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Expression and characterization of the nitrile reductase queF from *E. coli*

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ABSTRACT

The expression and characterization of a nitrile reductase from *Escherichia coli K-12* (EcoNR), a newly discovered enzyme class, is described. This enzyme has a potential application for an alternative nitrile reduction pathway. The enzyme activity towards its natural substrate, preQ$_0$, was demonstrated and optimal working conditions were found to be at 37 °C and at pH 7 with Tris buffer.

1. Introduction

Lee et al. recently reported the discovery of a new protein family which is involved in the nitrile metabolism [1]. The enzyme queF was identified as a biological nitrile reductase (NR) [1–5]. It was found that this enzyme catalyses the reduction of 7-cyano-7-deazaguanine (preQ$_0$) to the corresponding amine 7-amino-methyl-7-deazaguanine (preQ$_1$) during the biosynthesis of queuosine (Scheme 1). In this reaction queF is responsible for the reduction of the nitrile group of preQ$_0$ to the corresponding amine; an unique reaction in nature [1–6]. The enzyme uses NADPH as a cofactor to deliver all four electrons in the form of two hydride ions [1,2,4,6]. The intermediate imine does not leave the enzyme, resulting in high chemoselectivity [1,2,4]. In this it is very different from the chemical reduction, where the imine intermediate can lead to undesired side reactions [7,8]. Indeed it has very recently been shown for the NR from *Bacillus subtilis* that a covalent intermediate is formed [9].

Additionally the catalytic chemical reduction of nitriles to amines often required harsh reaction conditions like high pressures and temperatures [8,10–14]. Therefore this new enzyme is of great interest. In order to explore the possibilities that NR from *Escherichia coli* offers we cloned, overexpressed and characterized it.

2. Materials and methods

2.1. ^{1}H NMR and ^{13}C NMR spectra

^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz with a Bruker Avance 400 NMR spectrometer and at 300 MHz with Varian Inova-Unity 300 NMR spectrometer. The corresponding solvent signals were used as internal standard. For ^{1}H NMR DMSO-D6: 2.50 ppm and MeOD: 3.31 ppm. For ^{13}C NMR DMSO-D6: 39.52 ppm and MeOD: 49.00 ppm. NMR chemical shifts are given in the δ-scale (ppm) and the coupling constant J in Hz. The acronyms for spin multiplicity are: *s*: singlet; *d*: doublet; *t*: triplet; *q*: quartet and *m*: multiplet. UV–vis measurements were performed with the UV/Vis spectrometer UV-2401 PC from Shimadzu Corporation. Temperature settings were done with a Julabo type F12 thermostat.

2.2. Construction and expression of BsubNR and EcoNR expression vectors

Two candidate genes for nitrile reductases were chosen based on NCBI database, NP_389258 (from *B. subtilis*) (BsubNR) and NP_417274 (from *E. coli*) (EcoNR) (see Supporting Information). The synthetic genes sub-cloned into plasmid pET-30a(+) were obtained from GeneArt (a division of Life Technologies Corporation). The recombinant plasmids were transformed into chemocompetent *E. coli* BL21 (DE3) cells and plated onto lysogeny broth (LB) agar plates containing kanamycin (30 μg mL^{-1}). Colonies from LB agar plates were picked, plasmids purified and sent to sequencing service BaseClear for sequence validation.

500 mL cultures of transformed *E. coli* BL21 (DE3) cells were grown in LB media containing kanamycin 30 μg mL^{-1} at 37 °C until $OD_{600\,nm}$ reached 0.5. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was added into the medium for induction, followed by an overnight incubation at 17 °C. Co-expression with chaperones was carried out following the instruction of the Takara kit manual (Takara Bio Inc.) Cells were harvested by centrifugation (4 °C, 30 min, 7000 × *g*) and the cell pellet was resuspensed in 50 mM phosphate buffer (pH 7.5). Bug Buster (Novagen) solution was applied for cell lysis and the cell debris was removed by centrifugation (4 °C, 30 min, 7000 × *g*).

