Supporting Materials and Methods

Materials. DEAE-Sephacel and a low-molecular-weight standard kit were obtained from Amersham Biosciences. Standard proteins for high-performance gel filtration chromatography and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS were purchased from Oriental Yeast Co. (Tokyo) and Sigma, respectively. All other biochemicals used, except for *N*-formyl-L-kynurenine, were from commercial sources and of reagent grade. *N*-formyl-L-kynurenine was synthesized as described in ref. 1.

Culture Conditions for *Arthrobacter pascens* Strain F164. *A. pascens* strain F164 was collected from an agar plate and then inoculated for subculture. The subculture was carried out at 28°C for 24 h with reciprocal shaking in a 500-ml shaking flask containing 90 ml of 2× YT medium (16 g of tryptone/10 g of yeast extract/5 g of NaCl per liter of distilled water) supplemented with *N*-benzylformamide (NBFA) at a final concentration of 0.05% (wt/vol). Then 5 ml of the subculture was inoculated into a 2-liter shaking flask containing 500 ml of medium (pH 7.0) consisting of 10 g of glycerol, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.005 g of $FeSO_4 \cdot 7H_2O$, 1 ml of vitamin mixture (2), and NBFA at a final concentration of 0.05% (wt/vol) per liter of distilled water, followed by incubation at 28°C with reciprocal shaking. After 24 h of incubation, the cells were harvested by centrifugation at 13,000 × *g* at 4°C and then washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 10% (wt/vol) glycerol.

Enzyme Assay. All of the reactions were performed under linear conditions as to protein (≤1.5 μg/ml) and time (≤10 min). The standard assay A mixture comprised 0.1 M potassium phosphate buffer (pH 7.5), 10 mM NBFA, and an appropriate amount of enzyme [N-substituted formamide deformylase (NfdA)] in a total volume of 400 μl. The reaction was started by the addition of the enzyme and carried out at 25°C for 10 min. The reaction was stopped by the addition of 400 μl of cold acetonitrile to the reaction mixture, and a supernatant was obtained by centrifugation (12,000 × *g*, 10 min). Forty microliters of the supernatant was mixed with 80 μl of 0.2% (vol/vol) triethylamine and 40 μl of 0.4% (wt/vol) 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (3). The amount of GITC-derivatized benzylamine was determined by HPLC, which was performed with the same system as used for the measurement of NBFA under *Materials and Methods*, except that the wavelength of 250 nm was used for the monitoring.

In standard assay B, NfdA activity was also assayed by measurement of formate by HPLC. The reaction was started by the addition of the enzyme and carried out at 25°C for an appropriate time. The reaction was stopped by the addition of 400 μl of cold acetonitrile to the reaction mixture, and a supernatant was obtained by centrifugation (12,000 × *g*, 10 min). The amount of formate was determined by HPLC, which was performed with a Shimadzu LC-6A system equipped with a Unison US-C18 column (reversed-phase; 4.6 by 250 mm; Imtakt, Kyoto). The following solvent system was used: 100 mM KH_2PO_4-H_3PO_4 buffer (pH 2.0), at the flow rate of 0.6 ml/min and 37°C. Monitoring was conducted at 210 nm. This assay was used to determine the specificity of

the enzyme for various N-substituted formamides and the stoichiometry of the enzymatic reaction.

One unit of NfdA activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of *N*-benzylamine (or formate) per min from NBFA under the above conditions. The protein concentration was determined by the method of Bradford (4). The specific activity is expressed as units per milligram of protein.

Electrophoresis. SDS/PAGE was performed in a 12.5% polyacrylamide slab gel according to Laemmli (5). 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* (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Molecular Mass Determination. The purified enzyme sample was applied to a Superose 12 HR10/30 column (Amersham Biosciences), which was attached to an ÄKTA purifier (Amersham Biosciences), and then eluted with 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M KCl at the 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, i.e., glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome *c* (12.4 kDa).

