Discovery and Characterization of a Novel *N*-Substituted Formamide Deformylase

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Discovery and Characterization of a Novel *N*-Substituted Formamide Deformylase

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Contents

General introduction 1

Chapter I **Screening of *N*-substituted formamide-degrading microorganisms and optimum culture conditions for the production of *N*-substituted formamide deformylase by *Arthrobacter pascens* F164**

Section I Screening of *N*-substituted formamide-degrading microorganisms and identification of *Arthrobacter pascens* F164 4

Section II Optimum culture conditions for the production of *N*-substituted formamide deformylase by *Arthrobacter pascens* F164 10

Chapter II **Purification and characterization of a novel *N*-substituted formamide deformylase from *Arthrobacter pascens* F164** 22

Chapter III **Genetic analysis of *N*-substituted formamide deformylase from *Arthrobacter pascens* F164** 43

References 68

Conclusions 73

Acknowledgements 75

Publications 76

General introduction

Nitriles are very toxic and generally bio-undegradable organic compounds containing a C≡N moiety. Kobayashi et al. have studied nitrile metabolism (Kobayashi et al., 1992a; Yamada and Kobayashi, 1996; Kobayashi and Shimizu, 1998); they clarified the structures and functions of enzymes (i.e., nitrilase [Komeda et al., 1996a; Kobayashi et al., 1992b and 1993a; Kobayashi and Shimizu, 1994], nitrile hydratase [Komeda et al., 1996b and c; Kobayashi et al., 1995; Kobayashi and Shimizu, 1999], and amidase [Kobayashi et al., 1993b, 1997 and 1998]) involved in metabolism, and their genes and regulation mechanisms.

On the other hand, information is quite limited on the metabolism of an isonitrile (more generally called an isocyanide) containing an isocyano group (-N≡C), which is an isomer of a nitrile. Isonitriles as well as nitriles are generally highly toxic and produced in nature by various organisms, including bacteria, fungi, marine sponges, etc (Edenborough and Herbert,, 1988; Scheuer, 1992; Garson and Simpson, 2004). An isocyanide metabolite, xanthocillin, was first isolated from *Penicillium notatum* (Rothe, 1950)). This isonitrile exhibits a wide antibiotic activity spectrum (Hagedorn and Tonjes, 1957). Although parts of the metabolic intermediates of some isonitriles have been elucidated through incorporation experiments (Hagadone et al., 1984; Achenbach and Grisebach, 1965; Achenbach and König, 1972; Pfeifer et al., 1972; Simpson et al., 2004), their synthetic and degradative pathways remained entirely undetermined. Furthermore, none of the enzymes involved in isonitrile metabolism, except for the enzyme the author describe below, has yet been identified.

Recently, Goda et al. isolated a microorganism, *Pseudomonas putida* N19-2, that is able to degrade isonitriles from soil and discovered an isonitrile-metabolizing enzyme, designated as isonitrile hydratase (EC 4.2.1.103), in this strain (Goda et al., 2001 and 2002). This enzyme catalyzes the hydration of an isonitrile [R-N≡C] to the corresponding

N-substituted formamide [R-NH-CH(=O)], and confers the ability of isonitrile-degradation on the microorganism. However, there have been no reports on the metabolic pathway for *N*-substituted formamide produced from the isonitrile or the enzymes acting on the former in the metabolism.

The author is interested in how C-N hydrolases evolved. Because the structure of *N*-substituted formamide contains a nitrogen-carbon bond, an *N*-substituted formamide-degrading enzyme would also belong to the category of C-N hydrolases. A search for such an enzyme and its functional analysis would contribute to clarification of the metabolism of an isonitrile in nature and provide us with new knowledge about C-N hydrolases, which might facilitate elucidation of their functional and structural evolution.

In this study, the author discovered and characterized *N*-substituted formamide deformylase (NfdA), a novel enzyme involved in the metabolism of isonitriles. Chapter I describes the screening of *N*-substituted formamide- and isonitrile-degrading microorganisms from soil and the investigation of optimum culture conditions for the production of NfdA by *Arthrobacter pascens* F164. Demonstration of inducibility of the enzyme formation in this strain is also described. Chapter II describes the purification and characterization of NfdA, which catalyzes the hydrolysis of an *N*-substituted formamide to the corresponding amine and formate, from *A. pascens* F164. In Chapter III, gene cloning and genetic analysis of the NfdA enzyme are described. Putaive metal-binding amino acid residues of the enzyme and the evolutionary relationships between NfdA and its distantly related homologues are discussed. It is hoped that these studies will contribute to a better understanding of isonitrile metabolism at the protein and gene levels and development of a new enzymatic procedure to synthesize useful *N*-substituted formamides.

Chapter I

Screening of *N*-substituted formamide-degrading microorganisms and optimum culture conditions for the production of *N*-substituted formamide deformylase by *Arthrobacter pascens* F164

Section I

Screening of *N*-substituted formamide-degrading microorganisms and identification of *Arthrobacter pascens* F164

Introduction

Kobayashi et al. have been interested in the enzymes involved in the metabolism of nitrile (Kobayashi and Shimizu, 1998; Komeda et al., 1996a,b and c; Kobayashi et al., 1997) and its isomer, isonitrile. Isonitrile (more generally called isocyanide), like nitrile, is generally a highly toxic compound with an isocyano group (-N≡C). Although naturally occurring isonitriles are elaborated by various organisms, including bacteria, fungi, and marine sponges (Edenborough and Herbert, 1988; Scheuer, 1992), the metabolism of isonitriles was unknown at the protein and gene levels before Goda et al. discovered an isonitrile-degrading enzyme, which was designated isonitrile hydratase. It is the first known enzyme involved in the isonitrile metabolism (Goda et al., 2001 and 2002). The enzyme catalyzes the hydration of an isonitrile to the corresponding *N*-substituted formamide: R-N≡C + H_2O → R-NH-CH(=O). However, there have been no reports on an enzyme that is involved in the further metabolism of an *N*-substituted formamide produced from the corresponding isonitrile.

In this section, the author describes the screening of *N*-substituted formamide-degrading microorganisms from soil through an acclimatization culture. It is also described that identification of strain F164 as *Arthrobacter pascens*, which is able to degrade both an *N*-substituted formamide and the corresponding isonitrile.

Materials and methods

Materials

N-Benzylformamide (NBFA) and benzyl isocyanide were purchased from Aldrich (Milwaukee, WI, USA) and Sigma-Aldrich Co., respectively. Benzylamine was obtained from Nacalai Tesque (Kyoto). All other biochemicals used were from commercial sources and of reagent grade.

Isolation of bacteria catabolizing *N*-substituted formamide and isonitrile

N-Substituted formamide- and isonitrile-catabolizing microorganisms were isolated from soil samples by the enrichment culture technique described in the previous report (Goda et al., 2001) with some modifications. The following synthetic medium was used: basal medium consisted 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 a vitamin mixture (0.4 g of thiamine hydrochloride, 0.2 g of riboflavin, 0.4 g of pyridoxime hydrochloride, 0.4 g of nicotinic acid, 10 mg of folic acid, 0.4 g of calcium pantothenate, 2 mg of biotin, 2 g of inositol and 0.2 g of *p*-aminobenzoic acid) per 1 liter of distilled water (pH7.0) was used. A spoonful of a soil sample was added to a test tube containing 10 ml of the basal medium supplemented with NBFA as the sole nitrogen source at a final concentration of 0.02% (w/v). 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 one month of cultivation, the microorganisms were spread on agar plates and isolated.

Assaying of the *N*-substituted formamide- and isonitrile-degrading abilities of the isolated strains

Each of the isolated strains was inoculated into a test tube containing 10 ml of the basal medium supplemented with NBFA at a final concentration of 0.05% (w/v) as the sole nitrogen source, and then incubated at 28°C for 72 h with reciprocal shaking. Then the cells were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5), and suspended in 0.1 M potassium phosphate buffer (pH 7.5).

The NBFA-catabolizing abilities of the isolated strains were assayed by means of the resting-cell reaction. The reaction mixture was composed of 0.1 M potassium phosphate buffer (pH 7.5), 10 mM NBFA, and an appropriate amount of cell suspension in a total volume of 1 ml. The reaction was carried out at 25°C for 2 h, 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 NBFA in the reaction mixture was determined by HPLC with a Shimadzu LC-6A system (Kyoto) equipped with a Cosmosil $5C_{18}$-AR-II column (reversed-phase; 4.6 by 150 mm: Nacalai Tesque). The following solvent system was used: 10 mM KH_2PO_4-H_3PO_4 buffer (pH 2.7)/acetonitrile, 1:1 (v/v), at the flow rate 1.0 ml/min and 40°C. The absorbance was measured at 198 nm.

The isonitrile-catabolizing abilities of the isolated strains were assayed by means of the resting-cell reaction. The reaction mixture was composed of 0.1 M potassium phosphate buffer (pH 7.5), 10 mM benzyl isocyanide, and an appropriate amount of cell suspension in a total volume of 1 ml. The reaction was carried out at 25°C for 1 day. The reaction was stopped by the addition of cold acetonitrile to an aliquot of the reaction mixture (1:1) and then rapidly removing the cells by centrifugation at 0-4°C. The residual amount of benzyl isocyanide in the reaction mixture was determined by HPLC with the same system as used for the NBFA measurement.

Results

Isolation of *N*-substituted formamide-degrading microorganisms

At about one month from the start of the study, using the acclimatization culture method described under "*Materials and methods*", the author isolated thirteen microorganisms that are able to utilize NBFA as the sole nitrogen source. The author then analyzed the NBFA-degrading reaction of these microorganisms on HPLC chromatography. As the result of their resting cell reaction, these isolates were found to degrade NBFA and produce an unknown compound concomitantly with the *N*-substituted formamide-degradation (Fig.1). To identify the unknown compound produced from NBFA through their cell reaction, further examination by HPLC was performed. As a result, it was found that the retention time (1.8 min under the experimental conditions used) on the HPLC chromatography of the reaction product agreed with those of authentic benzylamine (Fig.1). Thus the author proposed the unknown product to be benzylamine, and that these isolates contain an NBFA-degrading enzyme which catalyzes the hydrolysis of NBFA to yield benzylamine and formate (Fig.2). From a type of the catalytic reaction, the author named this enzyme "*N*-substituted formamide deformylase (NfdA)".

Based on the above proposition, the author defined one unit of the enzyme activity as the amount of enzyme that catalyzes the formation of 1 μmol of benzylamine per min. The author then examined NBFA-degrading activities of 13 isolates, and finally discovered strain F164 to exibit the highest enzyme activity. This strain was also found to be able to grow on a culture medium containing benzyl isocyanide (an isonitrile) as the sole nitrogen source and to degrade the isonitrile into the corresponding *N*-substituted formamide, NBFA. Therefore, strain F164 was chosen as the representative source to study *N*-substituted formamide-degrading enzyme involved in isonitrile metabolism.

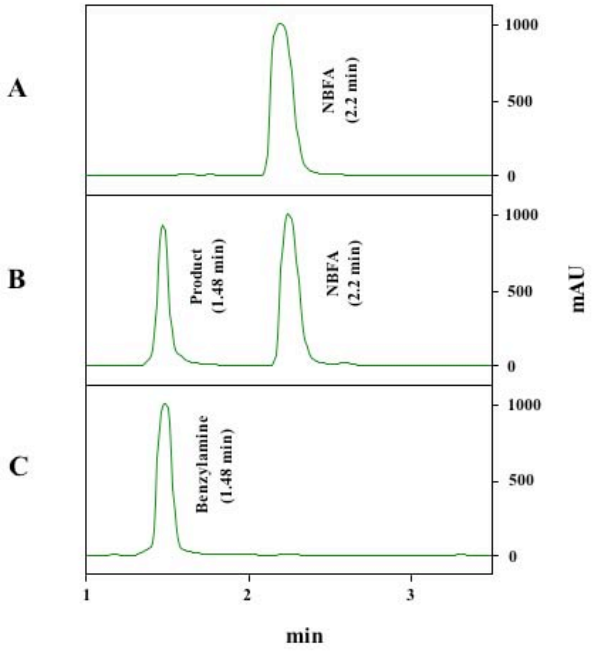

Fig.1. Typical HPLC profiles of the authentic NBFA (A), benzylamine (C), and the supernatant of resting cell reaction (B).

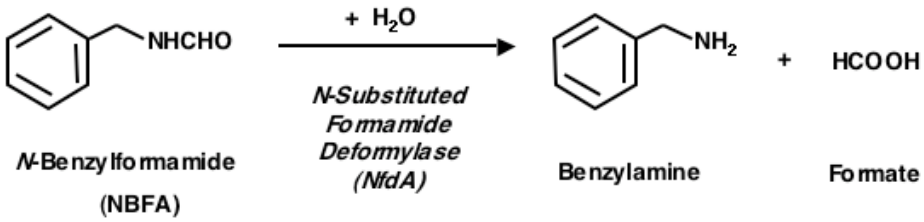

Fig. 2. Proposed NBFA degrading reaction by NBFA-degrading microorganisms.

Identification of strain F164 as *Arthrobacter pascens*

In order to identify strain F164, morphological test, physiological test, determination of GC content of the genomic DNA and 16S rDNA sequencing were performed. Morphologically, strain F164 is a Gram-positive rod, non-endospore forming, and non-motile (Fig.3). Its physiological characteristics are as follows: oxidase, negative; catalase, negative; oxidative/fermentative dissimilation of glucose, negative; reduction of nitrate, negative; pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, positive; β-glucuronidase, urease, negative; decomposition of aesculin and gelatin, positive; growth on sole carbon sources, negative with glucose, ribose, xylose, mannitol, maltose, milk sugar, white sugar and glycogen; growth at 42°C, negative; and rod-coccus cycle, positive. The GC content of the genomic DNA of this strain was determined as 63.0% with a DNA-GC kit (Yamasa Shoyu Co., Choshi) according to the same method as described previously (Goda et al., 2001). Based on these characteristics together with its 16S rRNA sequence, which was determined (data not shown) with a Microseq 500 16S rRNA-encoding DNA Bacterial Sequencing Kit (Applied Biosystems, CA, USA) according to the manufacturer's specifications, strain F164 was identified as *Arthrobacter pascens*.

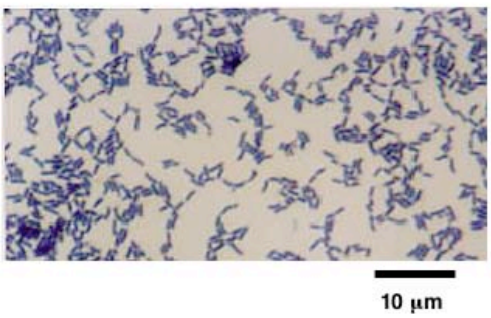

Fig. 3. Photograph of strain F164.

Section II

Optimum culture conditions for the production of *N*-substituted formamide deformylase by *Arthrobacter pascens* F164

Introduction

In Section I, *Arthrobacter pascens* F164 was initially isolated from soil samples through an acclimatization culture and found to be able to degrade NBFA (which is an *N*-substituted formamide) and the corresponding isonitrile, benzyl isocyanide. From HPLC analysis of the resting cell reaction, this strain was proposed to contain *N*-substituted formamide deformylase (NfdA) which catalyzes the hydrolysis of NBFA to produce benzylamine and formate. Further study was expected to purify and characterize the enzyme from this strain. However, the growth rate and the enzyme activity in the cells were low.

