JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 106, No. 2, 174–179. 2008
DOI: 10.1263/jbb.106.174

Novel Catalytic Activity of Nitrile Hydratase from *Rhodococcus* sp. N771

Kayoko Taniguchi,[1,2§] Kensuke Murata,[3] Yoshihiko Murakami,[2§§] Shunya Takahashi,[4] Takemichi Nakamura,[5] Koichi Hashimoto,[3] Hiroyuki Koshino,[4] Naoshi Dohmae,[5] Masafumi Yohda,[3] Takuji Hirose,[1] Mizuo Maeda,[2] and Masafumi Odaka[2,3]*

Department of Engineering, Saitama University, Sakura-ku, Saitama, Saitama 338-8570, Japan,[1] Maeda Bioengineering Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan,[2] Department of Biotechnology and Life Science, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Koganei, Tokyo 184-8588, Japan,[3] Molecular Characterization Team, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan,[4] and Biomolecular Characterization Team, RIKEN, Wako, Saitama 351-0198, Japan[5]

Received 29 January 2008/Accepted 22 May 2008

Nitrile hydratase (NHase) from *Rhodococcus* sp. N771 is a non-heme iron enzyme catalyzing the hydration of various nitriles to the corresponding amides. We report a novel catalytic activity of NHase. When NHase was incubated with an large excess of commercially available isovaleronitrile, the charge transfer band from the sulfur ligand to the Fe atom shifted from 710 nm to 820 nm, but recovered within 4 min. Similar UV-Vis absorption changes were observed after the addition of isobutylisonitrile (*i*BuNC), a major impurity in commercially available isovaleronitrile, suggesting that NHase catalyzes the conversion of *i*BuNC to other compounds. The reaction product was identified as isobutylamine (*i*BuNH$_2$) by liquid chromatography tandem mass spectrometry. NHase also converts *t*-butylisonitrile and 1,1,3,3,-tetramethylbutylisonitrile to the corresponding amines. Kinetic analysis of the conversion of *i*BuNC to *i*BuNH$_2$ showed a K_m value comparable to that for nitriles, while the V_{max} value was more than 10^5 times smaller than that for methacrylonitrile. This is the first report suggesting that NHase is a bifunctional enzyme catalyzing a reaction other than the hydration of nitriles.

[**Key words:** nitrile hydratase, non-heme iron, isonitrile, amine, bifunctional enzyme]

Nitrile hydratase (NHase, EC 4.2.1.84) catalyzes the hydration of nitriles to corresponding amides (1). The enzyme was first reported by Asano *et al.* (1) and was subsequently applied in the industrial production of acrylamide and nicotinamide (2, 3). NHase is the first example of a successful introduction of a bioconversion system for the manufacture of a chemical commodity. Recent studies have clarified that NHase is involved in the microbial degradation process of the aldoxime-nitrile pathway (4–7). Briefly, aldoxime dehydratase converts aldoxime to a corresponding nitrile, which is hydrated by NHase and subsequently hydrolyzed to carboxylic acid and ammonia by amidase. NHase is also attractive because of the unique structure of its metallocenter (8, 9). The enzyme consists of α and β subunits, has a molecular mass of 23 kDa, and contains non-heme iron (III) or noncorrin cobalt(III) at its catalytic center. The crystal structures of both Fe- (10–12) and Co- (13–15) type NHases have been elucidated. In all types, the overall structures are well conserved, including structures around the metal centers. The metal is bound to the metal-binding motif, Cys1-Xxx-Leu-Cys2-Ser2-Cys3 (Xxx is Ser1 in Fe-type NHase and Thr in Co-type NHase) of the α subunit. The metal retains a distorted octahedral geometry, and the ligand atoms are two main-chain amide nitrogens of Ser2 and Cys3, three cysteine sulfurs, and a solvent ligand. Among the three cysteine ligands, Cys2 and Cys3 are post-translationally modified to cysteine-sulfinic acid (Cys-SO$_2$H) and cysteine-sulfenic acid (Cys-SOH), respectively. Although these modifications are not identified in the crystal structures at relatively low resolutions (10, 14), this structure is likely common among Fe- and Co-type NHases, as well as in the related protein, thiocyanate hydrolase (16, 17). We reconstituted unmodified NHase of *Rhodococcus* sp. N771 from recombinantly expressed subunits (18). Initially, it exhibited no enzymatic activity, but became activated following aerobic oxidation of αCys112 and αCys114 (corresponding to Cys2 and Cys3 in the motif), to Cys-SO$_2$H and Cys-SOH, respectively. These modifications appeared to be responsible for the catalytic reaction. To characterize NHases, various experiments using spectroscopic techniques, including UV-Vis absorption (9, 19), electron spin resonance (8, 9, 20), Fourier-transform

