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Nitrile Reductase from *Geobacillus kaustophilus*: A Potential Catalyst for a New Nitrile Biotransformation Reaction

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Abstract: The cloning, expression and characterization of a nitrile reductase (NRed) from the thermophile *Geobacillus kaustophilus* is reported. The enzyme shows a 12-fold increase in activity in response to a temperature change from 25 °C to 65 °C. The substrate scope regarding its biocatalytic applicability was investigated by testing a range of common nitriles. The narrow substrate range observed for the wild-type enzyme prompted the rational design of *Gk*NRed active site mutants based on a previously published homology model from *Bacillus subtilis*. The activities of the mutants and the wild-type enzyme were investigated in their structure-function relationship regarding the natural substrate 7-cyano-7-deazaguanine ($preQ_0$) as well as a range of synthesized $preQ_0$-like substrate structures. A distinct dependence of the wild-type enzyme activity on specific structural modifications of the natural substrate was observed. Two non-natural nitriles derived from $preQ_0$ could be reduced to their corresponding amino compounds.

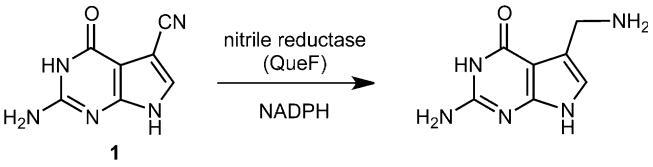
Keywords: active site mutants; amines; 7-cyano-7-deazaguanine reductase; enzyme catalysis; *Geobacillus kaustophilus*; nitrile reductase; nitriles; thermostability

Introduction

Recently, a novel enzymatic activity has been reported as part of the biosynthetic pathway to the modified nucleosides queuosine Q and archaeosine G^+. The nucleosides share an unusual 7-deazaguanine core structure and are among the most complex modified nucleosides in tRNA. They are ubiquitous in prokaryotes and eukaryotes (Q) and archaea (G^+). The new protein 7-cyano-7-deazaguanine reductase QueF involved in the nitrile metabolism catalyzes the NADPH-dependent reduction of the nitrile group to its corresponding amine and is so far the only enzyme known to be capable of reducing a nitrile group to the primary amine (Scheme 1). The genes from *B. subtilis* (*Bacillus subtilis*) and *E. coli* (*Escherichia coli*) were recently cloned and the recombinant enzymes were characterized.[1]

There are two related sequence subfamilies of QueF enzymes known: type I single domain enzymes (YkvM subfamily) found in *B. subtilis* and type II two domain proteins (YqcD subfamily) found in *E. coli*. Both proteins show sequence similarity to the family of GTP cyclohydrolases I.[1] Recently, a high resolution crystal structure together with molecular simulation studies of the NRed from *Vibrio cholerae* (a type II subfamily enzyme) was published providing further insight into the catalytic mechanism.[2]

Chemical nitrile reduction generally involves expensive or dangerous chemicals, such as $LiAlH_4$,



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Scheme 1. Reduction of 7-cyano-7-deazaguanine ($preQ_0$) to 7-aminomethyl-7-deazaguanine ($preQ_1$) depicted as substep from the biosynthetic pathway of queuosine.

borane or molecular hydrogen under high pressure in presence of Pt, Pd, Ru or Raney-Ni catalysts.[3]

Hence, a nitrile reductase would be a valuable contribution to the currently available biocatalytic tools of nitrile transforming enzymes (i.e., oxynitrilases,[4] nitrilases and nitrile hydratases[5]).

Recently, the isolation of an old yellow enzyme (OYE) from the thermophile *G. kaustophilus* (*Geobacillus kaustophilus*) has been reported.[6] This prompted us to search for nitrile reductase activity in this organism with all the benefits in enzyme isolation and purification of a thermostable enzyme.