This section deals with the optimum culture conditions for the production of NfdA by *A. pascens* F164. It is also described that NfdA is an inducible enzyme depending on NBFA.

Materials and methods

Organism, media and growth conditions

A. pascens F164, which was isolated from soil samples through an enrichment culture (Section I), was used. The subculture was carried out at 28°C for 24 h with reciprocal shaking in a test tube containing 10 ml of 2 x YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter of distilled water) supplemented with NBFA at a final concentration of 0.05% (w/v). Then 1 ml of the subculture was inoculated into a 500-ml shaking flask containing 90 ml of one of the various media to be examined in each experiment. Each cultivation was carried out at 28°C with reciprocal shaking. Two kinds of basal media were used. Basal medium I consisted of 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter of distilled water (pH 6.8). Basal medium II consisted 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 $FeSO_4 \cdot 7H_2O$, and 1 ml of vitamin mixture (Section I) per liter of distilled water (pH 7.0). In basal culture conditions, 1 ml of the subculture was inoculated into 90 ml of basal medium II supplemented with NBFA at a final concentration of 0.05% (w/v) and cultivated at 28°C for 72 h.

Preparation of cell-free extacts

After each cultivation, the cells were harvested by centrifugation at 10,400 x g at 4°C and then washed twice with 10 mM potassium phosphate buffer (pH 7.5). Washed cells were resuspended in 2 ml of 0.1 M potassium phosphate buffer (pH 7.5) and then disrupted by sonication at 200 W for 20 min with an Insonator model 201M (Kubota, Tokyo). The cell debris was removed by centrifugation. The supernatant solution was designated as the cell-free extracts.

Enzyme assay

The reaction mixture comprised 0.1 M potassium phosphate buffer (pH 7.5), 10 mM NBFA, and an appropriate amount of cell-free extracts in a total volume of 400 μl. The reaction was started by the addition of the extracts and carried out at 25°C for 15 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 x *g*, 10 min). Forty μl of the supernatant was mixed with 80 μl of 0.2% (v/v) triethylamine and 40 μl of 0.4% (w/v) GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate) (Nimura et al., 1980). 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 "*Isolation of bacteria catabolizing N-substituted formamide and isonitrile* (Section I)" except that the wavelength of 250 nm was used for the monitoring.

One unit of NfdA activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol benzylamine per min from NBFA under the above conditions. The specific activity was expressed as units per mg of protein.

Protein determination

The protein concentration was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin (Nacalai Tesque) as a standard.

Electrophoresis

SDS-PAGE was performed in a 12.5% polyacrylamide slab gel according to Laemmli (Laemmli, 1970). 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), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

N-terminal 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, CA, USA).

Results and Discussion

Comparison of the nutrient and synthetic media as basal media for NfdA production by *Arthrobacter pascens* F164

A. pascens F164 was able to grow on a synthetic medium with NBFA as a sole nitrogen source, but the growth rate and NfdA activity in the cells were low. First, the effect of the two kinds of basal media (I and II) supplemented with 0.05% (w/v) NBFA were investigated. In the examination used medium I and II, cultivation times after subculture were 24 h and 72 h, respectively. Compared with the nutrient medium (basal medium I, 0.033 units/mg), use of the synthetic medium (basal medium II) resulted in enhancement of NfdA activity (0.098 units/mg). No formation of the deformylase was detected without the addition of NBFA to each basal medium. Thus the synthetic medium with NBFA was found to be suitable for NfdA production.

Effects of cultivation time and culture temperature

Next, the cultivation time and temperature were examined. In the examination of cultivation time, each cultivation was carried out in basal medium II supplemented with 0.05% (w/v) NBFA at 30°C with reciprocal shaking. In the examination of culture temperature, each cultivation was carried out in the same medium for 24 h. The highest specific activity was observed in cells grown for 24 h at 30°C (Fig. 1).

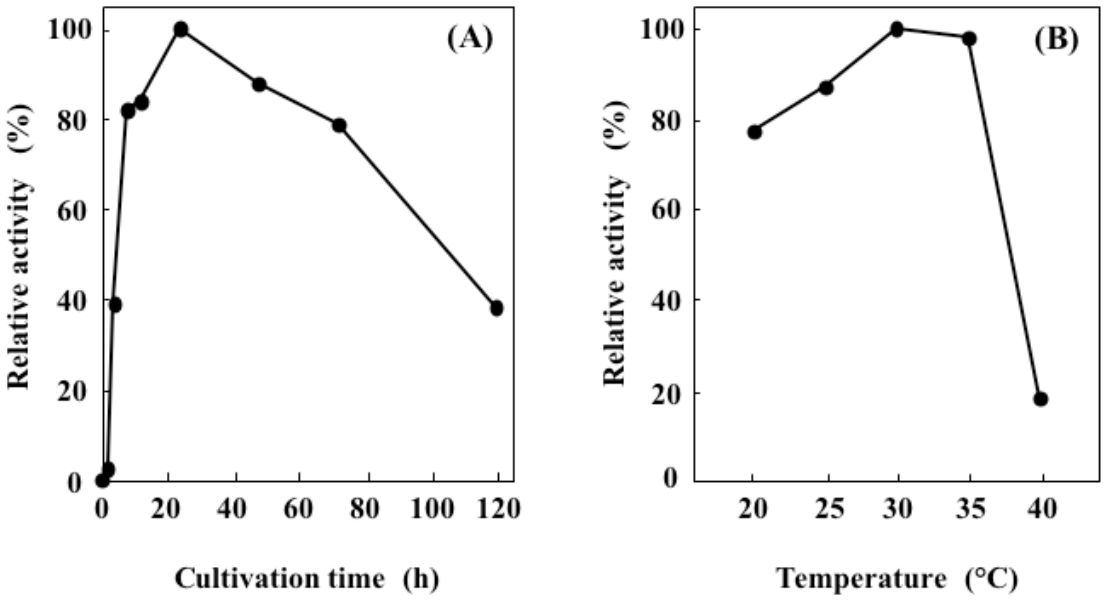

Fig.1. Effects of cultivation time and temperature on the NfdA formation by *A. pascens* F164.

Effects of nitrogen sources

To enhance NfdA activity, the effect of various nitrogen sources was examined. Of all the compounds tested, NBFA was found to be the most effective for enzyme activity (Table 1). This finding, together with the phenomenon of no formation of NfdA without the addition of NBFA to the medium, suggests that NfdA is an inducible enzyme depending on NBFA. The author observed on SDS-PAGE that the crude extract contained a protein that was not synthesized without the addition of NBFA to the culture medium (Fig. 2, *lane B*).

Table 1. Effect of Various Nitrogen Sources on the Formation of NfdA by *Arthrobacter pascens* F164. Each compound was used as sole nitrogen source and added at a final concentration of 0.05% (w/v) to basal medium II. The pH of each medium was adjusted to 7.0. Cultivation was carried out for 24 h at 30°C with shaking. The synthesis of benzylamine, corresponding to 0.252 units/mg of protein, was taken to be 100%.

Nitrogen sources	Relative activity	Nitrogen sources	Relative activity
	%		%
NBFA	100	Benzamide	11
Formanilide	2	Phenylacetamide	24
N-Cyclohexylformamide	1	ε-Caprolactam	21
N,*N*-Dimethylformamide	3	Formamide	20
N-Formylmorpholine	2	Acetamide	18
Cyclohexyl isocyanide	0	Propionamide	14
Benzyl isocyanide	11	*n*-Butyramide	14
Benzonitrile	21	Isobutyramide	14
Benzyl cyanide	15	*n*-Valeramide	20
Acetonitrile	39	Isovaleramide	40
Propionitrile	18	Capronamide	20
n-Butyronitrile	12	Methacrylamide	24
Isobutyronitrile	24	Urea	63
n-Valeronitrile	17	Thiourea	40
Isovaleronitrile	7	*N*-Methylurea	58
n-Capronitrile	7	Phenylurea	48
Methacrylonitrile	4	Aniline	17

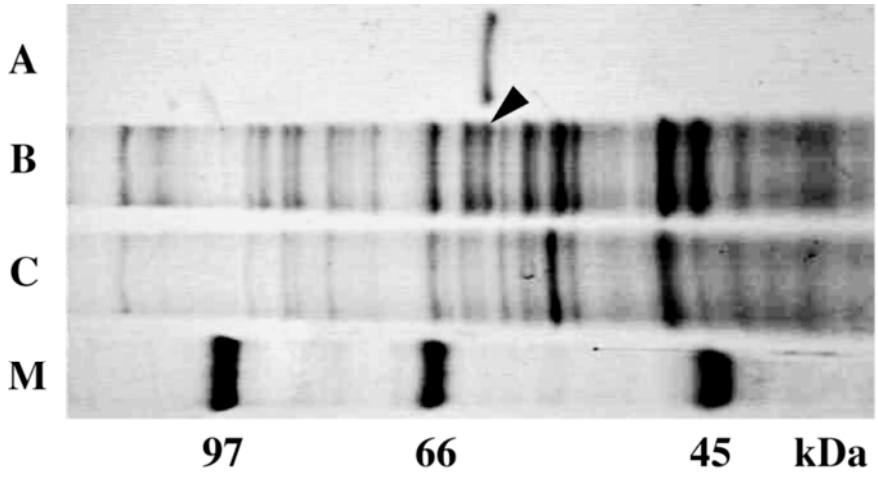

Fig. 2. SDS-PAGE of cell-free extracts of the cells cultured with and without NBFA. SDS-PAGE with 7% polyacrylamide gel was performed as described in *materials and method*s. Lane A, the purified NfdA (0.1 μg). Lane B, a cell-free extract (8 μg) of cells cultured with NBFA as sole nitrogen source. Lane C, a cell-free extract (8 μg) of cells cultured with ammonium sulfate as sole nitrogen source. Lane M, marker proteins: phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). The arrow indicates the protein band of NfdA. The N-terminal amino acid sequence of the band arrowed, which was determined by the method described in *materials and methods*, was the same as that of the purified NfdA (TQMRDLMIIN). This finding, together with the same mobility of the band and the purified NfdA band in SDS-PAGE, indicated that it was NfdA.

Effects of the concentration of NBFA

Next, the effect of the concentration of NBFA was investigated. Specific activity decreased with increasing concentrations of NBFA added when the final concentration was more than 0.05% (w/v). No significant increase in specific activity was confirmed when the final concentration of NBFA was within the range from 0.01 to 0.05% (w/v) (Fig. 3). Although the cells grown on the medium containing 0.05% (w/v) NBFA did not exhibit the highest specific activity, they showed the highest total activity (4.63 units). Hence the author determined the final concentration of NBFA to be 0.05% (w/v).

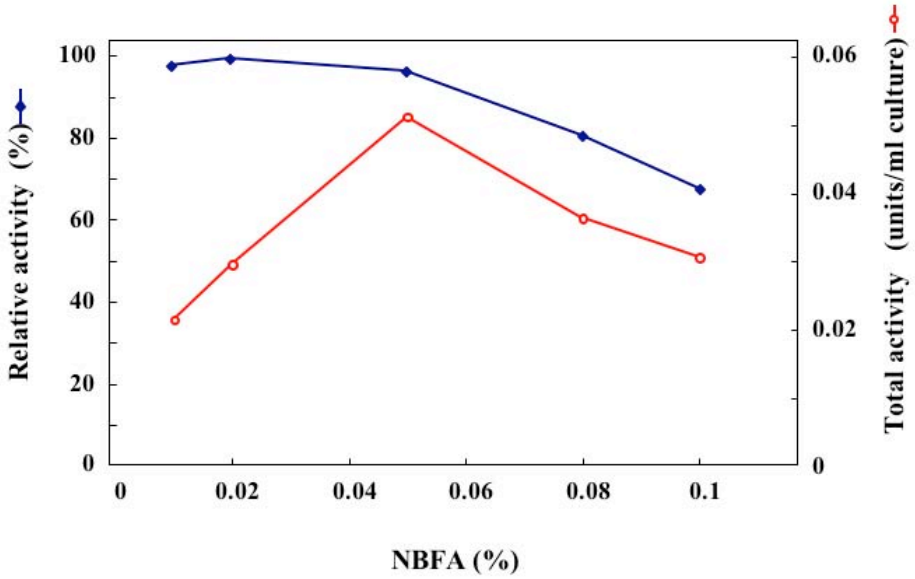

Fig.3. Effects of the NBFA concentration of NBFA on the NfdA production. Closed and open circles show specific activity and the total activity of NfdA in *A. pascens* F164 at various concentration of NBFA, respectively.

Effects of carbon sources

To increase NfdA activity, the author examined the effects of various carbon sources. The following compounds were used as sole carbon source: glycerol, glucose, galactose,

sorbitol, mannitol, sucrose, maltose, lactose, and soluble starch. Each compound (1.0%, w/v) was added to basal medium II with 0.05% (w/v) NBFA. Compared with glycerol, used as sole carbon source in the basal medium II (100%), none of the compounds tested was significantly effective (72-113%) (Table 2). Glucose strongly repressed the formation of NfdA, the inhibition being 28%, indicating that the enzyme, like other inducible enzymes so far known, is also subject to catabolite repression by glucose.

Table 2. Effect of various carbon sources on the formation of NfdA by *Arthrobacter pascens* F164. Each compound was used as the sole carbon source and added at a final concentration of 1.0 % (w/v) to basal medium II containing 0.05% (w/v) NBFA. The pH of each medium was adjusted to 7.5. Cultivation was carried out for 24 h at 30°C with reciprocal shaking. The NfdA activity of cell-free extracts of cells cultivated in the medium with glycerol was taken to be 100%.

Carbon soureces	Relative activity
	%
Glycerol	100
Glucose	72
Galactose	113
Sorbitol	95
Mannitol	107
Sucrose	93
Maltose	85
Lactose	96
Starch, soluble	96
Succinic acid	48

Effects of nitrogen sources in addition to NBFA and inorganic compounds

To enhance the cell growth of *A. pascens* F164, various nitrogen sources in addition to NBFA were added to the medium. The following compounds were tested: NZ amine, casitone, polypepton, tryptone peptone, malt extract, yeast extract, bonito extract, $(NH_4)_2SO_4$, NH_4Cl, $NH_4H_2PO_4$, and KNO_3. The addition of each nitrogen source at a final concentration of 0.1% (w/v) in addition to NBFA caused a remarkable decrease in the formation of the enzyme, the inhibition being 27 to 74%. These data suggest that NBFA-dependent induction of the enzyme is repressed by the presence of the other nitrogen sources. Moreover, the addition of various inorganic compounds (LiCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeSO_4$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $CuSO_4$, $ZnCl_2$, SrCl, RbCl, CsCl, and $NaMoO_4$) did not enhance NfdA formation.

Based on the above results, a medium containing 0.05% (w/v) NBFA, 1.0% (w/v) glycerol, 0.05% (w/v) K_2HPO_4, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, 0.0005% (w/v) $FeSO_4 \cdot 7H_2O$, and 0.1% (w/v) vitamin mixture in distilled water (pH 7.0) was found to be the most suitable for the preparation of cells with high NfdA activity. When *A. pascens* F164 was cultured for 24 h at 30°C in this optimum medium at an initial pH of 7.0, the enzyme productivity was high. The specific activity amounted to 0.292 units per mg of protein, which corresponded to approximately three times enhancement compared to the enzyme acitivity in the cells cultivated under the basal culture conditions. The maximum enzyme activity was also 5.20 units. Furthermore, the cultivation time after subculture was shortened to 24 h, which was 48 h shorter than that under the basal culture conditions.