* Corresponding author. e-mail: modaka@cc.tuat.ac.jp
phone/fax: +81-(0)42-388-7479
Present address: §Eco-Soft Material Research Units, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan and §§Department of Applied Chemistry, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Koganei, Tokyo 184-8588, Japan.

infra-red (21–23), resonance Raman (9, 24, 25), X-ray absorption (9, 26), and Mössbauer (20) spectroscopy have been performed, but the catalytic mechanism remains poorly understood.

Although most NHases show broad substrate specificities, there are no known reports that NHase catalyzes a substrate other than nitriles. Interestingly, two aliphatic nitriles, isobutyronitrile (IBN) and isovaleronitrile (IVN), function as competitive inhibitors for Fe-type NHases (27, 28). Previously, we studied the interaction between IBN and NHase, and reported that the inhibitor was not IBN, but was 2-cyano-2-propyl hydroperoxide (Cpx), a contaminating substance in commercially available IBN (29). We reported that Cpx irreversibly inactivated Fe-type NHase from *Rhodococcus* sp. N771 by oxidizing Cys114-SOH to Cys-SO_2H, and concluded that since no other residues were modified, Cys114-SOH was most likely involved in the catalytic reaction. Based on our study on the interaction between IVN and Fe-type NHase of *Rhodococcus* sp. N771, we found that NHase catalyzes the conversion of isobutylisonitrile to isobutylamine. This represents the first report suggesting that NHase possesses a novel catalytic activity to convert isonitriles to their corresponding amines.

MATERIALS AND METHODS

Materials NHase from *Rhodococcus* sp. N771 can be inactivated by nitrosylation in the dark and re-activated by photo-denitrosylation (30). Since the nitrosylated NHase is very stable, native NHase was purified as the nitrosylated form in the dark, as described previously (19). The purified NHase was stored in the nitrosylated state in a 60% saturated ammonium sulfate suspension in the dark. Prior to use, the nitrosylated NHase was dissolved in an appropriate buffer, desalted using Centriprep-30 (Millipore, USA) and denitrosylated by irradiation with 5000 lx of white light (71.0 W/m^2) from a 500 W spot photoreflector lamp (Toshiba, Tokyo) for 15 min in an ice bath. NHase concentration was determined by measuring the absorbance at 280 nm (ε_{280}=1.5 ml·mg^{-1}·cm^{-1}) after photodissociation of nitric oxide. High grade isovaleronitrile, *t*-butylisonitrile (*t*BuNC), 1,1,3,3,-tetramethylbutylisonitrile, cyclohexylisonitrile, isobutylamine (*i*BuNH_2), *t*-butylamine, and 1,1,3,3,-tetramethylbutylamine were purchased from Tokyokasei (Tokyo) or from Sigma (St. Louis, MO, USA). Isobutylisonitrile (*i*BuNC) was prepared as reported previously (31).

UV-Vis absorption spectra of NHase in the presence of nitrile or isonitrile compounds Photo-denitrosylated NHase was dissolved in 50 mM Tris–HCl, pH 7.5, containing 40 mM *n*-butyric acid. Nitrile or isonitrile compounds were added to the NHase solution at the indicated concentrations. UV-Vis absorption spectra were recorded with a UV-Vis spectrophotometer (Cary50; Varian, Palo Alto, CA, USA). In case of time course measurement, data collection started at the indicated times.