EcoNR could be purified using Ni-NTA spin column (Qiagen) following the protocol in the product manual. The purified enzyme was active against preQ$_0$.

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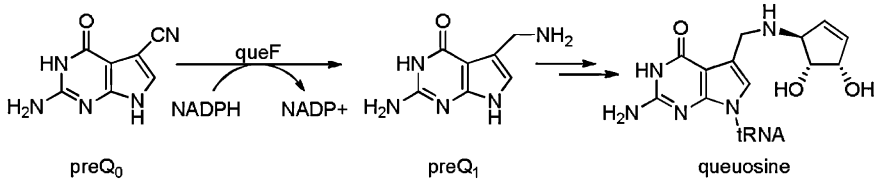


Scheme 1. Reaction catalysed by queF in the queuosine pathway [4].

2.3. Synthesis of chloroformylacetonitrile

Under nitrogen atmosphere and at 0 °C methyl formate (4.38 g; 4.52 mL; 73 mmol) was added to a stirred mixture of sodium methoxide (3.57 g; 66 mmol) in THF (60 mL). Then chloroacetonitrile (5.0 g; 4.17 mL; 66 mmol) was added dropwise over a period of 1 h. The mixture was allowed to stir for an additional 3 h. Afterwards 12 M HCl (6.07 mL) was added dropwise at 0 °C. The solvent was reduced by 50% in vacuo and the resulting slurry was used as obtained for the next reaction step [15,16].

2.4. Synthesis of the natural substrate preQ0

Sodium acetate (14 g; 170 mmol) was dissolved in water (110 mL) and 2,6-diaminopyrimidin-4-one (6.66 g; 53 mmol) was added. Then the reaction mixture was heated to 50 °C while chloroformylacetonitrile was added over a period of 1 h. The reaction mixture was stirred for 18 h. Afterwards THF was removed at reduced pressure before the mixture was refluxed at 110 °C for 1 h. Then the reaction mixture was allowed to cool to room temperature and the precipitated solid was filtered off, washed with copious amounts of ice water and redissolved by converting it into its potassium salt with 6 N KOH. After treatment with charcoal, the mixture was filtered over Celite and the solution was brought to pH 6 with conc. HCl. The precipitate was filtered and dried in a desiccator to yield 6.77 g (38.7 mmol, 73%) of the brown to yellow product [15,16]. ^{1}H NMR (DMSO): δ [ppm] = 11.95 (bs, 1H, 2-H), 10.71 (bs, 1H, 5-H), 7.55 (s, 1H, 6-H), 6.39 (s, 2H, NH_2). ^{13}C NMR (DMSO): δ [ppm] = 157.74 (C-1), 153.82 (C-3), 151.73 (C-4a), 127.86 (C6), 115.96 (CN), 98.82 (C-7a), 85.63 (C-7).

2.5. QueF activity assay

The activity of queF in *E. coli* crude extracts was analysed using UV–vis-spectroscopy, as described by Lee et al. [1]. A cuvette was filled with 1 mL of an aqueous solution containing 0.1 M KCl, 0.1 M Tris (pH 7.5), 1 mM Dithiothreitol (DTT), 0.1 mM NADPH and 20 μM preQ$_0$. The enzyme activity was monitored at 37 °C by recording the absorption of light at the wavelength of 340 nm for a defined period of time (usually 120–300 s). This wavelength was chosen because it is a characteristic absorption maximum of NADPH. With the molar extinction coefficient of 6.22 $\mu mol^{-1}\ cm^{-1}$ at 340 nm [17] the change in absorption can be converted into the change of concentration. In the absence of natural substrate blank measurements were carried out.

