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Biochemical properties of a new nitrile reductase cloned from *Pectobacterium carotovorum*

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ABSTRACT

Nitrile reductase is a newly discovered enzyme class that can catalyze the reduction of nitriles directly to amines, a very important reaction in synthetic organic chemistry. However, little progress has been made towards the application of this biocatalysis reaction for organic synthesis since the initial discovery of nitrile reductase for over ten years. One of the potential reasons may be due to the lack of structural information and biochemical properties, in addition to its inherent narrow substrate scope. Meanwhile, the highly conserved active site residues make it a great challenge to widen the substrate spectrum of nitrile reductase by means of protein engineering approaches. To explore more nitrile reductases with different characteristics (*e.g.* specific activity, thermostability, *etc*) for potential synthetic applications, a new nitrile reductase, designated as *Pc*NRed, was discovered from the genome of *Pectobacterium carotovorum*, with a moderate specific activity of 366 U/g protein towards its natural substrate, $preQ_0$. The *Pc*NRed was subsequently overexpressed in *Escherichia coli* BL21 (DE3), purified to homogeneity and then its biochemical properties were examined. The optimal reaction temperature and pH were determined to be 40 °C and 7.4, respectively. The thermal deactivation process of *Pc*NRed obeys first-order kinetics, with half-lives of 47.2, 13.6 and 3.9 h measured at 30, 40, and 50 °C, respectively. The kinetic constants were also determined with respect to both of the substrates NADPH and $preQ_0$, giving k_{cat} of 0.025 s^{-1} and 0.024 s^{-1}, and K_M of 0.229 mM and <0.25 μM. To shed some light on the structural information of *Pc*NRed, a homology model was built and compared with its counterparts, which is helpful for further engineering of this novel but less-elucidated enzymes.

1. Introduction

Nitrile compounds are important building blocks for the synthesis of a variety of pharmaceuticals, agrochemicals, fine chemicals, as well as bulk chemicals. Traditional biocatalysis approaches to nitrile chemistry comprise mainly four enzyme groups, including nitrilases, nitrile hydratases, nitrogenases, and oxygenases [1]. Of them, nitrilases and nitrile hydratases have been successfully employed as biocatalysts for the production of carboxylic acids and amides, respectively, from their nitrile precursors at industrial scale [2,3]. Besides these four nitrile-converting enzyme types, hydroxynitrile lyases represent another important group of biocatalysts involved in nitrile transformation, specifically in the synthesis of chiral cyanohydrins, which has also been running at commercial scale [4]. Very recently, a novel enzyme family involved in nitrile metabolism was discovered, which was found to catalyze the direct reduction of a nitrile group to a primary amine, specifically the NADPH-dependent reduction of 2-amino-5-cyanopyrrolo[2,3-*d*]pyrimidin-4-one ($preQ_0$) to the corresponding amine, 2-amino-5-aminomethylpyrrolo[2,3-*d*]pyrimidin-4-one ($preQ_1$), during the biosynthetic pathway of hypermodified nucleosides present in transfer RNA (tRNA) (Scheme 1) [5–8]. The environmentally benign and highly selective advantages of nitrile reductase-mediated reduction of a nitrile group to a primary amine would pave the way for developing environmentally sustainable nitrile-reducing biocatalysts to replace metal hydride catalysts since the traditional chemical approach always requires harsh reaction conditions, such as high pressures, high temperatures and the use of dangerous chemicals [9–12]. Meanwhile, the unsatisfactory enantioselectivity and high cost of chemical reduction will also result in low economic effectiveness. All of the characteristics mentioned above highlight the great interest in this emerging class of enzymes.

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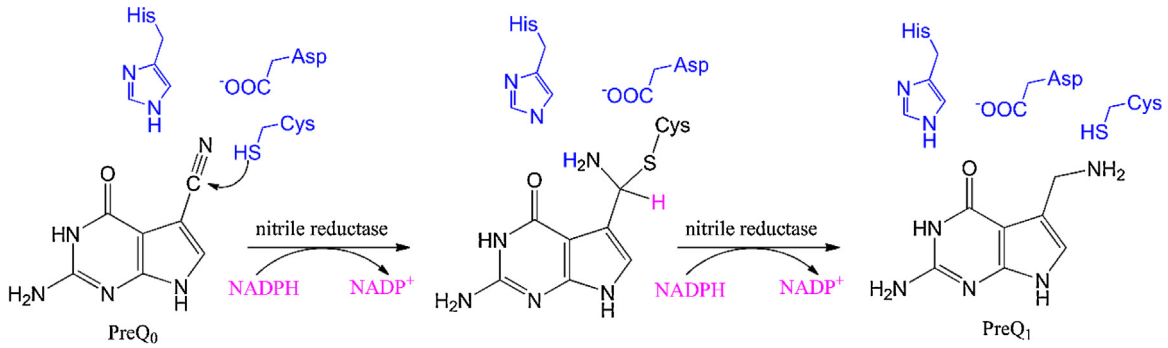

Scheme 1. Nitrile reductase-catalyzed reduction of 7-cyano-7-deazaguanine (preQ$_0$) to 7-aminomethyl-7-deazaguanine (preQ$_1$).