Reaction product identification after incubation of isonitrile compounds Photo-denitrosylated NHase was dissolved in 50 mM Tris–HCl, pH 7.5. The concentrations of NHase used were 2.9 μM and 60 μM for *i*BuNC and *t*BuNC, respectively. The reaction was started by the addition of isonitrile compounds at a final concentration of 40 mM. After incubation for a specific time at room temperature, 150 μl was sampled and the NHase was removed by centrifugal ultrafiltration using a Microcon10 (Millipore). The filtrate was diluted ten times with acetonitrile and analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS). The normal phase LC (hydrophilic interaction chromatography, HILIC) was operated at a flow rate of 200 μl/min on a ZIC^{TM}-HILIC column (2.1×150 mm; SeQuant, Sweden) connected to a model 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Solvent A was 20 mM ammonium acetate in water and solvent B was 100% acetonitrile. The column was equilibrated with 10% solvent A and 90% solvent B prior to sample injections. After each injection, the initial mobile-phase composition was maintained for 3 min before a linear gradient was started; the solvent B content in the mobile phase was then decreased from 90% to 20% over 15 min. At the end of 18 min, the linear gradient was obtained and the final mobile-phase composition was held for 5 min before column re-equilibration. The eluate was directly introduced to a 4000 QTRAP triple quadrupole ion trap mass spectrophotometer (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization probe. The spectrometer was operated in both Q3 (regular mass spectra) and product ion (MS/MS) scan modes. In the MS/MS mode, precursor ions were isolated at Q1, subjected to collision induced dissociation in Q2 (nitrogen collision gas; 25 eV collision energy), and the product ions were analyzed by Q3 scanning.

Kinetic analyses of the conversion of isonitriles to the corresponding amines Photo-denitrosylated NHase was dissolved in 50 mM sodium phosphate, pH 7.5, to a concentration of 3.2 μM. Twenty microliters of each isonitrile solution at the concentration of 0–4 mM was added to 20 μl of the NHase solution and incubated for 10 min at room temperature. The reaction was terminated by centrifugal ultrafiltration using a Microcon10 (Millipore). Twenty microliters of the flow-through fraction was mixed with the equivalent volume of 20 mM 4-fluoro-7-nitrobenzofurazan (NBD-F) and incubated at 60°C for 1 min. The reaction was terminated by the addition of 60 μl of 1 M HCl, and the solution was analyzed using a reversed phase HPLC at a flow rate of 1.0 ml/min on ODS-80TS (4.6×150 mm; Tosoh, Tokyo), connected to a Waters 2690 HPLC system with a fluorescent detector (Waters, Milford, MA, USA). Solvent A was CH_3CN/75 mM H_3PO_4 (16/84) and solvent B was CH_3CN/MeOH/50 mM KH_2PO_4 (21/39/40). The column was equilibrated with 100% solvent A. The content of solvent B was increased as follows: 0 to 3 min, 0%; from 3 to 23 min, 80%; from 23 to 24 min, 100%; from 24 to 31 min, 100%, from 31 to 36 min, 0%. The excitation wavelength was set at 470 nm, and the emission at 540 nm was monitored. The calibration curve was examined using the authentic *i*BuNC. A single unit of the enzymatic activity converting *i*BuNC to *i*BuNH_2 was defined as the quantity of NHase that produces 1 μmol of *i*BuNH_2 per minute.

Other methods Kinetic studies of NHase for the methacrylonitrile hydration were performed as described previously (18). A single unit of NHase activity hydrating methacrylonitrile to methacrylamide was defined as the quantity of NHase that produces 1 μmol of methacrylamide per minute. The inhibitor value K_i, of isobutylisonitrile for NHase was determined by Dixon plot (32).

RESULTS AND DISCUSSION

NHase specifically reacts with isobutylisonitrile, an impurity of commercially available isovaleronitrile

The interaction between Fe-type NHase of *Rhodococcus* sp. N771 and commercially available IVN was examined by measuring UV-Vis absorption spectra. NHase exhibited an absorption peak at 710 nm, the charge transfer band from a sulfur ligand to the Fe atom (red curve in Fig. 1A) (25). When commercially available IVN was added at a final concentration of 100 mM, the absorption peak shifted to approximately 820 nm, and recovered within a few minutes (Fig. 1A). These results suggested that IVN reacted very