2.6. Determination of the effects of temperature and pH on queF activity

Reactions were performed by varying the temperatures between 25 and 50 °C while the other parameters stayed constant. The typical composition of the reaction mixture was 0.1 M KCl, 0.1 M Tris (pH 7.5), 1 mM DTT, 0.1 mM NADPH, 20 μM preQ$_0$ [1]. In addition, reactions at all temperatures were done with varied enzyme concentrations (15 μL, 30 μL, 45 μL, 60 μL, 75 μL crude extract). To investigate the pH dependence the same reaction conditions and enzyme concentrations were applied at 37 °C. The only parameter that changed was the pH, which was tuned by the application of different buffer systems. For pH 6–7 sodium phosphate monobasic monohydrate buffer and for pH 7–9 Tris buffer was used. Both buffers were used at the same concentration (0.1 M). At pH 7 reactions with both buffers were performed to rule out significant differences between the enzyme activity in both systems. The change in light absorbance at 340 nm was followed for 120 s and fitted with a linear function.

2.7. Determination of the temperature and pH stability on queF activity

For the determination of temperature stability one standard reaction mixture with 0.1 M KCl, 0.1 M Tris (pH 7.5), 1 mM DTT, 0.1 mM NADPH, 20 μM preQ$_0$ [1] and 50 μL crude enzyme extract was incubated at different temperatures (37, 40, 45 and 50 °C) over a period of 4 h without NADPH and substrate. Every 30 min a sample was taken and the activity was tested upon addition of cofactor and substrate. The measurement was done according to the procedure described above at 25 °C. To investigate the pH stability the enzyme was incubated at 37 °C (with buffer, KCl and DTT) and different pH values for 4 h. Every 30 min samples were taken and the remaining activity was measured after addition of NADPH and substrate (25 °C).

2.8. Substrate specificity

Substrate specificity was assayed by using three different, non-natural nitriles. The three nitriles were acetonitrile, benzonitrile and benzyl cyanide at a concentration of 20 μM. The screening was started with an enzyme concentration of 75 μL (174.7 U mL^{-1}) and was increased stepwise to 500 μL (1162.7 U mL^{-1}).

2.9. Determination of K_M and k_{cat}

Purified EcoNR was used for the determination of K_M and k_{cat}.

For the determination of the K_M value of NADPH a cuvette was filled with 1 mL of an aqueous solution containing 0.1 M KCl, 0.1 M Tris (pH 7) and 1 mM DTT. Studies were done with a preQ$_0$ concentration of 70 μM. Measurements with different concentrations of the cofactor between 5 and 300 μM were performed. The volume of added purified EcoNR (3.38 mg/mL) was 15 μL. This low concentration of enzyme was used to ensure a slow enough reaction and a more accurate monitoring of the NADPH consumption at low concentrations. The absorption of NADPH at 340 nm was monitored by using UV–vis-spectroscopy at 37 °C, the measuring time was 120 s.

For the determination of the K_M value of preQ$_0$ the same conditions were chosen as for the determination of the K_M of NADPH (0.1 M KCl, 0.1 M Tris (pH 7), 1 mM DTT). This time the concentration of NADPH was kept constant at a value of 100 μM and the substrate concentration was varied from 0.1 to 70 μM. The enzyme solution (6.65 mg/mL) was diluted 60 times with a solution containing 0.1 M KCl and 0.1 M Tris (pH 7) to reduce the activity to a reasonable value. The volume of added diluted EcoNR in the reaction mixture was 15 μL. The absorption of NADPH at 340 nm was monitored by using UV–vis-spectroscopy at 37 °C for 120 s.

2.10. Homology model of EcoNR

A protein sequence comparison using BLAST as a tool provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed a high sequence identity (>60%) of EcoNR with a nitrile reductase from *Vibrio cholera* whose structure is published (PDB ID: 3BP1) [2,3]. This provides a good basis for building a homology model of EcoNR to gain more insights into the enzyme's structure as structural information of EcoNR. The model was built by YASARA structure software (version 11.9.18) using its built-in function and default parameters. The model was visualized using PyMOL software.