Table 3. Effects of NBFA and Other Nitrogen Sources on NfdA Formation. Each cultivation was carried out in basal medium II supplemented with a final concentration of 0.05% (w/v) of each nitrogen source for 24 h at 30°C with shaking.

Nitrogen sources	Specific activity	Fold
	units/mg	
$(NH_4)_2SO_4$	0.006	1
NBFA	0.242	40
$(NH_4)_2SO_4$+ NBFA	0.088	15

From these findings together with the results shown in Fig. 2 and Table 3, the author clarified for the first time that NfdA is an inducible enzyme, which is probably controlled by an intricate regulatory mechanism, including induction by NBFA and repression by ammonium or glucose, but it is not clear how the expression of the enzyme is regulated by these compounds at the gene and protein levels. Further analysis is necessary to elucidate the regulatory mechanism.

Chapter II

Purification and characterization of

a novel *N*-substituted formamide deformylase from *Arthrobacter pascens* F164

Introduction

In Chapter I, the author performed the screening of *N*-substituted formamide-degrading microorganisms from soil samples through an enrichment culture technique and finally selected *Arthrobacter pascens* F164. This strain was able to grow on a culture medium with NBFA (an *N*-substituted formamide) as the sole nitrogen source and degrade NBFA and the corresponding isonitrile, benzyl isocyanide. In the resting cell reaction, benzylamine was found to be produced from NBFA as substrate. This data suggested that strain F164 would have *N*-substituted formamide deformylase which catalyzes the hydrolysis of NBFA to yield benzylamine and formate. Moreover, the author investigated and determined the optimum culture conditions for the enzyme production in this strain. Further study was expected to elucidate enzymological properties of NfdA for a better understanding of isonitrile metabolism at the protein level.

This chapter describes the purification and characterization of NBFA-degrading enzyme obtained from the cells cultivated under the optimum culture conditions.

Materials and methods

Materials

DEAE-Sephacel and a low-molecular-weight standard kit were obtained from Amersham Biosciences (Piscataway, NJ, USA). Standard proteins for high-performance gel filtration chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were purchased from Oriental Yeast Co. (Tokyo) and Sigma Co., Ltd. (St. Louis, MO, USA), respectively. All other biochemicals used, except for *N*-formyl kynurenine, were from commercial sources and of reagent grade. *N*-Formyl-L-kynurenine was synthesized as described in the previous report (Auerbach and Knox, 1957).

Organism, media and culture conditions

A. pascens F164, which was isolated from the soil samples (Chapter I) was used. This strain 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 x YT medium (16 g of tryptone, 10 g of yeast extract and 5 g of NaCl per liter of distilled water) supplemented with NBFA at a final concentration of 0.05% (w/v). 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 glycerol, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 1 ml of vitamin mixture (2), and NBFA at a final concentration of 0.05% (w/v) per liter of distilled water, followed by incubation at 28°C with reciprocal shaking. After 24 h incubation, the cells were harvested by centrifugation at 13,000 x *g* at 4°C and then washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 10% (w/v) glycerol.

Purification of NfdA

All purification procedures were performed at 0-4°C. Potassium phosphate buffer (pH

7.5) containing 10% (w/v) glycerol was used throughout the purification, unless noted otherwise. Centrifugation was carried out for 15 min at 13,000 x *g*.

Step 1. Preparation of a cell-free extract. Washed cells from 12 l of culture broth were resuspended in 480 ml of 0.1 M buffer and then disrupted by sonication at 200 W for 60 min with an Insonator model 201M (Kubota, Tokyo). The cell debris was removed by centrifugation.

Step 2. Ammonium sulfate fractionation. The resulting supernatant solution was fractionated with ammonium sulfate (40-50% saturation), followed by dialysis against 10 mM buffer.

Step 3. DEAE-Sephacel column chromatography. The dialyzed solution was applied to a DEAE-Sephacel column (5 x 40 cm) equilibrated with 10 mM buffer. Protein was eluted from the column with 1 liter of the same buffer, the concentration of KCl being increased linearly from 0.2 to 0.5 M. The active fractions were collected and then ammonium sulfate was added to give 70% saturation. After centrifugation of the suspension, the precipitate was dissolved in 10 mM buffer, followed by dialysis against 10 mM buffer.

Step 4. Resource ISO column chromatography. The enzyme solution from step 3 was mixed with an equal amount of 10 mM buffer containing 50% saturated ammonium sulfate, and then placed on a Resource ISO column (1.6 x 3 cm; Amersham Biosciences) equilibrated with 10 mM buffer containing 25% saturated ammonium sulfate. The enzyme was eluted by lowering the concentration of ammonium sulfate (25 to 15% saturation) in 180 ml of the same buffer. The active fractions were combined and precipitated with ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation, dissolved in 10 mM buffer, and then dialyzed against 10 mM buffer.

Step 5. Resource Q column chromatography. The enzyme solution from step 4 was applied to a Resource Q column (0.64 x 3 cm; Amersham Biosciences) equilibrated with 10 mM buffer. Protein was eluted from the column with 60 ml of the same buffer, the concentration of KCl being increased linearly from 0.2 to 0.4 M. The active fractions were

pooled and then precipitated with ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation and then dissolved in 10 mM buffer. The resultant solution was dialyzed against 10 mM buffer containing 10% (v/v) glycerol, and then centrifuged. The homogeneity of the purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay

All of the reactions were performed under linear conditions as to protein (~ 1.5 μg/ml) and time (~ 10 min).

The enzyme activity of NfdA was determined by the following two methods (Standard assay A and B). The standard assay A mixture comprised 0.1 M potassium phosphate buffer (pH 7.5), 10 mM NBFA, and an appropriate amount of 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 x *g*, 10 min). Forty μl of the supernatant was mixed with 80 μl of 0.2% (v/v) triethylamine and 40 μl of 0.4% (w/v) GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate) (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 "*Isolation of bacteria catabolizing N-substituted formamide and isonitrile described* (Chapter I)" 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 x *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 *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 mg of protein.

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 AXIMA-CFR plus. The instrument was calibrated with bovine serum albumin (66 kDa) and cytochrome *c* (12.4 kDa) as standard proteins.

Identinfication of the compound produced from NBFA by NfdA

One product in the reaction mixture with the purified enzyme (NfdA) from *Arthrobacter pascens* F164 was extracted with ethylacetate, and then analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS was performed with a ThermoElectron TraceGC/PolarisQ GCMS (San Jose, USA) equipped with a DB-1ms capillary column (0.25 x 30 m; Agilent Technologies, Wilmington, DE, USA). The initial column temperature of 120°C was raised at 30°C/min to 200°C. The injection temperature was 300°C. The carrier gas was He, at the 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.

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*-formylkynurenine 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*-formylkynurenine) 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 the *N*-substituted formamides (except *N*-butylformamide and *N*-formylkynurenine) were measured by standard assay B described 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% (v/v) triethylamine and 0.13% (w/v) GITC in acetonitrile (to stop the reaction), and a supernatant was obtained by centrifugation (12,000 x *g*, 10 min). The amount of GITC-derivatized butylamine was determined by HPLC. 10 mM KH_2PO_4-H_3PO_4 buffer (pH 2.0)/acetonitrile, 61:125 (v/v), was used as the eluent. *N*-Formylkynurenine deformylase and amidase activities were assayed at 25°C as described previously (Bailey and Wagner, 1974; Kobayashi et al., 1993b). Allantoinase activity was measured by standard assay B described under "*Enzyme assay*" with modifications. The amount of hydantoin formed was determined by HPLC, the wavelength of 200 nm being used for the monitoring.

Other procedures

Electrophoresis and N-terminal amino acid sequencing of NfdA were performed as described in Section II in Chapter I.

Results

Purification of NfdA

The author found that the NfdA activity of *A. pascens* F164 was only induced when NBFA was added to the culture medium (Chapter I). Among the formamides tested, NBFA was the most effective inducer. Therefore, purification of the enzyme was carried out from an extract of NBFA-induced cells.

Through the purification steps described under *Materials and Methods*," the enzyme was purified 99.7-fold with a yield of 7.1% (Table I). The purified enzyme gave only one band on SDS-PAGE (Fig. 1), corresponding to a molecular mass of 61 kDa. The molecular mass of the enzyme subunit was also determined to be 58,556 Da by MALDI-TOF MS. The molecular mass of the native enzyme was 121 kDa according to the results of gel filtration chromatography (Fig. 2), indicating that the enzyme consists of two identical subunits. The purified enzyme showed specific activity of 28.9 units/mg (Table I). The N-terminal amino acid sequence of the enzyme was determined as TQMRDLMIINA. It exhibited no homology with the amino acid sequences of the reported proteins.

Table I. Purification of *N*-substituted formamide deformylase. Enzyme assaying was performed by means of standard assay A.

Step	Total protein	Total activity	Specific activity	Yield
	mg	units	units/mg	%
Cell-free extract	2430	705	0.29	100
$(NH_4)_2SO_4$	752	564	0.75	80.0
DEAE-Sephacel	53.2	277	5.2	39.3
Resource ISO	3.81	76.2	20.0	10.8
Resource Q	1.72	49.7	28.9	7.1

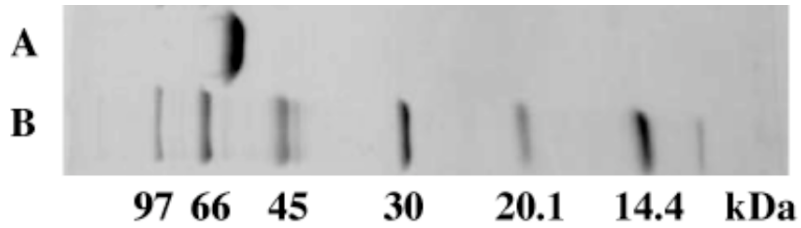

Fig. 1. SDS-PAGE of the purified *N*-substituted formamide deformylase. Lane A, the purified enzyme (4 μg). Lane B, marker proteins.

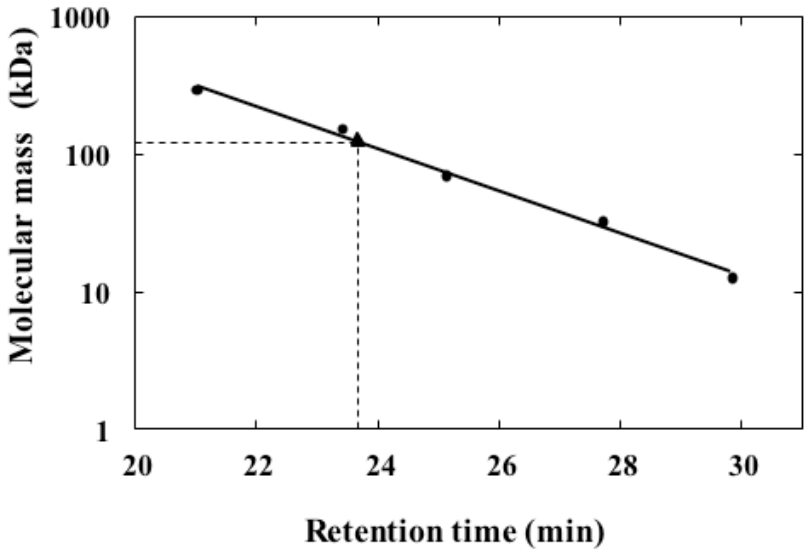

Fig. 2. Determination of the molecular mass of NfdA by gel filtration chromatography. The standard curve molecular mass versus retention time was derived from the elution profiles of the standard molecular weight markers on a Superose 12 HR10/30 gel filtration column. The peak position of NfdA is indicated by ▲. The standards (●) used were described in *Materials and methods*.

Identification of the reaction products and stoichiometry

The unknown compound produced from NBFA through the reaction of the enzyme purified from *A. pascens* F164 was examined by GC-MS. As a result, it was found that both the retention time (1.8 min under the experimental conditions used) on the GC and the MS spectrum of the reaction product agreed with those of authentic benzylamine (Fig. 3). Furthermore, the reaction product and authentic benzylamine retention times on HPLC chromatography were the same as each other, which was described in Chapter I. On the other hand, the other product that was not detected on GC-MS was found to be formate on HPLC analysis; the retention time of the reaction product agreed with that of authentic formate (6.1 min under the standard assay B conditions). No other compounds (which may be produced from NBFA), such as benzoic acid, benzylalcohol, formamide and ammonia, exhibited any similarity to the reaction product on HPLC or GC-MS analysis. Thus, the reaction products were identified as benzylamine and formate.

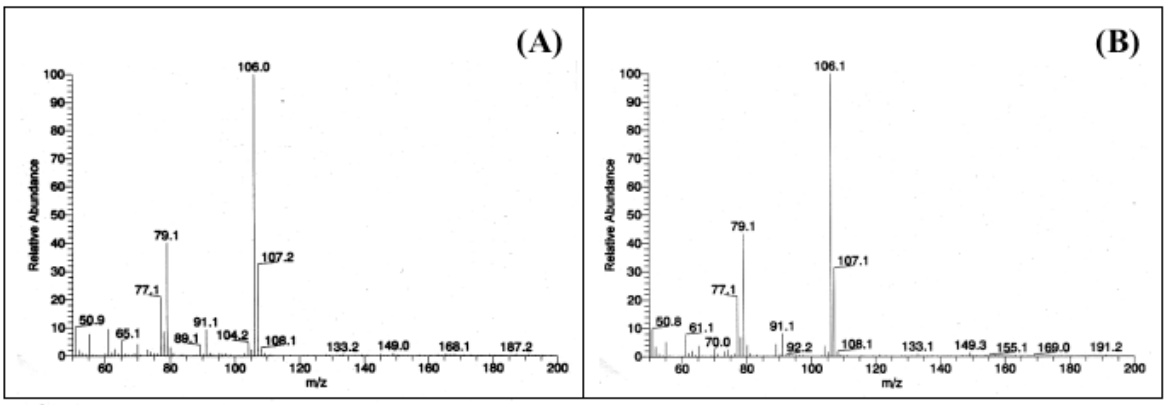

Fig. 3. MS spectra of the reaction product (A) and authentic benzylamine (B).