Here we wish to report the cloning, expression and characterization of the first nitrile reductase from thermophilic origin. The enzyme from *G. kaustophilus* belongs to the type I enzyme subfamily and showed an 82% sequence homology with *Bs*NRed (*B. subtilis* nitrile reductase). The substrate scope of *Gk*NRed (*Geobacillus kaustophilus* nitrile reductase) was investigated regarding its biocatalytic applicability by testing a range of common nitriles. These investigations were supported by the rational design of *Gk*NRed active site mutants based on a previously published homology model from *B. subtilis*. Compounds structurally derived from the natural substrate $preQ_o$ were synthesized and tested with wild-type enzyme and mutants.

Results and Discussion

Cloning and Expression

The WTNRed (wild-type) and six NRed mutants from *G. kaustophilus* were cloned, heterologously expressed in *E. coli* and purified to homogeneity as described in the Experimental Section. The thus prepared protein was used throughout the characterization studies, whereas for bioreduction reactions, a less tedious purification by thermoprecipitation was found to be sufficient.

Enzyme Characterization

Specific reductase activities of *Gk*NRedWT and mutants were assayed spectrophotometrically by monitoring the depletion of NADPH and by an HPLC-based activity measurement method using the natural substrate $preQ_0$. The same methods were applied for all screenings with non-natural substrates (see Experimental Section).

The overall behaviour of the new *Gk*NRed in terms of optimum pH and pH stability does not differ significantly from that of *B. subtilis* NRed, thus exhibiting a bell-shaped pH profile within a range of pH 5.5 to 10 with maximal activity at 7.5 (see the Supporting Information, Figure S4). The enzymatic activity increased 12-fold in response to a change in temperature from 25 to 65 °C. Half life times of 43 h and 15 h were determined at 55 °C, the latter with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Supporting Information, Figure S5).

The apparent kinetic parameters of *Gk*NRedWT of $preQ_0$ reduction are given in Table S1 (Supporting Information). Thus the K_m values of *Gk*NRed for $preQ_0$ and NADPH are 11 μM and 34 μM, respectively. The K_{mNADPH} is comparable to a value of 19 μM found in the literature for *Bs*NRed. The corresponding *Bs*NRed K_{mpreQ_0} value is 0.24 μM and therefore 46-fold smaller as that determined for *Gk*NRed. For *Gk*NRred a turnover number of 3.9 min^{-1} was obtained, a 5.5-fold increase as compared to published data for *Bs*NRed.[1b] The increased k_{cat} and higher K_{mpreQ_0} values of *Gk*NRred are ascribed to the 25 °C higher temperature in initial rate measurements. *Gk*NRed activities were determined spectrophotometrically by monitoring NADPH depletion at 55 °C (a 2:1 stoichiometry of NADPH:$preQ_0$ is required in a 4-electron reduction). The k_{cat} was calculated according to the previous assumption that the minimal biological unit is a dimer of 38.8 kDa.[1a,8]

Screening of *Gk*NRedWT

An initial substrate screening of different aliphatic, aromatic and heterocyclic nitriles (for structures see Figure S1 in the Supporting Information) with wild-type QueF from *Geobacillus kaustophilus* as well as the previously reported *Escherichia coli* and *Bacillus subtilis* QueF has revealed that these enzymes are highly specific for their natural substrate 7-cyano-7-deazaguanosine (**1**, Scheme 1).[7]

In a recent publication a three-dimensional homology model of the *B. subtilis* QueF active site has been presented based on sequence alignment with the structure of GTP cyclohydrolase I from *E. coli*,[8] however, a crystal structure is not yet available. The active site model shows a conserved Glu and an invariant Cys residue shared between QueF and GTP-CH I. The Cys residue is considered as relevant for the covalent catalytic mechanism postulated. Other residues presumably important for binding were identified as Glu97 and His96, both involved in hydrogen bonding to the 6-oxo and 2-amino substituents of 7-cyano-7-deazaguanine ($preQ_0$).[8]

With this model at hand, we made a sequence comparison of *B. subtilis* and *G. kaustophilus* QueF, (both type I enzymes) by using ClustalW2 multiple sequence alignment, which revealed an 82% sequence identity between the enzymes. These facts have suggested the design and expression of several active site mutants of *G. kaustophilus* QueF (Table 1).