Unfortunately, although this novel enzyme family has been discovered for over ten years, little progress has been made towards the application of this unique enzyme for organic synthesis except the elucidation of its crystal structure and reaction mechanism. One important reason for this is the inherent narrow substrate scope of nitrile reductases and the highly conserved active site residues make it a great challenge to expand its substrate spectrum by protein engineering. Hence, the discovery of new nitrile reductases with different characteristics (*e.g.* specific activity, thermostability, *etc*) would provide versatile starting points for further engineering to obtain industrial applicable biocatalysts. Here we report the molecular cloning, overexpression and biochemical characterization of a new nitrile reductase (*Pc*NRed) from *Pectobacterium carotovorum*. The biochemical properties of *Pc*NRed were systematically investigated, and a homology model was also constructed and compared with its counterparts to shed some light on the structure of *Pc*NRed.

2. Materials and methods

2.1. Chemicals and strains

2-Amino-5-cyanopyrrolo[2,3-*d*]pyrimidin-4-one (preQ$_0$) was obtained from Accela ChemBio Co., Ltd. (Shanghai, China). NADPH (sodium salt; >95% purity) was purchased from Aladdin (Shanghai, China). All other chemicals were also obtained commercially and used without further purification.

The microbial strains used for genome mining were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and China General Microbiological Culture Collection Center (CGMCC). *Escherichia coli* DH5α and *Escherichia coli* BL21 (DE3) were used for cloning and expression of nitrile reductases.

2.2. Cloning and expression of PcNRed gene in Escherichia coli

Genomic DNA was extracted from *Pectobacterium carotovorum*, using TIANamp Bacteria DNA Kit from Tiangen (Shanghai, China). Oligonucleotide primers with *Bam*H I and *Hind* III restriction sites were designed according to *Pc*NRed gene squence (GenBank Accession No. CP003776.1). The DNA fragment of *Pc*NRed gene was amplified and digested with *Bam*H I and *Hin*d III and then inserted into the expression vector pET-28a (Novagen, Shanghai). The resulting plasmid, pET-28a-*Pc*NRed, was then transformed into *Escherichia coli* BL21 (DE3) competent cells. The cells were cultivated at 37 °C in LB medium containing 50 μg/ml kanamycin. When the OD_{600} of the culture reached 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, and the cells were cultivated continuously at 16 °C for another 24 h.

2.3. Purification of PcNRed

Cells were harvested by centrifugation, washed twice with saline and resuspended in buffer A (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 10 mM imidazole) and disrupted with an ultrasonic oscillator (JY92-II, Scientz Biotech. Co., Ltd). The cell debris was removed by centrifugation (60 min at 9300*g*) at 4 °C. Then the supernatant was loaded onto a His trap Ni-NTA FF column (1 ml, GE Healthcare Corp.) pre-equilibrated with buffer A, and the proteins were eluted with an increasing gradient of imidazole from 20 mM to 500 mM in buffer A at a flow rate of 1 ml/min. The purity of fractions was estimated by SDS-PAGE. The fractions containing the target protein were combined and dialyzed against 20 mM sodium phosphate buffer (pH 7.4) for desalting. Finally, the enzyme sample was concentrated and stored at −80 °C with 20% glycerol for further use.

2.4. Enzyme assay

*Pc*NRed activity was assayed spectrophotometrically at 30 °C by recording the decrease in the absorbance of NADPH at 340 nm for a defined period of time (usually 300 s). The reaction mixture was composed of 0.1 M Tris-HCl (pH 7.4), 0.1 mM NADPH, 0.1 mM preQ$_0$, and an appropriate amount of enzyme in a total volume of 1 ml. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH per minute under the standard assay conditions.

2.5. pH and temperature optima and thermostability

The optimum pH of *Pc*NRed was determined with the following buffers (100 mM): sodium citrate (pH 4.0 – 6.0), sodium phosphate (pH 6.0 – 7.5), Tris-HCl (7.5–9.0), Na_2CO_3—$NaHCO_3$ (9.0–10.0). The optimum temperature was determined under the standard assay conditions at a range of temperatures from 20 °C to 70 °C. Thermal stability was examined by measuring the residual activity of *Pc*NRed towards preQ$_0$ after incubating the purified enzyme (1 mg/ml) at the desired temperatures (30, 40, and 50 °C) for a required period. All the assays were performed in triplicate.

2.6. Effects of metal ions and EDTA on PcNRed activity

Influence of various metal ions and EDTA on *Pc*NRed activity was investigated by including each compound (final concentrations, 10 μM and 100 μM) in the reaction mixture at 30 °C, and the enzyme activity was measured under the standard assay conditions. Control experiments were performed in the absence of any tested compound. All the assays were performed in triplicate.