The stoichiometry of *N*-substituted formamide consumption, and benzylamine and formate formation during the hydrolysis of *N*-substituted formamides was examined in a reaction mixture consisting of 100 mM potassium phosphate buffer (pH 7.5), 4 mM NBFA, and 1.67 μg/ml of the enzyme in a final volume of 800 μl. After 30 min incubation, the amounts of NBFA, benzylamine and formate were determined (Fig.4). The amounts of benzylamine and formate formed, and the NBFA remaining were 1.12 mM, 1.18 mM and 2.83 mM, respectively. The formation of other compounds was not noted. The results demonstrated that benzylamine and formate were formed stoichiometrically with the consumption of NBFA, and that the enzyme catalyzes the hydrolysis of an *N*-substituted formamide to the corresponding amine and formate: the deformylation of an *N*-substituted formamide.

$$\text{Ph-CH}_2\text{-NH-CH(=O)} + \text{H}_2\text{O} \rightarrow \text{Ph-CH}_2\text{-NH}_2 + \text{HCOOH}$$

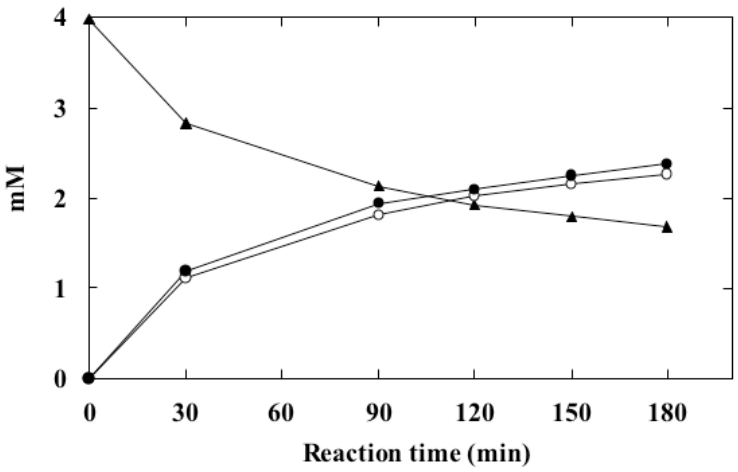

Fig. 4. Conversion of NBFA by NfdA. A reaction mixture (800 μl) containing 4 mM NBFA, 100 mM potassium phosphate buffer, pH 7.5, and 1.67 μg/ml of the enzyme was incubated at 25°C. Symbols: NBFA (▲); benzylamine (○); formate (●).

Effects of temperature and pH on the activity and stability of the enzyme

The effects of temperature and pH on the enzyme activity were examined. The optimal temperature was 35°C. The enzyme exhibited maximum activity at pH 7.0 (Fig. 5A and 5B).

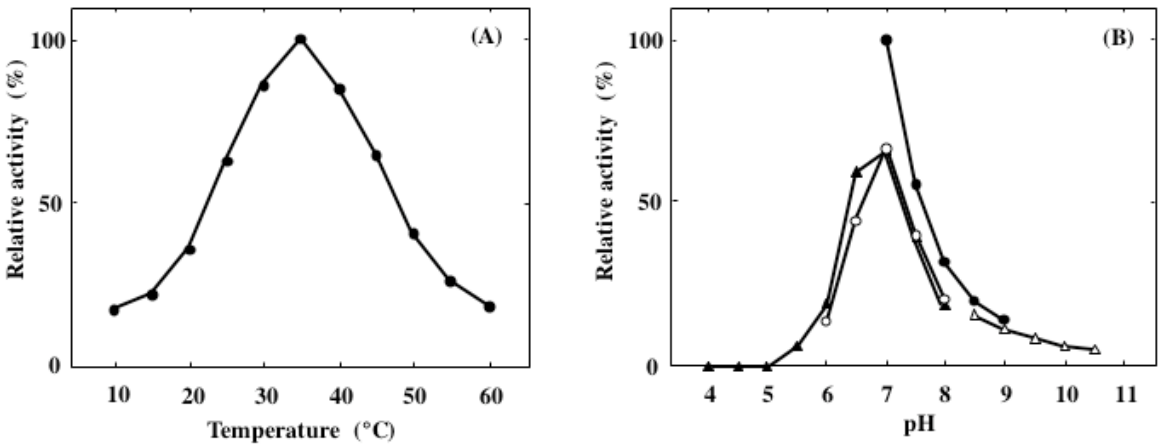

Fig. 5. Effects of temperature and pH on the activity of *N*-substituted formamide deformylase. A, reactions were carried out for 10 min at various temperatures. B, reactions were carried out for 10 min at 25°C in the following buffers (0.1 M): citrate-Na_2HPO_4 (▲), potassium phosphate (○), Tris/HCl buffer (●), and NH_4OH-NH_4Cl (△). Relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions used.

The stability of the enzyme was examined at various temperatures. After the enzyme had been preincubated for 30 min in 10 mM potassium phosphate buffer (pH 7.5) containing 10% (w/v) glycerol, an aliquot of the enzyme solution was taken, and then the enzyme activity was assayed under the standard assay A conditions. It exhibited the following activities: 60°C, 0%; 55°C, 0%; 50°C, 51%; 45°C, 75%; 40°C, 84%; 35°C, 94%; 30°C, 98%; 25°C, 100%; 20°C, 100%; and 10°C, 100%.(Fig. 6A)

The stability of the enzyme was examined at various pH values. After the enzyme had been incubated at 25°C for 30 min in the following buffers at a concentration of 0.1 M; citrate-Na_2HPO_4 buffer (pH 4.0-8.0), potassium phosphate buffer (pH 6.0-8.0), Tris/HCl buffer (pH 7.0-9.0), and NH_4OH-NH_4Cl buffer (pH 8.5-10.5), an aliquot of the enzyme solution was taken, and then the enzyme activity was assayed under the standard assay A conditions. NfdA was most stable in the pH range of 7.5-8.5 (Fig. 6B).

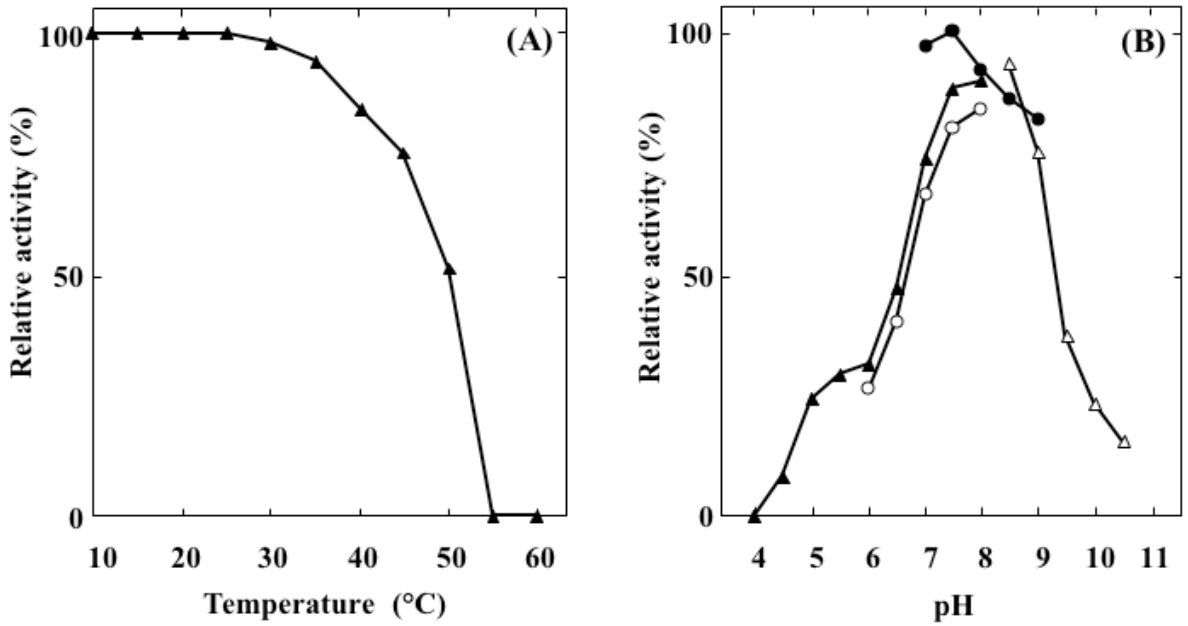

Fig. 6. Effect of temperature and pH on the stability of NfdA. NfdA activity was assayed under the standard assay A conditions after the enzyme was preincubated (A) for 30 min in 10 mM potassium phosphate buffer (pH 7.5) containing 10% (w/v) glycerol or (B) at 25°C for 30 min in the following buffers (0.1 M): citrate-Na_2HPO_4 (▲), potassium phosphate (○), Tris/HCl buffer (●), and NH_4OH-NH_4Cl (△). Relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions used.

Substrate specificity

The ability of the enzyme to catalyze the hydrolysis of various *N*-substituted formamides, amides and other compounds was examined. As described below, NfdA from *A. pascens* F164 has a narrow substrate spectrum. Among the tested *N*-substituted formamides, NBFA was the most suitable substrate for the enzyme. *N*-Butylformamide (3.4%) was hydrolyzed at significantly lower rates, compared with the activity towards NBFA (100%). The hydrolysis of NBFA followed Michaelis-Menten-type kinetics, the K_m and V_{max} values being 0.075 mM and 52.7 μmol/min/mg, respectively. On the other hand, The K_m and V_{max} values for *N*-butylformamide were 7.5 mM and 6.3 μmol/min/mg, respectively; *N*-butylformamide was turned over more slowly and bound with much lower affinity by the enzyme. Allylformamide, *N*-(2-cyclohex-1-enylethyl)formamide and *N*-(α-methylbenzyl)formamide were rather poor substrates (Table II).

Not only other *N*-substituted formamides but also all of the tested compounds (except the above mentioned ones), which are listed in "*Materials and methods*", did not act as substrates for our enzyme, despite the addition of a large amount of the enzyme and a long incubation period.

Table II. Substrate specificity of *N*-substituted formamide deformylase. All experimetnts were carried out using the assay conditions described under "Materials and Methods". The activity is given relative to the deformylation of NBFA.

Substrate	Relative activity
	%
NBFA	100
N-Butylformamide	3.4
Allylformamide	0.22
N-(2-Cyclohex-1-enylethyl)formamide	0.13
N-(α-Methylbenzyl)formamide	0.16

Inhibitors

Various compounds were investigated as to their inhibitory effect on the enzyme activity. 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 concentration of each of the tested compounds was 1 mM, unless otherwise stated. The enzyme was very sensitive to $HgCl_2$, CuCl (at 0.25 mM), $CuCl_2$, and $AgNO_3$, the inhibition being 100% inhibition. $ZnCl_2$ and $SnCl_2$ also showed inhibitory effects on the enzymatic activity (25% and 34%, respectively). The enzyme was completely inhibited by thiol-specific reagents such as *N*-ethylmaleimide and *p*-chloromercuribenzoate, whereas iodoacetate and 5,5'-dithio-bis-2-nitrobenzoate did not inhibit the activity at all. Carbonyl-specific reagents, *e.g.*, aminoguanidine and semicarbazide, hardly inhibited the enzyme, but phenylhydrazine caused partial inhibition (25%). Chelating agents, such as α,α'-dipyridyl, KCN, diethyldithiocarbamate and EDTA, did not influence the activity at all, but *o*-phenanthroline and 8-hydroxyquinoline caused appreciable inhibition (38% and 54%, respectively). The enzyme was unaffected by oxidizing reagents and serine-modifying reagents such as H_2O_2, ammonium persulfate, phenylmethanesulfonyl fluoride and diisopropyl fluorophosphates. However, reducing reagents such as dithiothreitol caused remarkable inhibition (90% inhibition). (Table III)

Table III. Effects of various compounds on the activity of *N*-substituted formamide deformylase. 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, unless otherwise stated.

Inhibitor	Relative acitivity
	%
None	100
LiCl, NaCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, $MnCl_2$, $AlCl_3$, $Pb(NO_3)_2$, $FeSO_4$, $FeCl_3$, RbCl, $SrCl_2$, CsCl, Na_2MoO_4, $CoCl_2$, and $NiCl_2$	82-110
$ZnCl_2$	75
$SnCl_2$	66
$CdCl_2$	34
CuCl (0.25 mM), $CuCl_2$, $AgNO_3$ and $HgCl_2$	0
Iodoacetate	93
5,5'-Dithio-bis-2-nitrobenzoate	85
N-Ethylmaleimide*	0
p-Chloromercuribenzoate	0
Semicarbazide	100
Aminoguanidine	98
Phenylhydrazine	75
α,α'-Dipyridyl	109
o-Phenanthroline*	62
8-Hydroxyquinoline*	46
EDTA	95
Diethyldithiocarbamate	107
NaN_3	128
KCN	109
Dithiothreitol	10
2-Mercaptoethanol	86
H_2O_2	90
Ammonium persulfate	109
Phenylmethanesulfonyl fluoride*	112
Diisopropyl fluorophosphates	117

* Methanol was added to the reaction mixture to the final concentration of 0.25-1% (v/v) to enhance the solubility of the tested compounds.

Discussion

Isonitriles are elaborated by various organisms, including bacteria, fungi, and marine sponges. Most of them show a wide antibiotic activity spectrum and have potential as possible agents of practical use; *e.g.,* a series of isocyanoterpenes, isolated from marine sponges, exhibit antimalarial activity (Angerhofer et al., 1992; Wright et al., 20001) and an antifouling effect similar to those of copper sulfate (Fusetani, 1997). Interest in the biosynthesis of these metabolites, particularly in the origin of the isocyano group, has led to some experimental studies on them (Hagadone et al., 1984; Achenbach and Grisebach, 1965; Achenbach and König, 1972; Pfeifer et al., 1972; Simpson et al., 2004). However, the metabolism of isonitriles had been completely unknown at the protein and gene levels before Goda et al. discovered an isonitrile-degrading enzyme, which was designated as isonitrile hydratase; it is the first known enzyme involved in the isonitrile metabolism (Goda et al., 2001). The enzyme catalyzes the hydration of an isonitrile to the corresponding *N*-substituted formamide, indicating that isonitrile hydratase is responsible for the initial committed step in isonitrile catabolism. However, no enzyme has been reported to be involved in the further metabolism of an *N*-substituted formamide produced from the corresponding isonitrile. The author initially isolated an *N*-substituted formamide-and isonitrile-degrading microorganism, *Arthrobacter pascens* F164, from soil sample by an enrichment culture (Chapter I) and characterized NfdA in the cells at the protein level for the first time (this chapter).

In living organisms, many enzymes (*e.g.*, amine oxidase (Yamashita et al., 1996; Frebort et al., 2003) are responsible for amine metabolism (Chattopadhyay et al., 2003). Among them, amine-synthesizing enzymes have received increasing attention in various fields. There are several types of amine-forming deformylases involved in the metabolism of *N*-substituted formamides which are present in nature. (*i*) Kynurenine formamidase (EC 3.5.1.9) involved in the tryptophan degradation pathway (Bailey and

Wagner, 1974) converts *N*-formyl kynurenine to kynurenine. This enzyme is known to be responsible for the synthesis of the ommochrome pigment in insects (Cochran, 1976; Glassman, 1956). It also plays a role in maintaining an appropriate level of NAD in chicken embryos and is an essential enzyme in their normal development (Seifert and Casida, 1978; Eto et al., 1980). (*ii*) Formylmethionine deformylase (EC 3.5.1.31) deformylates *N*-formylmethionine (which is the free formyl amino acid produced on degradation of proinflammatory *N*-formylpeptide), but does not act on formyl di- or tri-peptides (Woodhouse et al., 1987; Broom et al., 1989). On the other hand, *N*-formylmethionine, which is generated through enzymatic transformylation of methionyl-tRNA and is an important amino acid for the initiation of all protein synthesis in procaryotes, is deformylated by (*iii*) peptide deformylase (EC 3.5.1.88) (Ragusa et al., 1998; Sereo et al., 2003). (*iv*) 10-Formyltetrahydrofolate deformylase (EC 3.5.1.10) hydrolyzes 10-formyltetrahydrofolate to tetrahydrofolate and formate, resulting in regulation of the ratio of tetrahydrofolate and one-carbon tetrahydrofolate pools in prokaryotic cells (Nagy et al., 1995). (*v*) Formamidase (EC 3.5.1.49) hydrolyzes formamide (which is the shortest *N*-substituted formamide as well as the shortest amide) to formate and ammonium (Fraser et al., 2001). (*vi*) *N*,*N*-Dimethylformamidase (EC 3.5.1.56) deformylates *N*,*N*-dimethylformamide (which is a useful chemical compound in the chemical industry, but there is concern about it being a possible environmental pollutant due to its toxicity to human beings and other organisms (Schär et al., 1986). (*vii*) Formylaspartate deformylase (EC 3.5.1.8) involved in the histidine degradation pathway (Ohmura and Hayaishi, 1957) acts on *N*-formyl-L-aspartate. (*viii*) Formylglutamate deformylase (EC 3.5.1.68) involved in the terminal reaction of the five-step pathway for histidine utilization in *Pseudomonas putida* (Hu et al., 1987) degrades *N*-formylglutamate to glutamate and formate. However, the substrate specificity of NfdA enzyme is completely different from those of the above deformylases. The enzyme utilizes no amides as substrates. Moreover, the N-terminal amino acid sequence of NfdA showed no significant homology to ones of any other deformylases mentioned above. These findings together with the reaction stoichiometry of NfdA demonstrate that my enzyme is

a novel deformylase for *N*-substituted formamides. The enzyme designated as "N-substituted formamide deformylase" has been approved as a new enzyme by NC-IUBMB: EC 3.5.1.91. [http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/5/1/91.html].