Table 1. Screening results of wild-type and mutant QueF from *Geobacillus kaustophilus*.

G. kaustophilus	**1**	**2**[a]	**3**[b]	**4–9**
wild-type	+++	++	+	–
His96Ala	++	++	+	–
His96Phe	++	++	+	–
Glu97Ala	++	++	+	–
Glu97Ser	++	++	+	–
Phe95Ala	++	++	+	–
Cys55Ala	–			

[a] Activities observed were in an equal range of magnitude to those of the natural substrate **1**.
[b] Activities observed were significantly lower compared to substrates **1** and **2**.

We chose the initial replacement of Phe95, His96 and Glu97 by Ala with the intention to broaden the enzyme's substrate specificity by reducing strong polar residues in the putative binding site. The Cys55Ala mutation was intended to verify the Cys55 residue to be essential for covalent catalysis as described for *B. subtilis* QueF.[1b]

The subsequent screening of all nitriles depicted in Figure S1 (Supporting Information) has revealed that all QueF mutants tested were active with respect to the natural substrate **1**. To our disappointment, however, no reaction could be detected with any of the remaining non-natural substrates.

This prompted us to address the problem from a substrate-based approach as well. Thus, we synthesized compounds structurally closely related to the parent natural substrate preQ$_0$ (**1**) to systematically study the impact of these structural changes on binding and catalytic activity. The analogues are depicted in Figure 1.

The structural changes in analogues **2** and **3** were made regarding their purine ring substituents, in particular the the 2-amino (**2**) and 6-oxo (**3**) functionality,[9] both substituents assumedly essential for binding to the Glu97 and His96 residues. The 6-oxo group in compound **4** was replaced by an amino group and the pyrimidine ring in compound **5** was replaced by benzene. Compound **6**, devoid of the pyrimidine ring, is supposed to represent the pyrrole part of the bicyclic structure. Nitriles **7** and **8** are analogues bearing the presumably essential substituents of the pyrimidine ring in identical positions in a phenyl ring, but carry the nitrile group in an open chain fragment of comparable distance to the parent structure **1**. Analogue **9** is identically substituted to the natural substrate but has no pyrrole ring.

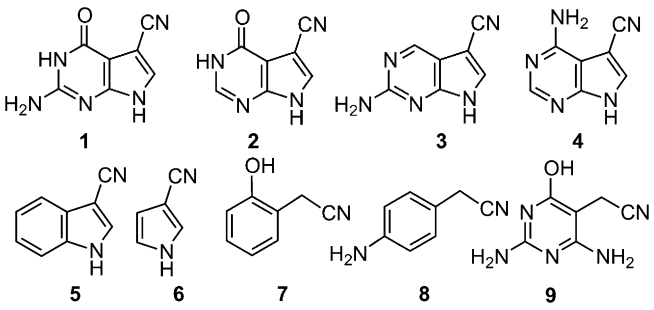

Figure 1. Substrate analogues for wild-type and mutant *Gk*NRed characterization studies.

The syntheses of compounds **1**, **2**, **4**, **6, 7** and **9** have been described, modifications made by us to previously published procedures are given in the Experimental Section. Compound **3** has not yet been reported in the literature. All experimental and spectroscopic data are given in the Experimental Section. The ^{13}C NMR data for some of the literature known compounds have not been published so far; these data are therefore included in the Experimental Section together with their corresponding spectra in the Supporting Information.