3. Results

The nitrile reductase from *E. Coli* (EcoNR), could be obtained in the soluble fraction (Fig. 02, Supporting information) and the activity towards preQ$_0$ was confirmed through the activity assay. The nitrile reductase observed from *B. subtilis* (BsubNR) was expressed mostly in the insoluble fraction (Fig. 01, Supporting Information) and efforts to increase the amount of soluble protein, like temperature variations, co-expression with chaperons etc., failed. The soluble fraction, which was obtained, displayed no activity towards preQ$_0$, the natural substrate (Fig. 05, Supporting Information). Therefore, EcoNR was chosen for further investigation.

3.1. Temperature dependence

Temperature dependence studies (Fig. 1) show that the relative initial rate is increasing linearly with increasing temperature up to 50 °C. The values of the specific activity for every temperature (in U/mg) were measured at several different protein concentrations. No significant differences in specific activity could be found, so that all the measurements were taken to calculate the average and the standard deviation (Fig. 1).

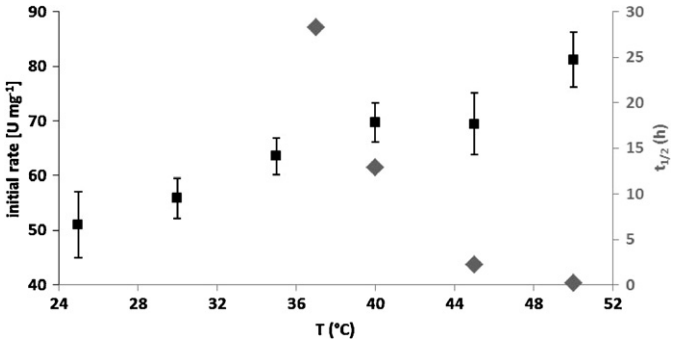


Fig. 1. Half life time (♦) and the initial rate (■) of the enzyme crude extract depending on the temperature in Tris buffer (pH 7.0).

3.2. Temperature stability

The temperature stability was investigated by incubation of the EcoNR at different temperatures. The results are summarized in Fig. 1. Below 40 °C there was only a slight reduction in activity observable within 4 h. On the other hand, the samples incubated at 45 °C and 50 °C showed a significant decrease of enzyme activity over time. The 37 °C and the 40 °C samples retained over 85% of their initial activity over the 4 h period resulting in a half life time ($t_{1/2}$) of 28.2 and 12.8 h, respectively. A further increase by 5 °C led to a considerable decrease in activity, with a $t_{1/2}$ of around 2.8 h. At 50 °C the half life time dropped to 6 min. This might in part also be due to the known temperature dependence of the Tris buffer.

3.3. pH dependence

The pH dependence was investigated in a range from pH 6 to 9. Sodium phosphate monobasic monohydrate buffer and Tris buffer at concentrations of 0.1 mol/L were used for the range of pH 6–7 and 7–9, respectively. The highest activity was obtained at pH 7, in both buffer systems. For easier evaluation the activity at pH 7 in Tris buffer was normalized to 100% and the other activities are expressed in relation to this value (Fig. 2).

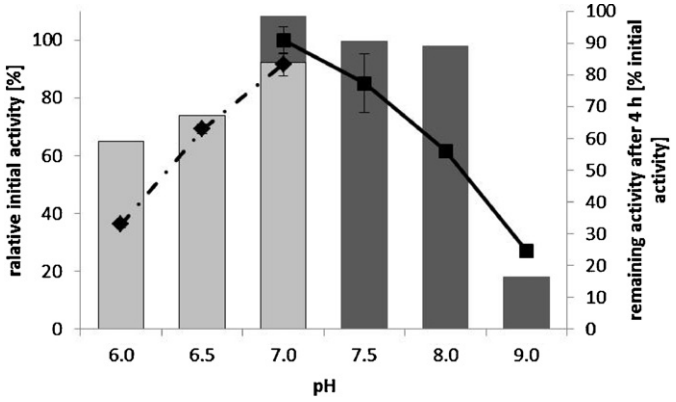
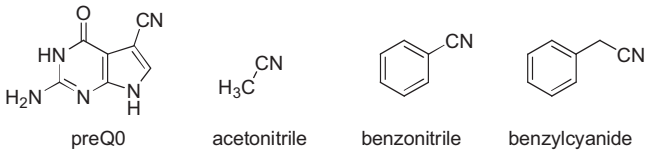