The author found that *A. pascens* F164 grew on a medium containing benzylisocyanide, which was the isonitrile corresponding to NBFA, as the sole nitrogen source. In the cell-free extract reaction, the isonitrile was confirmed to be hydrated to NBFA, which was further converted into benzylamine and formate by NfdA (unpublished data). This observation suggests that isonitrile hydratase and NfdA are closely related in sequential isonitrile metabolism. Although the isonitrile hydratase and NfdA genes have not been cloned from *A. pascens* F164 yet, there could be a genetic relationship between NfdA and isonitrile hydratase. It would be interesting to elucidate the potential genetic link between them for a greater understanding of the biological metabolism of isonitriles in nature.

Chapter III

Genetic analysis of *N*-substituted formamide deformylase from *Arthrobacter pascens* F164

Introduction

In Chapter II, the author purified and characterized *N*-substituted formamide deformylase (NfdA) from *Arthrobacter pascens* F164, which is able to degrade NBFA (an *N*-substituted formamide) and the corresponding isonitrile, benzyl isocyanide. NfdA stoichiometrically catalyzed the deformylation of an *N*-substituted formamide to yield the corresponding amine and formate: R-NH-CH(=O) + H_2O → R-NH_2 + HCOOH. Among the *N*-substituted formamides tested, NBFA was the most suitable substrate of the enzyme, whereas the enzyme does not act on other *N*-substituted formamides that are substrates for other known deformylases, including kynurenine formamidase (Bailey and Wagner, 1974), formylmethionine deformylase (Broom et al., 1989), peptide deformylase (Sereo et al., 2003), 10-formyltetrahydrofolate deformylase (Nagy et al., 1995), formamidase (Fraser et al., 2001), *N*,*N*-dimethylformamidase (Schär et al., 1986), formylaspartate deformylase (Ohmura and Hayaishi, 1957), and formylglutamate deformylase (Hu et al., 1987). Moreover, the N-terminal amino acid sequence of NfdA exhibited no significant homology to ones of any other deformylases mentioned above. These findings demonstrate that NfdA (which is involved in isonitrile metabolism) is a novel deformylase for *N*-substituted formamides, and its structure and reaction mechanism would be different from those of each of the other known deformylases. There is currently a great deal of interest in the catalytic mechanism of this enzyme. A more detailed understanding of it will be obtained by analysis of its three-dimensional structure. However, the yield of the enzyme obtained from the wild type strain was too low to perform such analyses. On the other hand, many enzymatic properties of NfdA have been cleared, however, the gene encoding NfdA (*nfdA* gene) from *A. pascens* F164 has been completely unknown. The gene cloning will allow understanding of the evolutionary relationships between NfdA and its homologues at the molecular level, and the overproduction of the enzyme in a heterologous cell, which will be necessary to study its reaction mechanism and three dimensional structure. This chapter describes the gene cloning

of NfdA enzyme from *A. pascens* F164 and the results of the homology search with BLAST server. This chapter also descibes the metal analysis of the recombinant enzyme purified from *Streptomyces* cells, which contained the *nfdA* gene and allowed the overproduction of NfdA in an active form, and the prediction of putative metal-binding sites in the amino acid sequence of NfdA through an novel alignment method, which was based on the camparison of predicted secondary structures of NfdA and amidohydrolase superfamily members. Furthermore, the author applied this method to regulatory proteins with significant overall sequence identity to NfdA, demonstrating that these proteins belong to amidohydrolase superfamily.

Materials and methods

Amino acid sequencing of NfdA

The N-terminal amino-acid sequence of NfdA was determined as described in Chapter II. 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 1:200 (mol/mol) of lysylendopeptidase (Takara Bio Inc., Otsu) in 20 mM Tris-HCl buffer (pH 9.0) or 1:50 (mol/mol) of endoproteinase Glu-C (Roche Diagnostics, Mannheim, Germany) 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.

Cloning and nucleotide sequencing of the *N*-substituted formamide deformylase gene

Escherichia coli DH10B (Invitrogen, Carlsbad, CA, USA) was used as the host for pUC plasmids (Sambrook et al., 1989). *E. coli* transformants were grown in 2 × YT medium (Sambrook et al., 1989).

Genomic DNA was prepared from *A. pascens* F164 as follows: the strain was cultured at 28°C for 24 h in 500 ml of 2 x 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% (w/v) 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; v/v/v), precipitated with isopropanol, treated with RNase, and then reprecipitated with ethanol.

An oligonucleotide sense primer (29-mer, 4608 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 Inc.) was subjected to PCR (94°C 30 s, 51°C 30 s, 72°C 60 s; 30 cycles), and the amplified DNA fragment (1057-bp) was gel-purified. The DNA fragment was then used as a probe for Southern hybridization and colony hybridization to clone the full-length *N*-substituted formamide deformylase gene (*nfdA*).

Southern hybridization was carried out using an Alkphos Direct Labelling 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 used for Southern hybridization.

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

Materials

DEAE-Sephacel and a low-molecular-weight standard kit were obtained from Amersham Biosciences (Piscataway, NJ, USA). Standard proteins for high-performance gel filtration chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) were purchased from Oriental Yeast Co. (Tokyo) and Sigma Co., Ltd. (St. Louis, MO, USA), respectively. *N*-Benzylformamide (NBFA) was obtained from Aldrich (Milwaukee, WI). Isovaleronitrile was from Tokyo Kasei Co. (Tokyo). Bacto-agar was from Difco (Detroit, WI, USA). Restriction enzymes were purchased from Takara (Tokyo), Toyobo (Osaka), and New England Biolabs (Beverly, MA, USA), and used according to the manufacturer's directions. DNA polymerase and T4 DNA ligase were obtained from Toyobo (Osaka). All other biochemicals used were from commercial sources and of reagent grade.

Bacterial strains and plasmids

The *Escherichia coli* strains used in this study were as follows: JM109 and DH5α were purchased from Takara; Origami B, Rosetta-gami B, Rosetta-gami B(DE3), and BL21(DE3) from Novagen; BL21(DE3) CodonPlus-RIL, JM110, and XL1-Blue from Stratagene; DH10B from Invitrogen; JM109(DE3) from Promega; and ER2566 from New England Biolabs. *Streptomyces lividans* TK24, *Streptomyces coelicolor* A3(2) M145, and *Streptomyces avermitilis* K139 were supplied by Dr. T. Fujii (National Institute for Agro-Environmental Sciences) and Prof. H. Ikeda (Kitasato University). The plasmids used and constructed in this work are shown in Table 1. The expression vector for *Streptomyces* cells, pSH19, was prepared as reported previously (Herai et al., 2004).

Table 1. Plasmids used in this study. Ap^r, Km^r, and Ts^r denote resistance to ampicillin, kanamycin, and thiostrepton, respectively.

	Plasmids	Description	Source
E. coli	pUC19	Cloning vector, Ap^r	Takara
	pHSG298	Cloning and expression vector, *lac* promoter, Km^r	Takara
	pHSG-*nfdA*	pHSG298 with 1.6 kb insert containing *nfdA*, Km^r	This work
	pET-24a(+)	Expression vector, T7 promoter, Km^r	Novagen
	pET-*nfdA*	pET-24a(+) with 1.6 kb insert containing nfdA, Km^r	This work
	pTYB1	Intein-CBD fusion expression vector, Ap^r	New England Biolabs
	pTYB1-*nfdA*	pTYB1 with 1.6 kb insert containing *nfdA*, Ap^r	This work
	pTYB11	Intein-CBD fusion expression vector, Ap^r	New England Biolabs
	pTYB11-*nfdA*	pTYB11 with 1.6 kb insert containing *nfdA*, Ap^r	This work
Streptomyces	pSH19	Expression vector, *nitA* promoter, Ts^r	Herai et al., 2004
	pSH19-*nfdA*	pSH19 with 1.6 kb insert containing *nfdA*, Ts^r	This work

Recombinant DNA methods

Purification of *E. coli* plasmids and *in vitro* DNA manipulation for cloning into *E. coli* cells were performed as described by Sambrook et al. (Sambrook et al., 1989). The following plasmids were introduced into appropriate *E. coli* strains: pHSG-*nfdA* was transformed into *E. coli* JM109, DH10B, Origami B, JM110, Rosetta-gami B, DH5α, and XL1-Blue; pET-*nfdA* into *E. coli* JM109(DE3), BL21(DE3), BL21(DE3) CodonPlus-RIL, and Rosetta-gami B(DE3); and pTYB1-*nfdA* or pTYB11-*nfdA* into *E. coli* BL21(DE3) and ER2566.

Plasmid DNA from *Streptomyces* cells was purified with a Plasmid Purification Kit (Qiagen, USA). Preparation of *Streptomyces* protoplasts and cloning of plasmids into *Streptomyces* cells were carried out as described by Kieser et al. (Kieser et al., 2000). Regeneration of *Streptomyces* protoplasts was achieved on regeneration agar plates (pH 7.2) containing 104 g of sucrose, 10 g of glucose, 0.25 g of K_2SO_4, 10 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of casamino acid, 5 g of yeast extract, 3 g of L-aspartic acid, 3 g of L-proline, 2 ml of a trace element solution (Kieser et al., 2000), 0.05 g of KH_2PO_4, 3 g of $CaCl_2$, 5.8 g of *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and 22 g of Bacto-agar per

liter of distilled water. Selection of transformants was performed by flooding the agar plates with 1 ml of 200 μg/ml thiostrepton after 16 h incubation at 28°C. The resultant colonies were inoculated onto medium I agar plates (1 g of yeast extract, 1 g of malt extract, 2 g of NZ amine type A, 10 g of maltose, and 15 g of Bacto-agar per liter of distilled water, adjusted to pH 7.2 with NaOH) supplemented with 20 μg/ml thiostrepton, and then incubated at 28°C. When plasmid pSH19-*nfdA* was transformed into *Streptomyces* protoplasts, further screening for positive clones with the *nfdA* gene on the plasmid was carried out by colony PCR as follows: two oligonucleotide primers for *nfdA*, a sense primer (5'-CCTGTGCGCAAGCGGTACTT-3') and an antisense primer (5'-TGAGCCTCGGCCCGAGGCAT-3'), were used. A reaction mixture (10 μl) comprising 1 μl of the cell suspension, 2 pmol of each primer, and KOD-Dash polymerase was subjected to PCR (94°C 35 s, 60°C 35 s, and 72°C 60 s; 30 cycles), and the amplified DNA fragment of *nfdA* was confirmed by gel electrophoresis.

Construction of expression plasmids

The *nfdA* gene (**AB164325**) was amplified by PCR with pNFD10 as a template. The primers used in the reaction are listed in Table 2. To construct each insert of plasmids, the following four primer pairs were used: (*i*) for pET-*nfdA* or pHSG-*nfdA*, the S1/AS1 primer pair was used; (*ii*) for pTYB1-*nfdA*, the S1/AS2 primer pair; (*iii*) for pTYB11-*nfdA*, the S2/AS3 primer pair; (*iv*) for pSH19-*nfdA*, the S3/AS1 primer pair. The PCR mixture was heated at 94°C for 2 min and then subjected to 30 cycles of amplification (94°C 15 s, 64°C 30 s, and 68°C 60 s). The amplified DNA was subcloned into vector pUC19 (when the DNA amplified by using primer pair (*i*) or (*iv*) was used) or pHSG298 (when the DNA amplified by using primer pair (*ii*) or (*iii*) was used). The nucleotides of the resultant plasmids were sequenced and checked by the dideoxy chain-terminating method using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The insert DNA was digested with appropriate restriction enzymes and then inserted into the corresponding vector.

Table 2. Primers used in this study. Restriction enzyme sites and ribosomal binding sites (RBS) are shown in lower case and bold letters, respectively. Start and stop codons are framed. The RBS of primers S1 and S3 are for *E. coli* and *Streptomyces*, respectively.

Name	Sequence	Restriction enzyme site	Note
S1	5'-GgaattcT**AAGGAG**GA ATAGcatatg ACACAA ATGCG TGACCTA ATG-3'	*Eco* RI, *Nde* I	RBS for *E. coli*
S2	5'-gctcttcTAACACACAA ATGCGTGACCTA ATGATC-3'	*Sap* I	No start codon of *nfdA*
S3	5'-aagcttAGCAAC**GGAGG**TACGGAC ATG ACACAA AT GCGTGACCTA ATG-3'	*Hin* dIII	RBS for *Streptomyces*
AS1	5'-CGggatcc CTA CGATCCGGTGCGTTCGTAGCGG-3'	*Bam* HI	
AS2	5'-gctcttcT GCA CGATCCGGTGCGTTCGTAGCGG-3'	*Sap* I	No stop codon of *nfdA*
AS3	5'-ctgcag CTA CGATCCGGTGCGTTCGTAGCGG-3'	*Pst* I	

Growth conditions

Each *E. coli* transformant was subcultured at 37°C in a test tube containing 5 ml of 2 x YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter of distilled water) (Sambrook et al., 1989) supplemented with 50 μg/ml kanamycin (when *E. coli* strains with pHSG-*nfdA* or pET-*nfdA* were cultured) or ampicillin (when *E. coli* strains with pTYB1-*nfdA* or pTYB11-*nfdA* were cultured). After overnight cultivation, 1 ml of the subculture was inoculated into a 500-ml shaking flask containing 100 ml of the same medium, followed by cultivation at 37°C as follows: an *E. coli* strain transformed with pHSG-*nfdA*, in which *nfdA* was under the control of the *lac* promoter, was cultured in the medium supplemented with 0.1 mM IPTG for 6 h. On the other hand, an *E. coli* strain harboring pET-*nfdA*, pTYB1-*nfdA*, or pTYB11-*nfdA*, in which *nfdA* was under the control of the bacteriophage T7 promoter, was cultured for 2 h, followed by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to the medium to a final concentration of 0.1 mM and then further cultivation for 4 h.

The *Streptomyces* transformant was cultured under the conditions given under *Results and Discussion*.

Purification of recombinant NfdA

All purification procedures were performed at 0-4°C. Potassium phosphate buffer (pH 7.5) containing 10% (w/v) glycerol was used throughout the purification, unless noted otherwise. Centrifugation was carried out for 15 min at 13,000 x *g*.

