, in a final volume of 200 μl. The reaction was carried out at 25 °C. After a 10-min incubation, the amounts of residual benzyl isocyanide and *N*-benzylformamide were determined. The *N*-benzylformamide formed and the benzyl isocyanide remaining amounted to 1.59 and 3.41 mM, respectively. No formation of other compounds was observed. The results indicated that benzyl isocyanide was stoichiometrically hydrated to *N*-benzylformamide.

**Effects of pH and Temperature on the Activity and Stability of the Enzyme**—The effects of pH and temperature on the enzyme activity were examined. The enzyme exhibited maximum activity at pH 7.3–7.7, as shown in Fig. 5A. As to the effect of pH, the substrate is partly decomposed into *N*-cyclohexylformamide on spontaneous and chemical (nonenzymatic) reaction under acidic conditions under pH 6.0. Thus, the enzyme activity under pH 6.0 shown in Fig. 5A was calculated from the modified amount of product; we subtracted the amount of *N*-cyclohexylformamide formed in the control (the reaction mixture without the enzyme) from that in each sample before the calculation. The substrate was stable over pH 6.5. The optimal temperature was 35 °C, and enzyme activity was rapidly lost above 40 °C (Fig. 5B). The substrate was very stable under 50 °C.

The stability of the enzyme was examined at various pH values. After the enzyme had been incubated at 25 °C for 30 min in six buffers at a concentration of 100 mM (citrate/KOH buffer (pH 4.0–6.0); citrate/K₃HPO₄ buffer (pH 5.5–7.0); KPB (pH 6.0–8.0); Tris-HCl buffer (pH 7.5–9.0); NH₄OH/NH₄Cl buffer (pH 8.5–10.0); and KHCO₃/KOH buffer (pH 10.0–11.0)), an aliquot of the enzyme solution was taken, and then enzyme activity was assayed under the standard conditions. The enzyme was the most stable in the pH range of 5.5–9.7. The stability of the enzyme was examined at various temperatures. After the enzyme had been preincubated for 30 min in 100 mM KPB (pH 7.5) containing 10% glycerol and 1 mM dithiothreitol, an aliquot of each enzyme solution was taken, and then the enzyme activity was assayed under the standard conditions. It exhibited the following activity: 25 °C, 0.890 ± 0.008 units/mg; 30 °C, 0.876 ± 0.023 units/mg; 35 °C, 0.826 ± 0.025 units/mg; 40 °C, 0.691 ± 0.022 units/mg; 45 °C, 0.144 ± 0.007 units/mg; 50 °C, 0.071 ± 0.008 units/mg; and 60 °C, not detected.

**Substrate Specificity**—The ability of the enzyme to catalyze the hydration of various isonitriles was examined. Among the seven tested isonitriles, six were substrates for the purified isonitrile hydratase, isocyanomethyl phosphonic acid diethyl ether not being a substrate (Table 2). These findings indicated that *Arthrobacter* isonitrile hydratase used not only aromatic isonitriles but also aliphatic isonitriles as substrates. The hydration of cyclohexyl isocyanide followed Michaelis-Menten-type kinetics, the *Kₘ* and *Vₘₐₓ* values being 0.95 ± 0.05 mM and 1.00 ± 0.03 unit/mg, respectively. On the other hand, these
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TABLE 3
Effects of various compounds on the activity of isonitrile hydratase

| Inhibitor                      | Relative activity |
|-------------------------------|-------------------|
| None                          | 100               |
| LiCl, NaCl, MgCl, CaCl, MnCl, RbCl, CsCl, SrCl | 95–103           |
| NaNO3 (0.25 mM)               | 94.0              |
| FeSO4 (0.25 mM)               | 92.4              |
| Pb(NO3)2                      | 88.1              |
| FeCl3                         | 80.3              |
| AlCl3                         | 78.6              |
| SrCl2                         | 58.3              |
| ZnCl2                         | 48.1              |
| CdCl2                         | 28.4              |
| CoCl2                         | 10.5              |
| NiCl2                         | 7.0               |
| HgCl2                         | 0                 |
| CuCl2                         | 0                 |
| AgNO3                         | 0                 |
| Iodoacetate                   | 96.4              |
| 5,5'-Dithiobis-2-nitrobenzoate| 65.1              |
| N-Ethylmaleimide              | 51.9              |
| p-Chloromercuribenzoate        | 0.2               |
| Hydroxylamine                 | 108               |
| Phenylhydrazine               | 98.2              |
| Semicarbazide                 | 100               |
| Aminoguanidine                | 97.9              |
| o,o-Dipryidyl                 | 105               |
| o-Phenanthroline              | 108               |
| 8-Hydroxyquinoline            | 73.9              |
| EDTA                          | 108               |
| Diethylidithiocarbamate       | 103               |
| NaN3                          | 95.8              |
| KCN                           | 100               |
| Dithiothreitol                | 110               |
| 2-Mercaptoethanol             | 98.8              |
| NaNO2                         | 105               |
| H2O2                          | 55.0              |
| Ammonium persulfate           | 94.8              |
| Phenylmethanesulfonate fluoride| 50.6             |
| Diisopropyl fluorophosphate   | 98.5              |