The molecular mass was also determined by MALDI-TOF MS with a Shimadzu (Kyoto) AXIMA-CFR plus. The instrument was calibrated with BSA (66 kDa) and cytochrome *c* (12.4 kDa) as standard proteins.

Metal Analysis. All glassware was soaked in 2 M HCl overnight and then exhaustively rinsed with distilled water before use. Before analysis, the enzyme was dialyzed against 1 mM potassium phosphate buffer (pH 7.5). The enzyme sample containing 1.38 mg protein per ml was analyzed with an inductively coupled radiofrequency plasma spectrophotometer [Shimadzu ICPS-8000 (27.120 MHz)]. The metal contents of the enzyme sample were determined from the calibration curves for standard solutions.

Amino Acid Sequencing of NfdA. The N-terminal amino acid sequence of the intact enzyme was determined with a sample electroblotted onto a polyvinylidene difluoride (PVDF) membrane after SDS/PAGE using a Procise protein sequencer (Applied Biosystems). To determine its internal sequences, the enzyme was incubated in 20 mM Tris·HCl buffer (pH 9.0) or 50 mM ammonium acetate buffer (pH 4.0) containing 8 M urea at 37°C for 24 h, and then digested with a 1:200 (mol/mol) ratio of lysylendopeptidase (Takara Bio, Otsu, Japan) in 20 mM Tris·HCl buffer (pH 9.0) or a 1:50 (mol/mol) ratio of endoproteinase Glu-C (Roche Diagnostics) in 50 mM ammonium acetate buffer (pH 4.0), respectively. The fragments were separated by SDS/PAGE, electroblotted onto a PVDF membrane, and then examined with a protein sequencer, and

the internal sequence of each fragment was determined by automated Edman-degradation.

Substrate Specificity. The following N-substituted formamides, amides, and other compounds were used for the measurement of substrate specificity of the enzyme. (A) N-substituted formamides: NBFA, *N*-butylformamide, allylformamide, *N*-*tert*-butylformamide, *N*-isopropylformamide, *N*-methylformamide, *N*-*sec*-butylformamide, formamide, formic hydrazide, *N*,*N*-dimethylformamide, *N*-formylethylamine, *N*,*N*-diethylformamide, *N*-(2-cyclohex-1-enylethyl)formamide, *N*-cyclohexylformamide, *N*-(1-cyclohexenyl)formamide, 1-formylpiperidine, 1-formylpiperazine, *N*-formylmorpholine, *N*-(α-methylbenzyl)formamide, formanilide, *N*-formyl-L-alanine, *N*-formyl-L-lysine, *N*-formyl-L-tyrosine, *N*-formyl-L-methionine, *N*-formyl-L-phenylalanine, *N*-formyl-D-phenylalanine, *N*-formyl-DL-alanine, *N*-formyl-L-aspartate, *N*-formylglycine, *N*-formyl-L-valine, *N*-formyl-L-leucine, *N*-formyl-L-isoleucine, *N*-formyl-L-tryptophan, *N*-formyl-L-histidine, *N*-formylurea, 2-formylamino-α-(methoxyimino)-4-thiazoleacetic acid, formyl anthranilic acid, *N*-formyl-L-kynurenine, and *N*-formylthiosemicarbazide. (B) Amides: urea, *n*-butyramide, acetamide, propionamide, isobutyramide, *n*-valeramide, isovaleramide, *n*-capronamide, methacrylamide, phenylacetamide, benzamide, *ε*-caprolactam, and phenylurea. (C) Others: adenine, cytosine, and allantoin.