The cells were harvested by centrifugation at 5,070 x *g* at 4°C and then washed twice with 10 mM buffer. The washed cells were resuspended in 0.1 M buffer and then disrupted by sonication at 200 W for 20 min with an Insonator model 201M (Kubota, Tokyo). The cell debris was removed by centrifugation. The resultant supernatant was fractionated with ammonium sulfate (40-50%), followed by dialysis against 10 mM buffer. The dialyzed solution was applied to a DEAE-Sephacel column (5 x 40 cm) equilibrated with 10 mM buffer containing 0.2 M KCl. Protein was eluted from the column with 1 liter of the same buffer, the concentration of KCl being increased linearly from 0.2 to 0.5 M. The active fractions were collected and then ammonium sulfate was added to give 70% saturation. After centrifugation of the suspension, the precipitate was dissolved in 10 mM buffer, followed by dialysis against 10 mM buffer. The obtained enzyme solution was brought to 25% ammonium sulfate saturation, and then placed on a Resource ISO column (1.6 x 3 cm; Amersham Biosciences) equilibrated with 10 mM buffer containing 25% saturated ammonium sulfate. The enzyme was eluted with a linear gradient of ammonium sulfate (from 25 to 15% saturation) in 10 mM buffer. The active fractions were combined and precipitated with ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation, dissolved in 10 mM buffer, and then dialyzed against 10 mM buffer. The homogeneity of the purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

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 1 mM potassium phosphate buffer (pH 7.5). Qualitative and quantitative analyses of the following metals in the enzyme solution (1.38 mg protein/ml) were performed with an inductively coupled radiofrequency plasma spectrophotometer, Shimadzu ICPS-8000 (27.120 MHz).: Be, B, Mg, Al, Si, P, S, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Sr, Zr, Mo, Pd, Ag, Cd, Sn, Sb, Ba, Ta, W, Pt, Au, Hg, Pb, La and Ce. The metal contents of the enzyme sample were determined from the calibration curves for standard solutions.

Enzyme assay and other procedures

NfdA activity assay was performed by the standard assay A method as described in Chapter II. One unit of NfdA activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of benzylamine per min from NBFA. Specific activity is expressed as units per mg of protein. Additionally, protein concentration determination; electrophoresis; and N-terminal amino acid sequencing and molecular mass determination of NfdA were performed as described in Chapter II.

Results and Discussion

Cloning and nucleotide sequencing of the *N*-substituted formamide deformylase gene

N-Substituted formamide deformylase was purified to homogeneity from *A. pascens* F164, and the amino acid sequences of peptides were determined by digesting the enzyme with lysyl endopeptidase or endoproteinase Glu-C. Two oligonucleotide primers were synthesized based on the N-terminal and internal sequences (corresponding to the amino acid residues 3-11 and 347-354 deduced from the *nfdA* gene, respectively) and used for PCR amplification with genomic DNA of *A. pascens* F164 as a template, resulting in the generation of a 1057-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 (Fig. 1).

To obtain the entire *nfdA* gene, after digestion of the genomic DNA with several restriction enzymes, Southern hybridization was performed using the 1057-bp fragment as a probe. A single 9-kb *Bam*HI fragment was positively detected, and this fragment was recovered and ligated with *Bam*HI-digested pUC19 to transform *E. coli* DH10B. After screening of the recombinant plasmids by colony hybridization, a positive clone, designated as pNFD10, was obtained.

The nucleotide sequencing of pNFD10 revealed a 1629-bp open reading frame encoding 542 amino acids (DNA Data Bank of Japan accession number AB164325), which precisely included those determined with the purified NfdA (Fig. 1.). Although the molecular mass of the protein encoded by this gene (*nfdA*) was calculated to be 58,694 Da, which was consistent with that of the enzyme subunit (M_r = 58,556) determined by MALDI-TOF MS (Chapter II), it was slightly different from that of the enzyme subunit (M_r = 61 kDa) determined on SDS-PAGE. This discrepancy may be explained by the unusual mobility of the enzyme protein on SDS-PAGE, which was caused by its small SDS-binding capacity, since some

proteins exhibit greater resistance to binding than others (Nelson, 1971).

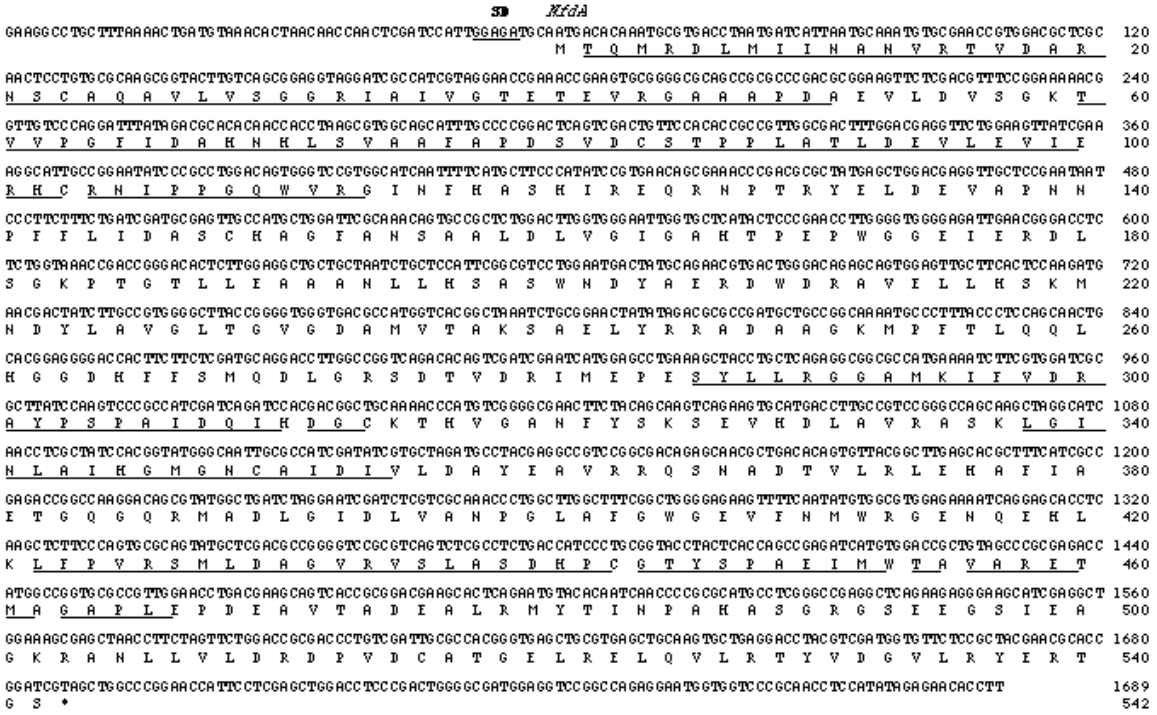

Fig.1 Nucleotide and amino acid sequences of the *N*-substituted formamide deformylase gene. The underlined amino acid and sequences were determined by Edman degradation. A potential ribosome-binding sequence is indicated by *S. D.* (Shine-Dalgarno), and a relevant stop codon is indicated by an *asterisk*. The nucleotide sequence has been deposited in the DNA Data Bank Japan under DDBJ accession number AB164325.

Homology search of the deduced amino acid sequence of NfdA

A search of protein sequence databases with the BLAST server revealed that NfdA exhibits less than 30% overall amino acid sequence identity with known proteins. Interestingly, the highest identities were observed with regulatory proteins, *e.g.* LAF3 isoform 1 (LAF3-1) from *Arabidopsis thaliana* (GenBank™ AAP55749; 26%) (Hare et al., 2003) and AepA from *Brucella melitensis* 16M (GenBank™ NP_541100.1; 28%) (Paulsen et al., 2002) (Fig. 2). On the other hand, only the N-terminal region (residues 58-72) of NfdA showed significant sequence identity (27-73%) to those of members of the amidohydrolase superfamily (Holm and Sander, 1997) including imidazolonepropionase (Nierman et al. 2001), atrazine chlorohydrolase (de Souza et al., 1996), cytosine deaminase (Ireton et al., 2002), dihydroorotase (Thoden et al., 2001), and urease (Jabri et al., 1995)(Fig. 2), although there is no similarity in the overall sequence except in the above limited region. The result suggests that NfdA would be a member of amidohydrolase superfamily. Further analyses for the enzyme is necessary to be performed in order to make more clear the relationship between NfdA and amidohydrolase superfamily members.

NfdA also didn't show no homology with known deformylases described in Chapter II. This findings, together with data on reaction stoichiometry and substrate specificity of NfdA (Chapter II), supported that NfdA is a novel deformylase for *N*-substituted formamides, and its structure and reaction mechanism would be different from those of each of the other known deformylases. There is currently a great deal of interest in the catalytic mechanism of this enzyme. A more detailed understanding of it will be obtained by analysis of its three-dimensional structure. However, the yield of the enzyme obtained from the wild type strain was too low to perform such analyses. To increase the knowledge on the enzymology of NfdA and to shed light on the structure/function relationship of this enzyme, the author tried to overexpress the recombinant NfdA in heterologous cells.

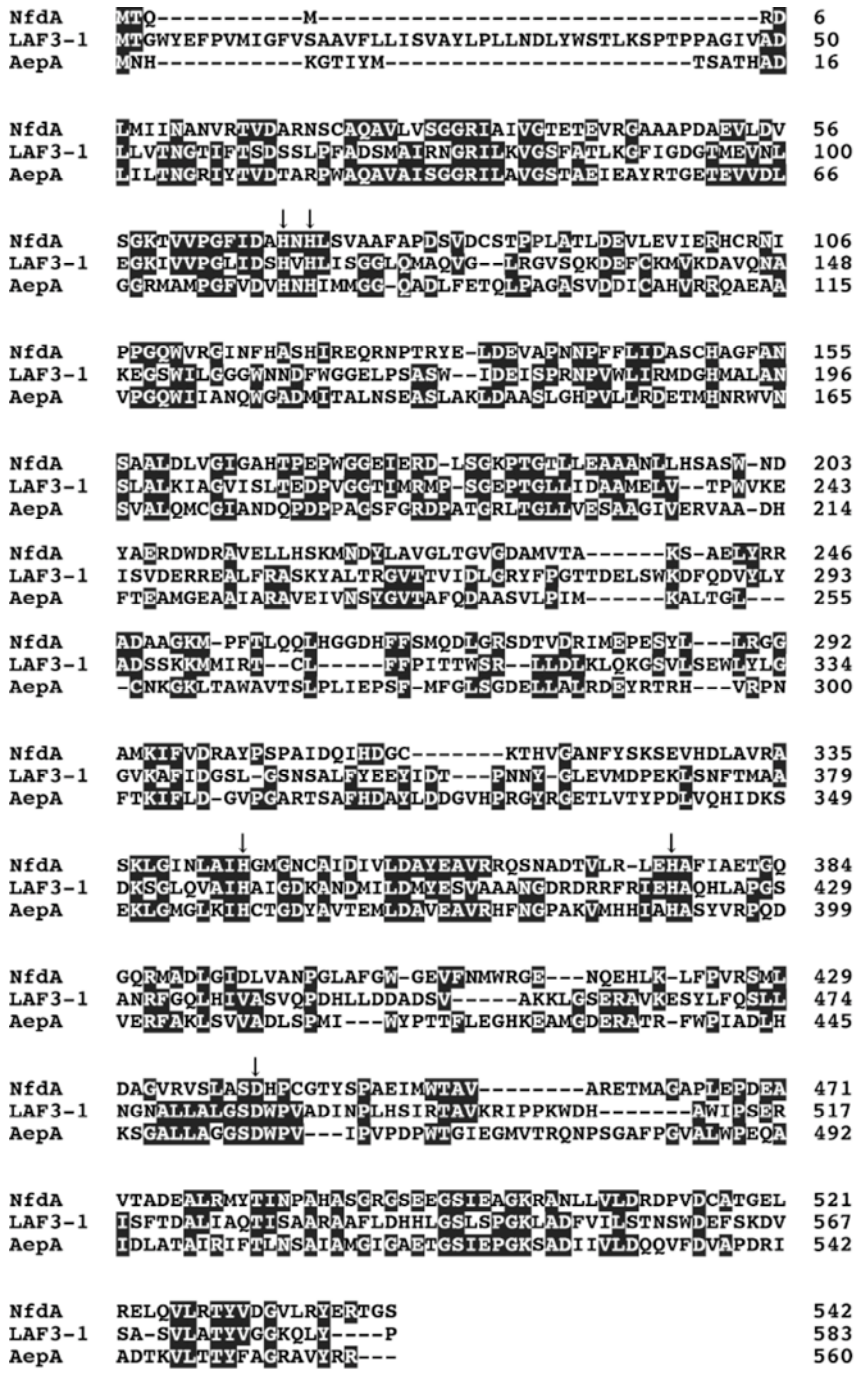

Fig. 2. Alignment of the amino acid sequences of NfdA and regulatory proteins. Residues that are conserved between NfdA and either of the regulatory proteins (LAF3-1 and AepA) are highlighted in reverse type. Arrows indicate the residues corresponding to highly conserved histidine and aspartate residues in the amidohydrolase superfamily.

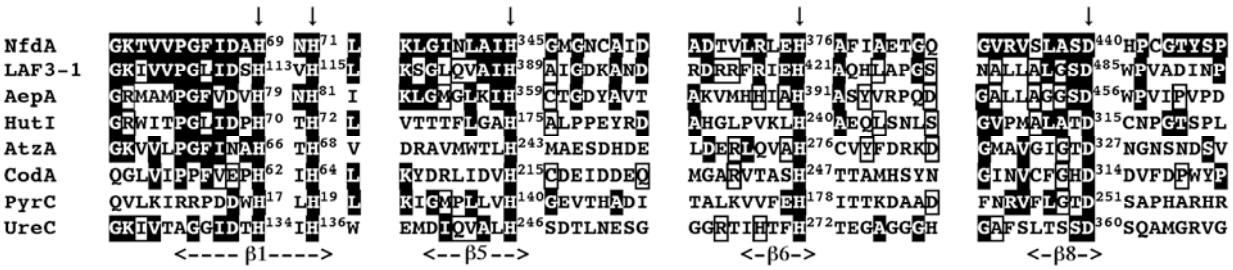

Fig. 3. Sequence alignment of NfdA with distantly related proteins. Each region of the amino acid sequences of cytosine deaminase (CodA), dihydroorotase (PyrC) and urease α subunit (UreC) contains β-strand secondary structure in which the functional residues (histidine and aspartic acid) map to the C-terminus of strands 1, 5, 6 and 8 in each enzyme (Holm and Sander, 1997). For NfdA, LAF3-1, HutI, and AtzA, the regions containing the predicted secondary structures that correspond to β1, 5, 6 or 8 of CodA, PyrC, and UreC are shown. LAF3 isoform 1 (LAF3-1) is from *A. thaliana* (GenBank accession no. AAP55749), AepA precursor (AepA) from *B. melitensis* 16M (NP_541100.1), imidazolonepropionase (HutI) from *Caulobacter crescentus* (SwissProt accession number P58079), atrazine chlorohydrolase (AtzA) from *Pseudomonas* sp. ADP (SwissProt accession number P72156), cytosine deaminase (CodA) from *E. coli* (SwissProt accession number P25524), dihydroorotase (PyrC) from *E. coli* (SwissProt accession number P05020), and urease α subunit (UreC) from *Klebsiella aerogenes* (SwissProt accession number P18314). The residues with amino acid numbers and vertical arrows are metal ligands established by X-ray crystallography of CodA (PDB ID code 1k6w), PyrC (PDB ID code 1J79), and UreC (PDB ID code 2kau), and the corresponding residues in the other proteins. The residues highlighted in reverse type are conserved in NfdA and all of the other proteins. Except for the residues highlighted in reverse type, identical amino acid residues in either of the regulatory proteins and the members of the amidohydrolase superfamily are boxed.