values for benzyl isocyanide were 0.30 ± 0.02 mM and 6.51 ± 0.05 units/mg, respectively. Benzyl isocyanide was turned over more rapidly and bound with much higher affinity by the enzyme.

The ability of the enzyme to catalyze the hydrolysis of nitriles to the corresponding acids was examined. Among the tested nitriles, the following were substrates for the purified Arthrobacter isonitrile hydratase: propionitrile (1.15 ± 0.07 units/mg), acrylonitrile (4.25 ± 0.12 units/mg), n-butyronitrile (1.45 ± 0.16 units/mg), benzonitrile (0.45 ± 0.003 units/mg), and 4-cyanopyridine (1.62 ± 0.07 units/mg). Other nitriles which are described under “Experimental Procedures” were inert as substrates of Arthrobacter isonitrile hydratase.

Inhibitors—Various compounds were investigated as to their inhibitory effects on the enzyme activity (Table 3). The enzyme was very sensitive to CdCl2, CoCl2, NiCl2, HgCl2, CuCl2, and AgNO3. Hydrophobic thiol reagents such as p-chloromercuribenzoate, 5,5'-dithiobis-nitrobenzoate, and N-ethylmaleimide obviously inhibited the activity of the enzyme. By comparison, the hydrophilic thiol reagent iodoacetate did not influence the activity. These results indicate that the active amino acid residue involved in enzymatic catalysis may be located in a hydrophobic environment. Carbonyl reagents (e.g. hydroxylamine, phenylhydrazine, semicarbazide, and aminoguanidine) did not inhibit the enzyme activity. One chelating reagent, 8-hydroxyquinoline, caused partial inhibition, but other chelating reagents and reducing reagents did not inhibit the activity. However, oxidizing reagent H2O2 caused appreciable inhibition. The enzyme was partially sensitive to a serine-modifying reagent, phenylmethanesulfon fluoride.

Immunological Studies on Isonitrile Hydratase—Western blot analysis of InhA (0.2, 2.0, 20, and 200 ng) and Arthrobacter isonitrile hydratase (0.2, 2.0, 20, and 200 ng) demonstrating the cross-reactivity of the polyclonal antibodies with the two enzymes, respectively. A, Arthrobacter isonitrile hydratase and anti-(Arthrobacter isonitrile hydratase) antibody. B, Arthrobacter isonitrile hydratase and anti-InhA antibody. C, InhA and anti-InhA antibody. D, InhA and anti-(Arthrobacter isonitrile hydratase) antibody.
neighboring well. As for the anti-InhA serum, a precipitin band was only formed with InhA. In contrast, there was no formation of a precipitin band between those of the Arthrobacter enzyme and anti-InhA serum or between the wells of InhA and anti-(Arthrobacter isonitrile hydratase) serum. Furthermore, we also analyzed the cross-reactivity of the two isonitrile hydrolases using an immunological technique. The enzyme solutions were incubated with various concentrations of anti-InhA or anti-(Arthrobacter isonitrile hydratase) serum, respectively. After centrifugation, the remaining activity in each supernatant solution was measured. On immunotitration analysis using the purified Arthrobacter isonitrile hydratase and antibodies, the Arthrobacter enzyme activity was found to dose-dependently decrease on incubation with the anti-(Arthrobacter isonitrile hydratase) antibody. However, the enzyme activity was not affected on incubation with the antibody against InhA. When the purified InhA was incubated with the anti-InhA antibody, InhA also dose-dependently decreased. Moreover, when the anti-(Arthrobacter isonitrile hydratase) antibody was incubated with InhA, no significant decrease in the activity was observed. These results also support that the isonitrile hydratase from A. pascens F164 was not immunologically related to InhA from P. putida N19-2 at all.