Fig. 2. Initial activity of the enzyme in phosphate (♦, dashed line) and Tris buffer (■, solid line) depending on pH. Error bars represent the standard deviation of 5 measurements. pH 6–7 was measured in phosphate buffer, pH 7–9 in Tris. 100% activity in Tris buffer = 58.1 U mg^{-1}. Columns represent the remaining activity after 4 h of incubation at different pH values. The columns at pH 7 represent the two different values, for Tris buffer and for phosphate buffer. Results of measurements in phosphate buffer are displayed in light grey and results in Tris buffer are shown in dark grey.



Scheme 2. Substrates used for the activity screening.

3.4. pH stability

To investigate the influence of the pH value on the activity, the enzyme was incubated over a period of 4 h. No decrease of activity was observed at pH 7, whereas at pH 9 less than 20% of the initial activity was retained.

3.5. Substrate scope

The preliminary screening for more substrates was performed with three other nitriles. Acetonitrile, benzonitrile and benzylcyanide were used as representatives for aliphatic, aromatic and benzylic substrates (Scheme 2). However, no activity could be observed, even with higher enzyme concentrations (Table 1).

3.6. K_M and k_{cat} of NADPH and preQ$_0$

The determination of K_M for the cofactor NADPH was difficult, caused by limitations in monitoring the enzyme activity at cofactor concentrations smaller than 5 μM. The data obtained give a value of <0.2 μM for K_M, possibly even smaller. A k_{cat} of 0.0033 s^{-1} was determined.

The determination of K_M for the natural substrate preQ$_0$ was again limited by substrate concentrations smaller than 0.5 μM. A value of <1.5 μM and a k_{cat} of 0.1268 s^{-1} were estimated. Limitations in the measuring procedure were already reported before by Lee et al. [1] In contrast to the earlier descriptions of K_M the here determined values are lower for NADPH. All previous publications report a significant difference between the K_M values of the substrate and the cofactor, the one of the substrate preQ$_0$ being 3–80 times lower [1,3,6].

4. Discussion

The recently described nitrile reductase is a biocatalyst with a great potential for nitrile reduction to amines under more environmentally friendly conditions. This new class of enzymes has not been widely investigated and not many enzymes have been described. Therefore, the characterization of the nitrile reductase from *E. coli* will provide more insights about this enzyme class as well as basic knowledge for further understanding.

The results of the temperature dependence study displayed a linear trend of increased enzyme activity when the reaction temperature varied from 25 °C to 50 °C. This trend is different than an expected exponential change as observed by Wilding et al. [3] with a nitrile reductase from *Geobacillus kaustophilus*. The *G. kaustophilus* NR is derived from a thermophilic organism, which results in a high temperature stability. In contrast the here described EcoNR is much

Table 1
Screening results of preQ$_0$, acetonitrile, benzonitrile and benzyl cyanide at pH 7 in Tris buffer, 37 °C and a measuring period of 120 s.