Overexpression of NfdA in *E. coli* strains

To overproduce NfdA in *E. coli* strains, the author constructed four types of expression vectors carrying the *nfdA* gene (i.e., pHSG-*nfdA*, pET-*nfdA*, pTYB1-*nfdA*, and pTYB11-*nfdA*), and transformed each plasmid into *E. coli* strains as described under *Materials and Methods*. Identical parallel cultures of the cells, containing the parental plasmids without *nfdA* (i.e., pHSG298, pET-24a(+), pTYB1, and pTYB11), were used as negative controls. In these experiments, none of the samples showed NfdA activity. SDS-PAGE also revealed no differences in soluble protein profile between the cells transformed with pHSG-*nfdA* and those transformed with pHSG298. Moreover, overexpression of NfdA in the following *E. coli* transformants (i.e., *E. coli* BL21(DE3) and BL21(DE3) CodonPlus-RIL harboring pET-*nfdA*; and *E. coli* BL21(DE3) and ER2566 harboring pTYB1 or pTYB11) resulted in the accumulation of inclusion bodies in the insoluble fraction (data not shown). In order to solubilize the inclusion bodies, the author examined various conditions: the concentration of IPTG (0.1-1 mM), induction temperature (15, 20, 28, or 37°C), volume of the medium (100 or 500 ml), richness of the medium (M9 medium [Sambrook et al., 1989], or 2 x YT medium), and addition of various metal ions (e.g., LiCl, $MgCl_2$, $FeSO_4$, $FeCl_3$, $CoCl_2$, $NiCl_2$, or $ZnCl_2$) to a final concentration of 0.2 mM to the 2 x YT medium. However, NfdA was not expressed in an active and soluble form under any of the above culture conditions.

Several explanations can be given for the accumulation of insoluble protein in *E. coli*, as recently reviewed by Balbas (Balbas, 2001), however, it was difficult to identify the crucial factor that facilitates the expression of soluble NfdA in *E. coli*. Recently, Herai et al. developed a hyper-inducible protein expression system, designated as the P_{nitA}-NitR system, for *Streptomyces* cells as a host (Herai et al., 2004). This system was demonstrated to be very useful for the overexpression of a gene derived from either Gram-negative or Gram-positive bacteria. Like *Arthrobacter*, *Streptomyces* also belongs to the order Actinomycetales, and both microorganisms are Gram-positive and have a high G+C content (Madigan et al., 2000). Because of these similarities, together with the availability of the strong protein

expression system for *Streptomyces*, the author decided to express NfdA in an alternative host, *Streptomyces*.

Overexpression of NfdA in *S. lividans*

S. lividans TK24 was transformed with pSH19-*nfdA*, in which the *nfdA* gene was inserted downstream of the ε-caprolactam- or isovaleronitrile-inducible nitrilase (*nitA*) promoter. The ribosomal binding site recognized in *Streptomyces* was provided by designing the sequence on PCR primer (Table 2). The recombinant *S. lividans* TK24 collected from medium I agar plates was inoculated and then cultivated under the following basal culture conditions: the subculture was carried out to full growth at 28°C with 135 rpm rotary shaking in a 500-ml baffled flask containing 100 ml of YEME medium (3 g of yeast extract, 5 g of polypeptone, 3 g of malt extract, 10 g of glucose, 34 g of sucrose, and 2 ml of 2.5 M $MgCl_2 \cdot 6H_2O$ per liter of distilled water) (Kieser et al., 2000) supplemented with 0.5% (w/v) glycine and 20 μg/ml thiostrepton. Then 1 ml of the subculture was inoculated into a 500-ml baffled flask containing 100 ml of the same medium, followed by incubation under the same conditions. After 48 h incubation, isovaleronitrile or ε-caprolactam as an inducer was added to the medium to a final concentration of 0.1% (v/v) or 0.1% (w/v), respectively, and then further cultivation was carried out at 28°C for 72 h. Identical parallel cultures of the cells, containing the parental plasmid without the *nfdA* gene (i.e., pSH19), were used as negative controls. When *S. lividans* TK24 harboring pSH19-*nfdA* was cultured in the medium supplemented with isovaleronitrile or ε-caprolactam, significant NfdA activity was observed in the cell-free extract: isovaleronitrile, 4.53 U/mg; ε-caprolactam, 1.73 U/mg. Although NfdA activity was detected even in the absence of inducers, the specific activity of the cell-free extract was very low (0.40 U/mg). The author also analyzed each cell-free extract by SDS-PAGE, and detected a 61 kDa protein band that corresponded to the subunit of the *A. pascens* F164 enzyme (data not shown). This protein band, together with NfdA activity, was not detected in the control strain, i.e. *S. lividans* TK24 harboring pSH19 (data not shown). Because the level of NfdA expression in the cells induced by isovaleronitrile was higher than that in those induced by

ε-caprolactam, the author decided to use the former compound as an inducer. The author also introduced pSH19-*nfdA* into other *Streptomyces* strains; *S. coelicolor* A3(2) M145 and *S. avermitilis* K139, it being found that the *S. lividans* TK24 transformant exhibited the highest specific activity (*S. lividans* TK24, 5.1 U/mg; *S. coelicolor* A3(2) M145, 0.9 U/mg; and *S. avermitilis* K139, 0.1 U/mg). Thus the author chose *S. lividans* TK24 as the most suitable strain.

In order to increase the NfdA production and to shorten the total cultivation time, the author examined the effects of the concentration of isovaleronitrile, the addition of glycine, the time of induction, and the induction duration. Finally, the author found the optimum culture conditions were as follows: the recombinant *S. lividans* TK24 was subcultured to full growth at 28°C with 135 rpm rotary shaking in a 500-ml baffled flask containing 150 ml of YEME medium supplemented with 0.5% (w/v) glycine and 20 μg/ml thiostrepton. Then 1 ml of the subculture was inoculated into a 500-ml baffled flask containing 150 ml of the same medium, except that no glycine was added. After 24 h incubation under the same conditions, isovaleronitrile was added to the medium to a final concentration of 0.1% (v/v), and then further cultivation was carried out at 28°C for 48 h. The maximum specific activity of NfdA in the cell-free extract amounted to 8.5 U/mg of protein, which corresponded to approximately two times enhancement compared to the NfdA activity in the cells cultivated under the basal culture conditions. The value was also 29-fold higher than the highest specific activity obtained in a crude extract of *A. pascens* F164 (0.29 U/mg of protein). Moreover, the induced expression level was found to be extremely high on SDS-PAGE. As judged on quantitative evaluation of the gel track, the enzyme corresponded to ≈20% of the total soluble protein in the cells cultivated under the optimum conditions (data not shown). Furthermore, the total cultivation time from subculture to harvest was shortened to 72 h, which was 48 h shorter than that under the basal culture conditions. These findings, together with the previous report by Herai et al. (Herai et al., 2004) demonstrate that the P_{nitA}-NitR system for streptomycetes would be an alternative useful tool for overexpressing a gene that cannot be expressed in *E. coli*.

Purification of recombinant NfdA

The recombinant NfdA in *S. lividans* TK24 cells was purified to homogeneity through ammonium sulfate fractionation and only two-step column chromatography procedures (data not shown). The purification scheme was essentially identical to that for wild type NfdA (Chapter II), but the author were able to eliminate the third chromatographic step necessary for purification of the native enzyme. The specific activity of the purified recombinant enzyme was 28.8 U/mg (Table 3), which was comparable to that of the wild type enzyme (28.9 U/mg). The K_m and V_{max} values for NBFA with the recombinant enzyme were 0.067 mM and 47.1 U/mg, respectively, the V_{max}/K_m value being 703 U/mg/mM. These values were very close to those of the wild type enzyme (K_m, 0.075 mM; V_{max}, 52.7 U/mg; and $V_{max}/K_{m,}$ 703 U/mg/mM). There was no detectable difference in apparent molecular mass between the recombinant and wild type NfdA on SDS-PAGE (data not shown). The molecular mass of the recombinant enzyme subunit was also determined to be 58,773 Da by MALDI-TOF MS, this value being almost the same as that of the wild type enzyme (58,556 Da). Moreover, the N-terminal amino acid sequence of the recombinant NfdA, which was determined by automated Edman degradation, was identical with that of the wild type enzyme (TQMRDLMIINA). Other enzymological properties, including the effects of pH, temperature, and inhibitors, of the recombinant NfdA were also not different from those of the wild type NfdA. These data indicate that the recombinant NfdA produced by the *S. lividans* TK24 transformant is enzymologically identical to the *A. pascens* F164 NfdA.

Table 3. Purification of recombinant NfdA.

Step	Total protein	Total activity	Specific activity	Yield
	mg	units	units/mg	%
Cell-free extract	18,100	120,000	6.63	100
$(NH_4)_2SO_4$ (0.40-0.50)	4,060	71,900	17.7	60
DEAE-Sephacel	953	27,100	28.4	23
Resource ISO	318	9,160	28.8	7.6

Metal analysis of the recombinant NfdA

Since the purified enzyme could not be obtained in a large amount from *Arthrobacter* and metal analysis required a large amount of the enzyme, the author used the recombinant enzyme to perform the metal analyses with an inductively coupled radiofrequency plasma spectrophotometer. As a result, it was found that the enzyme contained 2.94 mol of zinc/mol of subunit. On the other hand, none of the other 34 metals described in *materials and methods* was detected within the limits of the assay (10 ng/ml). The absorption spectrum of the purified enzyme in 10 mM potassium phosphate buffer (pH 7.5) showed maximum absorbance at 282 nm. No other absorption peak or shoulder was observed, suggesting that no co-factor other than zinc would be bound to the enzyme.

The metal analysis of the recombinant NfdA showed for the first time that NfdA is an metalloenzyme which contains three zinc ions per subunit. However, it has been unclear which amino acid residues of the enzyme are involved in bindind to the zinc ions. The homology search with BLAST server suggested that NfdA would be a member of amidohydrolase superfamily including imidazolonepropionase (HutI) (Nierman et al. 2001),

atrazine chlorohydrolase (AtzA) (de Souza et al., 1996), cytosine deaminase (CodA) (Ireton et al., 2002), dihydroorotase (PyrC) (Thoden et al., 2001), and urease α subunit (UreC) (Jabri et al., 1995). The amidohydrolase superfamily is characterized by members with a structural context of alternating α-helix and β-strand secondary structure elements, designated as $(\alpha/\beta)_8$ barrel structure, where the highly conserved residues (His and Asp) locate in the C-terminal end of β strands 1, 5, 6 and 8 of CodA, PyrC, and UreC (Holm and Sander, 1997) (Fig. 3 in Section I of this chapter). These signature residues consisted of four histidines and one aspartate (where two histidines in the N-terminal region of each enzyme are designated as the HXH motif) are involved in metal-binding, catalytic function and stability of the enzyme structure (Holm and Sander, 1997). Thus the author suggested that five highly conserved amino acid residues bound to zinc ions would be located in the amino acid sequence of NfdA.

The BLAST search revealed that NfdA has the HXH motif and the signature aspartate, which correspond to the metal-binding residues present in β strands 1 and 8 of each member of the amidohydrolase superfamily (*e.g.* urease α subunit), respectively. The author then attempted to compare the secondary structure of NfdA with those of CodA, PyrC, and UreC to identify possible residual signature residues of NfdA. The secondary structural prediction of NfdA with the PSI-Pred (Cuff et al., 1998) and PROFsec (Rost, 1996; Rost and Liu, 2003) programs revealed that the distribution pattern of the predicted secondary structure for NfdA was similar to those of CodA, PyrC and UreC. The residual signature histidine residues were also found to exist in the predicted β strands of NfdA which correspond to β5 and β6 of the amidohydrolase superfamily members, respectively (Fig. 4). Therefore, it was suggested that these five conserved residues in NfdA would coordinate zinc ions, and concerned with the catalytic acitivity and stability of the enzyme structure. Mutant analyses will be expected to be performed in order to verify whether these amino acid residues actually serve as the zinc-binding ligands and play an essential role in the catalytic acitivity and stability of the enzyme structure. Moreover, these findings proposed that NfdA is a novel member of the amidohydrolase superfamily from these findings. Although all other members of this

superfamily contain one or two metal ions per subunit, NfdA contains theree zinc ions per subunit; there have been no reports on another member containing three metal ions in this superfamily (Holm and Sander, 1997). Determination of the enzyme structure will provide the information to clarify the reaction mechanism, together with knowledge on new type of enzyme structure in amidohydrolase superfamily.

Fig. 4. Secondary structure prediction of NfdA and regulatory proteins. The structural predictions were performed with the program PSI-Pred and PROFsec. The predictions for secondary structure (PSIPRED; RROF_sec) (H, α helix; E, β strand), the confidence of PSI-Pred prediction (Confidence), and the reliability of PROFsec prediction (Rel_sec) for four regions of NfdA, LAF3-1, and AepA, are shown. The confidence or reliability of these predictions varies between 0 (low) and 9 (high). For a comparison, the common β secondary structures (β1, β5, β6, and β8) of CodA, PyrC, and UreC are shown at the bottom. The residues highlighted in reverse type are highly conserved amino acids in the amidohydrolase superfamily, and the corresponding residues in NfdA, LAF3-1, and AepA. Numbers in parentheses are the length of the intervening sequence.

Furthermore, to our surprise, NfdA also exhibits the highest overall sequence identity with known regulatory proteins such as LAF3 isoform 1 (26%) and AepA (28%). LAF3 is one participant in the light signaling pathway that is essential for photoperiod sensing that regulates plant growth and development. This protein is involved in activation of the transcription of *XTR7*, which encodes a xyloglucan endotransglycosylase-related protein that breaks down the cell wall to allow seedling stems to elongate, and is an important for termination of hypocotyl elongation (Hare et al., 2003). AepA is produced by a plant-pathogenic bacterium, *Erwinia carotovora*. subsp. *carotovora* strain Ecc71. It is an activator protein that regulates the production of extracellular enzymes, which are required for maceration of plant tissues, and triggers the transcription of *pel-1*, which specifies a pectate lyase isozyme (Liu et al., 1993). As shown in Fig. 2, sequence alignment of NfdA and these proteins for the first time revealed that all the possible five conserved metal-binding residues are present in these proteins. On the other hand, each distribution pattern of their predicted secondary structure was found to be an $(\alpha/\beta)_8$ barrel structure, which was perfectly consistent with those of the amidohydrolase superfamily members (Fig. 4). These findings together with the sequence alignment support that the assignment of β secondary structure of NfdA as described above is valid, and suggests that these regulatory proteins belong to the amidohydrolase superfamily as well as NfdA. Their precise roles in the regulation of gene expression are not yet known. Based on the analogy to NfdA, they may be involved in the binding and degradation of a molecule chemically related to NBFA.