Cloning and Nucleotide Sequencing of the Arthrobacter Isonitrile Hydratase Gene—The isonitrile hydratase gene was purified to homogeneity from A. pascens F164, and the amino acid sequences of peptides were determined by digesting the enzyme with endoproteinase Asp-N. Two oligonucleotide primers were synthesized based on the NH2-terminal and internal sequences (corresponding to amino acids 1–7 and 92–98, respectively) and used for PCR amplification with genomic DNA of A. pascens F164 as a template, resulting in the generation of a 293-bp fragment. The deduced amino acid sequence of the amplified fragment was consistent with the internal sequences of the enzyme determined by Edman degradation, indicating that the fragment was a portion of the enzyme gene.

To obtain the entire isonitrile hydratase gene, after digestion of the genomic DNA with several restriction enzymes, Southern hybridization was performed using the 293-bp fragment as a probe. A single 2.2-kb BspEI fragment was positively detected, and this fragment was recovered and ligated with XmaI-digested pUC18 to transform E. coli DH10B. After screening of the recombinant plasmids by colony hybridization, a positive clone, designated as pAPINH10, was obtained.

The nucleotide sequencing of pAPINH10 revealed a 1077-bp open reading frame encoding 359 amino acids, which corresponded precisely to those determined with the purified isonitrile hydratase. The molecular mass of the protein encoded by this gene was calculated to be 38,880 Da, which was a little bit different from that of the enzyme subunit (molecular mass = 43 kDa) determined on SDS-PAGE.

A search of protein sequence databases with the BLAST server revealed that Arthrobacter isonitrile hydratase exhibits significant similarity to some nitrilases from the following strains: Methylibium petroleiphilum PM1 (GenBank™ accession number ABM93955.1, 64% identity); Bradyrhizobium sp. ORS278 (GenBank™ accession number CAL78276.1, 62% identity); Pseudomonas syringae pv. syringae B728a (GenBank™ accession number AAY35081.1, 57% identity); Rhodococcus rhodochrous K22 (GenBank™ accession number D12583, 51% identity) (16); and Rhodococcus rhodochrous J1 (GenBank™ accession no. D11425, 49% identity) (17). On the other hand, no sequence similarity was observed between Arthrobacter isonitrile hydratase and InhA at all.

Isonitrile Hydratase Activity in A. pascens F164—To investigate the function of isonitrile hydratase in vivo, A. pascens F164 was grown in two culture media. Although A. pascens F164 grew in the medium containing 0.4% (w/v) glycerol and 0.07% (w/v) (NH4)2SO4 as the carbon and nitrogen sources, respectively, and exhibited full growth at 51 h (Fig. 7A), the isonitrile hydratase was not observed during the cultivation (Fig. 7B). On the other hand, A. pascens F164 grew when a small amount of benzyl isocyanide was fed five times (at 0 h, 0.006% (v/v); 3 h, 0.02% (v/v); 15 h, 0.04% (v/v); 27 h, 0.04% (v/v); and 39 h, 0.04% (v/v), respectively) as the sole carbon and nitrogen sources (Fig. 7A), although the strain did not grow when 0.05% (v/v) of benzyl isocyanide was used. On replacement of glycerol and (NH4)2SO4 with benzyl isocyanide, isonitrile hydratase activity was detected in the cells (Fig. 7B). Moreover, the formation of isonitrile hydratase and NfdA in A. pascens F164 cells was examined (Fig. 7, B and C). Cell-free extracts were prepared from the strain cultured in the presence or absence of benzyl isocyanide, and then subjected to SDS-PAGE (Fig. 7D). When the strain was grown with benzyl isocyanide, remarkable bands of isonitrile hydratase and NfdA were detected on staining with Coomassie Brilliant Blue, which migrated to positions corresponding to the purified isonitrile hydratase (43 kDa) and NfdA (61 kDa), respectively. However, neither isonitrile hydratase nor NfdA was formed in the absence of benzyl isocyanide in the culture medium.