The assaying of substrate specificity was carried out in a reaction mixture (400 μl) consisting of 100 mM potassium phosphate buffer (pH 7.5), 10 mM substrate, and an appropriate amount of enzyme. The reaction was carried out at 25°C for an appropriate time and stopped by adding 400 μl of cold acetonitrile [when N-substituted formamides (except *N*-butylformamide and *N*-formyl-L-kynurenine) and allantoin were used as substrates] or 400 μl of 330 mM sodium phenoxide (when amides, adenine, and cytosine were used as substrates) to the reaction mixture. The levels of product formation were determined as follows. The enzyme activities for all of the N-substituted formamides (except *N*-butylformamide and *N*-formyl-L-kynurenine) were measured by standard assay B described above under *Enzyme Assay*. The activity toward *N*-butylformamide was measured by standard assay A with modifications. To increase the detection limit and accuracy for the butylamine product, 40 μl of the reaction mixture was taken and then added into 120 μl of a solution comprising 0.13% (vol/vol) triethylamine and 0.13% (wt/vol) GITC in acetonitrile (to stop the reaction), and a supernatant was obtained by centrifugation (12,000 × *g*, 10 min). The amount of GITC-derivatized butylamine was determined by HPLC. KH_2PO_4-H_3PO_4 buffer (10 mM), pH 2.0/acetonitrile, 61:125 (vol/vol), was used as the eluent. *N*-formylkynurenine deformylase and amidase activities were assayed at 25°C as described in refs. 6 and 7. Allantoinase activity was measured by standard assay B described above under *Enzyme Assay* with modifications. The amount of hydantoin formed was determined by HPLC with a wavelength of 200 nm being used for the monitoring.

Cloning and Nucleotide Sequencing of the *nfdA* gene. *Escherichia coli* DH10B (Invitrogen) was used as the host for pUC plasmids (8). *E. coli* transformants were grown in 2× YT medium (8). Genomic DNA was prepared from *A. pascens* strain F164 as follows: the strain was cultured at 28°C for 24 h in 500 ml of 2× YT medium with

reciprocal shaking. Cells were harvested by centrifugation, washed with 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA and 100 mM NaCl, and then suspended in 10 ml of 50 mM Tris buffer containing 10 mM EDTA and 15% (wt/vol) sucrose. The suspension was incubated with 7 mg/ml of lysozyme at 37°C for 30 min, and then 2 ml of 0.5 M EDTA (pH 8.0), 2 ml of 10% SDS and 2.7 mg of proteinase K were added to the solution, followed by incubation at 55°C for 3 h. DNA was purified by extracting the lysate with phenol/chloroform/isoamylalcohol (25/24/1; vol/vol/vol), precipitated with isopropanol, treated with RNase, and then reprecipitated with ethanol.

An oligonucleotide sense primer (29-mer, 4,608 variants, 5'-CARATGMGNGANYTNATGATHATHAAYGC-3') and an antisense primer (23-mer, 576 variants, 5'-ATRTCDATIGCNCARTTNCCCAT-3'; I = inosine) were synthesized based on the N-terminal (QMRDLMIINA) and internal amino acid (MGNCAIDI) sequences of the enzyme, respectively. A reaction mixture (50 µl) comprising 35 ng of genomic DNA, 300 pmol of each primer and *Ex Taq* polymerase (Takara Bio) was subjected to PCR (94°C 30 s, 51°C 30 s, 72°C 60 s; 30 cycles), and the amplified DNA fragment (1,057 bp) was gel-purified. The DNA fragment was then used as a probe for Southern hybridization and colony hybridization to clone the full-length *nfdA* gene.

Southern hybridization was carried out by using an Alkphos Direct Labeling and Detection System with CDP-*Star* (Amersham Biosciences) 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 was used for Southern hybridization.

Nucleotides were sequenced by the dideoxy-chain terminating method using an Applied Biosystems Prism 310 genetic analyzer.

Identification of the Compound Produced from NBFA by NfdA. One product in the reaction mixture with the purified enzyme from *A. pascens* (which was described under *Results*) was extracted with ethylacetate, and then analyzed by GC-MS. GC-MS was performed with a TraceGC/PolarisQ GCMS (Thermo Electron, San Jose, CA) equipped with a DB-1ms capillary column (0.25 × 30 m; Agilent Technologies, Wilmington, DE). The initial column temperature of 120°C was raised at 30°C per min to 200°C. The injection temperature was 300°C. The carrier gas was He at a flow rate of 1 ml/min.

The other product in the above reaction mixture was analyzed by HPLC. The HPLC conditions were the same as those for standard assay B.

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