Unexpectedly, the author have found that the histidine-aspartate signature in the amidohydrolase superfamily are perfectly conserved in NfdA, and the regulatory proteins (Fig. 5), suggesting that these enzymes and proteins might have evolved from a common ancestral gene. It would be of interest to determine how these proteins diverged and became differentiated as to biological function. Further studies on each enzyme and protein from the standpoints of its reaction mechanism and its three-dimensional structure could provide information on their evolutionary relationships.

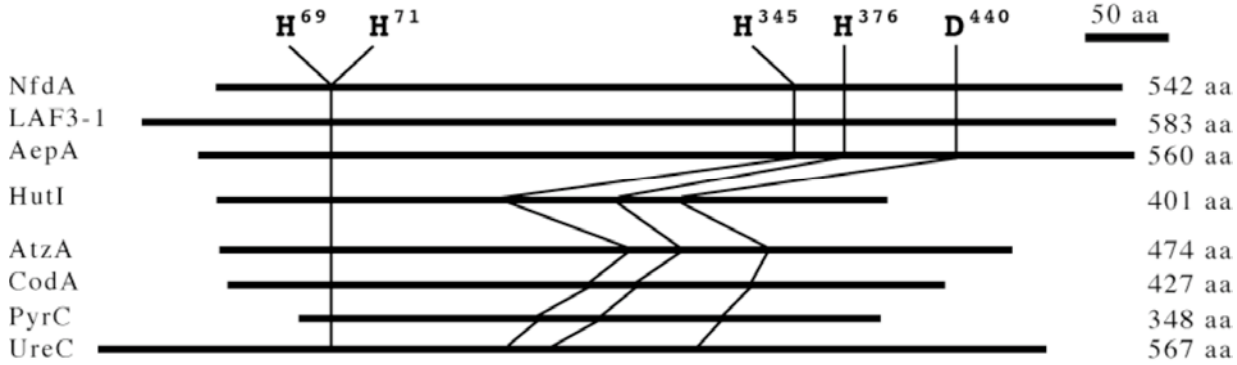

Fig. 5. Schematic representation of *N*-substituted formamide deformylase (NfdA) and distantly related proteins. The five highly conserved residues are indicated, and the number for each histidine and aspartate residue indicates the deduced amino acid number in NfdA. Numbers on the right indicate the total numbers of amino acid residues in each molecule.

References

Achenbach, H., and Grisebach, H. (1965) *Z. Naturforsch.* **20B,** 137-140.

Achenbach, H., and König, F. (1972) *Chem. Ber.* **105,** 784-793.

Angerhofer, C. K., Pezzuto, J. M., König, G. M., Wright, A. D., and Sticher, O. (1992) *J. Nat. Prod.* **55,** 1787-1789.

Auerbach, V. H., and Knox, W. E. (1957) *Methods Enzymol.* **3**, 620-623.

Bailey, C. B., and Wagner C. (1974) *J. Biol. Chem.* **249**, 4439-4444.

Balbas, P. (2001) *Mol. Biotechnol.* **19**, 251-267.

Bradford, M. (1976) *Anal. Biochem.* **72,** 248-254.

Broom, M. F., Sherriff, R. M., Tate, W. P., Collings, J., and Chadwick, V. S. (1989) *Biochem. J.* **257**, 51-56.

Chattopadhyay, M. K., Tabor, C. W., and Tabor, H. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 13869-13874.

Cochran, D. G. (1976) *Insect Biochem.* **6**, 267-272.

Cuff, J. A., Clamp, M. E., Siddigni, A. S., Finlay, M., and Barton, G. J. (1998) *Bioinformatics* **14**, 892-893.

de Souza, M. L., Sadowsky, M. J., and Wackett, L. P. (1996) *J. Bacteriol.* **178**, 4894-4900.

Edenborough, M. S., and Herbert, R. B. (1988) *Natl. Prod. Rep.* **5,** 229-245.

Eto, M., Seifert, J., Engel, J. L., and Casida, J. E. (1980) *Toxicol. Appl. Pharmacol.* **54**, 20-30.

Fraser, J. A., Davis, M. A., and Hynes, M. J. (2001) *Genetics*, **157**, 119-131.

Frebort, I., Sebela, M., Hirota, S., Yamada, M., Tamaki, H., Kumagai, H.,

Adachi, O., and Pec, P. (2003) *Biol. Chem.* **384**, 1451-1461.

Fusetani, N. (1997) *Curr. Org. Chem.* **1,** 127-152.

Garson, M. J., and Simpson, J. S. (2004) *Nat. Prod. Rep.* **21**, 164-179.

Glassman, E. (1956) *Genetics* **41**, 566-574.

Goda, M., Hashimoto, Y., Shimizu, S., and Kobayashi, M. (2001) *J. Biol. Chem.* **276**, 23480-23485.

Goda, M., Hashimoto, Y., Takase, M., Herai, S., Iwahara, Y., Higashigbata, H., and Kobayashi, M. (2002) *J. Biol. Chem.* **277**, 45860-45865.

Hagedorn, I., and Tonjes, H. (1957) *Pharmazie* **12,** 567-580.

Hagadone, M. R., Scheuer, P. J., and Holm, A. (1984) *J. Am. Chem. Soc.* **106**, 2447-2448.

Hare, P. D., Møller, S. G., Huang, L.-F., and Chua, N.-H. (2003) *Plant Physiol.* **133**, 1592-1604.

Herai, S., Hashimoto, Y., Higashibata, H., Maseda, H., Ikeda, H., Omura, S., and Kobayashi, M. (2004) *Proc. Natl. Acad. Sci. USA.* **101**, 14031-14035.

Holm, L., and Sander, C. (1997) *Proteins* **28**, 72-82.

Hu, L., Mulfinger, L. M., and Phillips, A. T. (1987) *J. Bacteriol.* **69**, 4696-4702.

Ireton, G. C., McDermott, G., Black, M. E., and Stoddard, B. L. (2002) *J. Mol. Biol.* **315**, 687-697.

Jabri, E., Carr, M. B., Hausinger, R. P., and Karplus, P. A. (1995) *Science* **268**, 998-1004.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., Practical *Streptomyces* Genetics, The John Innes Foundation, Norwich, 2000.

Kobayashi, M., Nagasawa, T., and Yamada, H. (1992a) *Trends in Biotechnol.* **10,**

402-408.

Kobayashi, M., Komeda, H., Yanaka, N., Nagasawa, T., and Yamada, H. (1992b) *J. Biol. Chem.* **267,** 20746-20751.

Kobayashi, M., Izui, H., Nagasawa, T., and Yamada, H. (1993a) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 247-251.

Kobayashi, M., Komeda, H., Nagasawa, T., Nishiyama, M., Horinouchi, S., Beppu, T., Yamada, H., and Shimizu, S. (1993b) *Eur. J. Biochem.* **217,** 327-336.

Kobayashi, M., and Shimizu, S. (1994) *FEMS Microbiol. Lett.* **120,** 217-224.

Kobayashi, M., Suzuki, T., Fujita, T., Masuda, M., and Shimizu, S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 714-718.

Kobayashi, M., Fujiwara, Y., Goda, M., Komeda, H., and Shimizu, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11986-11991.

Kobayashi, M., and Shimizu, S. (1998) *Nature Biotechnol.* **16,** 733-736.

Kobayashi, M., Goda, M., and Shimizu, S. (1998) *FEBS Lett.* **439,** 325-328.

Kobayashi, M., and Shimizu, S. (1999) *Eur. J. Biochem.* **261,** 1-9.

Komeda, H., Hori, Y., Kobayashi, M., and Shimizu, S. (1996a) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10572-10577.

Komeda, H., Kobayashi, M., and Shimizu, S. (1996b) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4267-4272.

Komeda, H., Kobayashi, M., and Shimizu, S. (1996c) *J. Biol. Chem.* **271,** 15796-15802.

Laemmli, U. K. (1970) *Nature* **227,** 680-685.

Liu, Y., Murata, H., Chatterjee, A., and Chatterjee, A. K. (1993) *Mol. Plant-Microb. Interact.* **6**, 299-308.

Madigan, M.T., Martinko, J.M., and Parker, J., Brock Biology of Microorganisms, ninth ed., Prentice Hall, NJ, 2000.

Nagy, P. L., Marolewski, A., Benkovic, S. J., and Zalkin, H. (1995) *J. Bacteriol.* **177**, 1292-1298.

Nelson, C.A. (1971) *J. Biol. Chem.* **246**, 3895-3901.

Nierman, W. C., et al. (2001) *Pro. Natl. Acad. Sci. U. S. A.* **98**, 4136-4141.

Nimura, N., Ogura, H., and Kinoshita, T. (1980) *J. Chromatogr.* **202**, 375-379.

Ohmura, E., and Hayaishi, O. (1957) *J. Biol. Chem.* **227**, 181-190.

Paulsen, I. T., et al. (2002) *Proc. Nat. Acad. Sci. U. S. A.* **99**, 13148-13153.

Pfeifer, S., Bär, H., and Zarnack, J. (1972) *Pharmazie* **27,** 536-542.

Ragusa, S., Blanquet, S., and Meinnel, T. (1998) *J. Mol. Biol.* **280**, 515-523.

Rost, B. (1996) *Methods Enzymol.* **266**, 525-539.

Rost, B., and Liu, J. (2003) *Nucleic Acids Res.* **31**, 3300-3304.

Rothe, W. (1950) *Pharmazie* **5**, 190.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schär, H.-P., Holzmann, W., Rasmostombo, G. M., and Ghisalba, O. (1986) *Eur. J. Biochem.* **158**, 469-475.

Scheuer, P. J. (1992) *Acc. Chem. Res.* **25,** 433-439.

Seifert J., and Casida, J. E. (1978) *Biochem. Pharmacol.* **27**, 2611-2615.

Sereo A., Giglione, C., Sardini, A., Maritinezsanz, J., and Meinnel, T. (2003) *J. Biol. Chem.* **278**, 52953-52963.

Simpson, J. S., Brust, A., and Garson, M. J. (2004) *Org. Biomol. Chem.* **2**, 949-956.

Thoden, J. B., Phillips, G. N., Neal, T. M., Raushel, F. M., and Holden, H. M. (2001) *Biochemistry* **40**, 6989-6997.

Woodhouse, A. F., Anderson, R. P., Myers, D. B., Broom, M. F., Hobson, C. H., and Chadwick, V. S. (1987) *J. Gastroenterol. Hepatol.* **2**, 35-43.

Wright, A. D., Wang, H., Gurrath, M., König, G. M., Kocak, G., Neumann, G., Loria, P., Foley, M., and Tilley, L. (2001) *J. Med. Chem.* **44**, 873-885.

Yamada, H., and Kobayashi, M. (1996) *Biosci. Biotech. Biochem.* **60,** 1391-1400.

Yamashita, M., Azakami, H., Yokoro N., Roh J. H., Suzuki, H., Kumagai, H., and Murooka, Y. (1996) *J. Bacteriol.* **178**, 2941-2947.

Conclusions

In this thesis, the author describes the discovery and characterization of a novel enzyme, *N*-substituted formamide deformylase (NfdA), involved in isonitrile metabolism. The findings in this study are summarized as follows:

In Chapter I, the author performed the screening of *N*-substituted formamide- and isonitrile-degrading microorganisms from soil samples through an acclimatization culture, resulting that thirteen microorganisms were isolated. From the HPLC analysis, these isolates were proposed to contain an enzyme which catalyzes the hydrolysis of N-benzylformamide (NBFA), wichi is a kind of *N*-substituted formamides, to produce benzylamine and formate. Based on this finding, the enzyme was designated as "*N*-substituted formamide deformylase (NfdA)". Of these isolates, strain F164 identified as *Arthrobacter pascens* exhibited the highest activity for NBFA degradation. This strain was also found to be able to degrade benzyl isocyanide into the corresponding *N*-substituted formamide, NBFA. Thus *A. pascens* F164 was chosen as the representative source to further study *N*-substituted formamide-degrading enzyme involved in isonitrile metabolism. The author then investigated the optimum culture conditions for the production of NfdA in this stain. The highest enzyme activity was obtained when this strain F164 was cultivated in a synthetic medium with NBFA as the sole nitrogen source. Moreover, it was also demonstrated that the enzyme is an inducible enzyme depending on NBFA.

In Chapter II, the author purified and characterized NfdA from *A. pascens* F164. The enzyme had a molecular mass of about 61 kDa and consisted of two identical subunits. It stoichiometrically catalyzed the hydrolysis of NBFA to yield benzylamine and formate. The substrate specificity of the enzyme is the highest for NBFA and different from known deformylases, including kynurenine formamidase, formylmethionine deformylase, peptide deformylase, 10-formyltetrahydrofolate deformylase, formamidase, *N*,*N*-dimethylformamidase, formylaspartate deformylase, and formylglutamate deformylase. Moreover, N-terminal amino

acid sequence of the enzyme showed no significant homology to ones of any other deformylases mentioned above. These findings demonstrated that NfdA is a novel deformylase for *N*-substituted formamides.

In Chapter III, cloning of the gene encoding NfdA enzyme (*nfd*A) from *A. pascens* F164 and genetic analysis of the *nfdA* gene were described. The deduced amino acid sequence of *nfdA* showed no homology with ones of any other deformylases described above and the less than 30% overall amino acid sequence identity with known proteins. Interestingly, the highest identities were observed with regulatory proteins, i. e. LAF3-1 and AepA. This finding in addition to the results described in Chapter II, supported that NfdA is a novel enzyme. Only the N-terminal region (residues 58-72) of NfdA also showed significant sequence identity (27-73%) to that of each member of the amidohydrolase superfamily, suggesting that NfdA would be belong to this superfamily. On the other hand, a heterologous overexpression system of *nfdA* gene from *A. pascens* F164 in *Streptomyces lividans* TK24 with the P_{nitA}-NitR system was established. The purified recombinant NfdA was enzymologically identical to the wild type enzyme. In addition, metal analysis initially revealed that the recombinant NfdA contains three zinc ion per subunit. In order to predict the metal-binding amino acid residues of NfdA, an alignment between NfdA and members of amidohydrolase superfamily including CodA, PyrC, and UreC was performed by using secondary structural prediction programs, PSI-Pred and PROFsec. As a result, it was revealed that all of the signature residues (four histidines and one aspartate), which are concerned with metal ions in this superfamily, are present in the predicted β strands of NfdA. This observation suggested that these five conserved amino acid residues would coordinate zinc ions, and concerned with the catalytic acitivity and stability of the enzyme structure.

Mutant analyses will elucidate whether these amino acid residues actually play such roles and three-dimensional structure of NfdA will provide the information to clarify the reaction mechanism.

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Publications

1. Fukatsu, H., Hashimoto, Y., Goda, M., Higashibata, H. & Kobayashi, M. "Amine-synthesizing enzyme *N*-substituted formamide deformylase: screening, purification, characterization, and gene cloning" ***Proc. Natl. Acad. Sci. USA***, **101**, 13726-13731 (2004).

2. Fukatsu, H., Goda, M., Hashimoto, Y., Higashibata, H. & Kobayashi, M. "Optimum culture conditions for the production of *N*-substituted formamide deformylase by *Arthrobacter pascens* F164" ***Biosci. Biotechnol. Biochem***., **69**, 228-230 (2005).

3. Fukatsu, H., Herai, S., Hashimoto, Y., Maseda, H., Higashibata, H. & Kobayashi, M. "High-level expression of a novel amine-synthesizing enzyme, *N*-substituted formamide deformylase, in *Streptomyces* with a strong protein expression system" ***Protein Exp. Purif***., **40**, 212-219 (2005).