DISCUSSION

In previous studies (6, 8), we discovered two novel enzymes, isonitrile hydratase (InhA) from P. putida N19-2 and N-substituted formamide deformylase (NfdA) from A. pascens F164, and we revealed the overall isonitrile metabolic pathway. These studies also demonstrated that InhA exhibits low amino acid sequence similarity to a kind of protease (7), and NfdA belongs to a certain amidohydrolase superfamily (8). It is interesting that isonitrile metabolic enzymes exhibit similarity to C–N bond-hydrolyzing ones. However, only one isonitrile hydratase and one N-substituted formamide deformylase have been isolated, respectively, and therefore their detailed characteristics remain unknown. Moreover, the crystal structures of the two enzymes have never been determined, and it has never been clarified how they act on a compound to cleave the C–N bond. It is necessary to screen and analyze other enzymes involved in isonitrile metabolism to compare their properties in detail.

We reported as unpublished data4 that A. pascens F164 showed isonitrile-degrading ability. This isonitrile-degrading enzyme coexists with NfdA in this strain. It is proposed that these enzymes are involved in the consecutive metabolism of isonitrile. However, we have never carried out detailed analysis

4 H. Sato, Y. Hashimoto, H. Fukatsu, and M. Kobayashi, unpublished data.
of this isonitrile-degrading enzyme; we did not purify or analyze the enzyme; we only observed the activity. To clarify the unknown enzyme properties, and the relationship between isonitrile-degrading enzyme and N-substituted formamide deformylase, we embarked on analysis of the isonitrile-degrading enzyme in *A. pascens* F164.

In the case of nitrile, which is an isomer of isonitrile, its degradation is known to proceed through two enzymatic pathways as follows: nitrile hydratase (18–22) and nitrilase (16, 17, 23–25). Nitrile hydratase catalyzes the hydration of a nitrile to the corresponding amide; on the other hand, nitrilase catalyzes the direct hydrolysis of a nitrile to the corresponding acid and ammonia. Both enzymes can utilize nitrile as a substrate, but the reactions are different, *i.e.* addition of one (hydration) or two (hydrolysis) water molecules. Considering these cases, there could be several pathways for the degradation of isonitrile. In the reaction using cell-free extracts of *A. pascens* F164, N-substituted formamide and amine were produced from benzyl isocyanide. Formation of the two reaction products would be due to the presence of the N-substituted formamide deformylase in the cell-free extracts and the possibility that the cell-free extracts contained another type of isonitrile-degrading enzyme that hydrolyzes an isonitrile to the corresponding amine. Replacement of benzyl isocyanide with cyclohexyl isocyanide resulted in formation of only one reaction product; N-substituted formamide was produced, and amine was not formed. These findings demonstrated that the isonitrile-degrading enzyme from *A. pascens* F164 is an isonitrile hydratase but not an enzyme-converting isonitrile directly into amine.

Based upon the properties of the purified *Arthrobacter* isonitrile hydratase, we performed a comparative study of the *Arthrobacter* enzyme and InhA to obtain detailed information on isonitrile-degrading enzymes. InhA from *P. putida* N19-2, which is different from *A. pascens* F164 in genus, has a molecular mass of ~60 kDa and consists of two identical subunits (one of which has a molecular mass of about 29 kDa) (6). InhA prefers only aromatic isonitriles as substrates. InhA is quite inhibited by a thiol reagent; a hydrophilic thiol reagent, iodoacetate, inhibits the enzyme but a hydrophobic one, *p*-chloromercuribenzoate, does not. InhA, which shows sequence similarity to cysteine protease, is known to have an active cysteine in its catalytic center (7). The active SH residue in InhA involved in enzymatic catalysis may be located in a less hydrophilic environment. To summarize, *Arthrobacter* isonitrile hydratase is similar to InhA in the following characteristics. (i) Both enzymes do not need any metal or cofactor for catalysis. (ii) They are inhibited by a thiol reagent. These similarities indicate that *Arthrobacter* isonitrile hydratase would have an active cysteine residue in its catalytic center. On the other hand, there are several differences between the two enzymes. The calculated molecular mass of the *Arthrobacter* enzyme subunit is about 14 kDa larger than that of InhA, and the subunit numbers are different. Moreover, we observed differences in the environment of the active site in the thiol inhibitor experiments. The active site of *Arthrobacter* isonitrile hydratase would be located in a more hydrophobic environment than that of InhA. Substrate specificity analysis also supports this difference in the environment of the active site. To clarify the difference in sec-
ondary structure between the two isonitrile hydratases, we compared their CD spectra (Fig. 4), because many common conformational motifs, including α-helices and β-sheets, have characteristic far-UV CD spectra in general. The CD spectra suggest that the Arthrobacter enzyme exhibits a difference in secondary structure from InhA. Next, we prepared polyclonal antibodies for Arthrobacter isonitrile hydratase and InhA, and examined the cross-reactivity to the two enzymes, respectively, using immunological techniques. In our study, immunological analysis revealed that Arthrobacter isonitrile hydratase does not share any antigenic determinants with InhA. This finding indicates that the Arthrobacter enzyme would greatly differ in amino acid sequence from InhA, because no cross-reaction was seen. Arthrobacter isonitrile hydratase has a different primary structure from that of InhA. Furthermore, we cloned the Arthrobacter isonitrile hydratase gene and revealed no sequence homology between the enzyme and InhA. These findings strongly support that these two isonitrile hydratases are unrelated structurally to each other, although both enzymes catalyze the same reaction, isonitrile hydration. On the other hand, the Arthrobacter enzyme exhibits similarity to nitrilases. Whereas InhA does not act on nitriles at all (6), Arthrobacter isonitrile hydratase has been found to act on nitriles weakly. Taking these findings and observations together, Arthrobacter isonitrile hydratase is expected to have a novel catalytic mechanism different from that of InhA. Because the two isonitrile hydratases are different from each other, we named the isonitrile hydratase from A. pascens F164 InhB. These findings shed new light on the biological metabolism of compounds containing carbon-nitrogen bonds. Further analyses, including identification of the active center of the enzyme, are required to clarify the differences in reaction mechanism between InhB and InhA.