Substrate	Spec. activity [U mg^{-1}]
PreQ$_0$	58.1
Acetonitrile	0
Benzonitrile	0
Benzyl cyanide	0

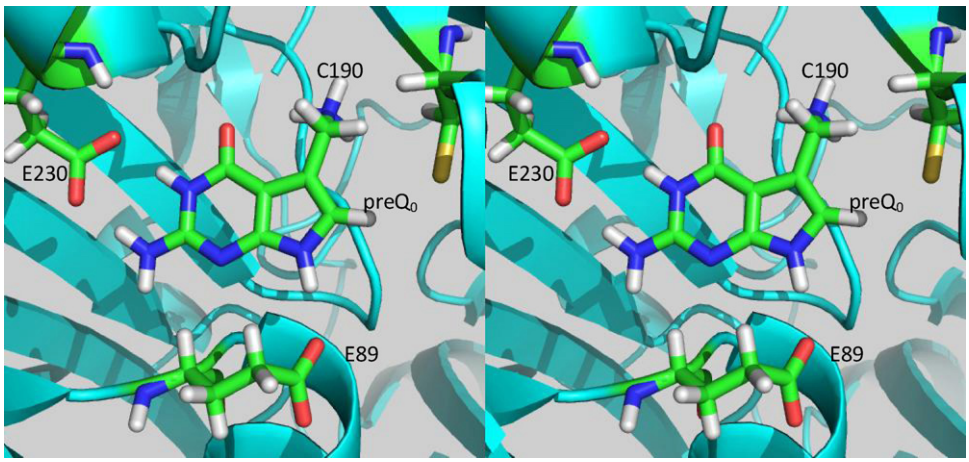


Fig. 3. Stereo view of the homology model of EcoNR using the YASARA structure software with $preQ_1$ in the active site. The picture was created by PyMOL software (http://www.pymol.org/). Active site residues and $preQ_1$ are labelled as such.

less temperature resistant and the exponential increase of the activity with temperature is superimposed by an increasing deactivation at elevated temperatures. If both effects are added up, the increase in initial activity is no longer exponential but rather linear.

The ability of EcoNR to retain its activity at different reaction temperatures starting from 37 °C and up to 50 °C (Fig. 1) is decreased as suggested from the results of the half life time study. Therefore, the follow-up studies of pH dependence and substrate scope were carried out at 37 °C.

The maximal activity of EcoNR was observed at pH 7 in the pH dependence study as shown in Fig. 2. A similar optimal pH range (7–8), was also observed in previous studies of Lee et al. [1] and Wilding et al. [3] with a nitrile reductase from *B. subtilis* and the one from *G. kaustophilus* (pH 7.5).

The substrate scope was explored to evaluate the potential of using wild type EcoNR for nitrile reduction in addition to its natural activity towards $preQ_0$. As shown in Table 1, EcoNR has a limited substrate scope as none of the tested nitriles could be converted. The nitrile reductase from *G. kaustophilus* and its variants in the study of Wilding et al. [3] also displayed a very limited activity towards other nitriles.

The homology model of EcoNR was built based on a structure with the PDB ID: 3RZP, which is a mutant of 3BP1 (C194A) complexed with $preQ_1$ in its active site (Fig. 3). Based on the homology model, residues in the active site of EcoNR, in this case, E230 and E89, are polar amino acids and help to accommodate polar compounds like $preQ_0$ and $preQ_1$ in the active site. These residues also support $preQ_0$ to orientate its nitrile group towards the catalytic residue C190 in mechanistic studies described by Kim et al. [2] and the recently described covalent intermediate [9]. Therefore, with non-polar compounds like benzonitrile, benzyl cyanide and acetonitrile, it would be difficult for the compounds to have interaction with residues in the active site of EcoNR.

In conclusion, a nitrile reductase from *E. coli* was expressed and characterized. The enzyme showed optimal working conditions at 37 °C and pH 7. Its activity towards the natural substrate $preQ_0$ was confirmed. Despite the narrow substrate scope, the preliminary data from enzyme characterizations may serve as a basis for further studies of nitrile reductases and their potential as a biocatalyst for an environmentally friendly nitrile reduction pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2012.12.003.

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