InhB from A. pascens F164 showed the most significant activity and affinity toward benzyl isocyanide ($K_m = 0.30 \pm 0.02 \text{ mM}$), yielding N-benzylformamide through its reaction. The N-substituted formamide deformylase (NfdA) of this strain exhibits the highest activity toward N-benzylformamide as a substrate. Therefore, we observed a similar trend in substrate specificity for InhB and NfdA, which co-exist in A. pascens F164. We measured the activities of InhB and NfdA in cell-free extracts of A. pascens F164 cells grown in two different culture media. Neither InhB nor NfdA was produced in the medium containing glycerol and (NH$_4$)$_2$SO$_4$ (Fig. 7, B–D). In the benzyl isocyanide-containing medium, the peak specific activity of NfdA was found at 27 h, i.e. 12 h later than that of InhB (15 h) (Fig. 7, B and C). These findings indicate that both InhB and NfdA were concomitantly induced by isonitrile. Furthermore, difference in growth of the cells cultured in media changing isonitrile concentration suggests that benzyl isocyanide at a high concentration would be toxic for A. pascens F164, but the strain can use a low concentration of benzyl isocyanide as the sole carbon and nitrogen sources for growth. Considering the formation of InhB in cooperation with NfdA, InhB would be physiologically involved in assimilation and/or detoxification of isonitrile for cell growth.

Under the optimum culture conditions for InhB, the highest isonitrile hydratase activity was obtained when A. pascens F164 was cultured in the medium containing NBFA. These findings indicate that InhB was inducibly produced with not only isonitrile but also N-substituted formamide. The finding that InhB was induced by its reaction product is very unusual. A similar observation has been reported for a nitrile hydratase. A high molecular mass nitrile hydratase is inducibly formed with an amide (the product of the nitrile hydratase reaction) (18, 19). Of particular interest is the mechanism underlying this unusual induction of InhB by a substrate and its reaction product.

In conclusion, our work on the isonitrile degradation in A. pascens F164 provides the first evidence of a close relationship between isonitrile hydratase and N-substituted formamide deformylase in isonitrile metabolism and/or defense mechanism. On the other hand, InhB will differ from InhA in primary and secondary structures, indicating that InhB would have evolved from a different ancestral gene and have a novel reaction mechanism. We discovered that InhB shows not only isonitrile hydratase activity but also nitrilase activity. These findings provide interesting new insights into the relationship between –N=C hydration and –C=N hydrolysis. Further research on this enzyme will provide us with novel knowledge about C–N hydration and lead to a better understanding of the catalytic mechanism.

Acknowledgments—We thank Dr. K. Shiraki for the CD spectra analysis. Special thanks are also due to Dr. Z. Zhou and S. Nonoyama for help in the preparation of antibodies.

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