Investigation of the Substrate Scope

The results given in Table 1 suggest the indispensable presence of an appropriately substituted pyrimidine ring for binding. An estimate of the activities of compounds **2** and **3**, differing from the natural substrate only in one functional group suggests that the impact of the 6-oxo functionality seems to be more important than that of the 2-amino group (Table 1) since the conversion of nitrile **3** was significantly lower. This can also be deduced from comparison of the respective K_m and k_{cat} values. While k_{cat} (for compound **2**) is in an equal range of magnitude compared to the natural substrate **1**, the active site binding is diminished, as indicated by the K_m value (Table S1 in the Supporting Information). Interestingly, replacement of the 6-oxo functionality by an amino group (nitrile **4**) resulted in a complete loss of activity. In addition to that, the reduction of preQ$_0$ by *Gk*NRed was not inhibited by **4** (Table S2 in the Supporting Information) further suggesting the 6-oxo group as essential anchor for substrate binding. The phenyl-replaced nitriles **7** and **8** – both have the oxo or amino group placed in equivalent ring positions compared to the pyrimidine ring in the natural substrate **1** – are not accepted as substrates, neither is analogue **9**, though this compound has exactly the substitution pattern of the natural substrate. These facts indicate that the ring nitrogen atoms of the pyrimidine and pyrrole rings are probably necessary for active site binding. Interestingly, compound **8** showed strong inhibition on preQ$_0$ reduction, while compound **7** did not inhibit it (Supporting Information, Table S2). Nitrile **6** representing the pyrrole ring fragment of the parent deazapurine as

well as indole-3-carbonitrile **5** with the complete bicyclic system but devoid of both substituents and pyrimidine ring nitrogens are neither accepted as substrates nor do they act as inhibitors. A range of additional nitriles (see Figure S2 in the Supporting Information) was screened with the mutants but they were not accepted as substrates.

Conclusions

The cloning, expression and characterization of an NRed from *G. kaustophilus* – the first nitrile reductase from a thermophile – was accomplished. The substrate scope regarding its biocatalytic applicability was investigated by testing a range of common nitriles. The narrow substrate tolerance observed for the wild-type enzyme prompted the rational design of active site mutants based on a previously published homology model from *B. subtilis*.

The activities of the mutants and the wild-type enzyme were investigated in their structure-function relationship regarding a range of synthesized substrates, which were structurally closely related to $preQ_0$. A distinct dependence of specific structural elements on catalytic activity of *Gk*NRedWT and mutants could be observed, in particular the presence of the 6-oxo group in the pyrimidine ring was recognized to be essential for a reduction reaction just as any fragmentation of the parent deazapurine ring resulted in complete loss of catalytic activity (compounds **6** and **9**). Two non-natural nitriles (**2** and **3**) differing in their substitution pattern of the pyrimidine ring could be reduced to the corresponding amino compounds.

Studies are in progress to elucidate the role of the individual ring nitrogen atoms, in particular of position N-9 (purine numbering), since the homology model is suggesting a possible binding interaction. However, to draw a clear picture of the *Gk*Nred active site an exact crystal structure of the enzyme is needed. Consequently, work is in progress in this direction.

Given the generally low (water) solubility of all substrates with the 7-deazapurine core structure, an enhanced thermostabilty of the enzyme can be very useful. Apart from this specific solubility behaviour of this compound class, the possibility of running a biocatalytic reaction at elevated temperature is often beneficial in terms of higher k_{cat} values.[10]

The present work has demonstrated a straightforward preparation of a nitrile reductase capable of reducing nitriles closely related to the natural substrate as well as first insights into the demands regarding the structural properties of possible substrates. Nevertheless, further investigations including different approaches in catalyst design will be necessary to generate a more broadly applicable biocatalyst.

Experimental Section

Chemicals, Strains and General Procedures

NADPH (sodium salt; $\geq$ 97% pure) was obtained from Roth (Karlsruhe, Germany). All other chemicals were purchased from Sigma–Aldrich/Fluka (Gillingham, Dorset, U.K.) or Roth (Karlsruhe, Germany), and were of the highest purity available. The microorganism used was *E. coli* BL21 (DE3).

Reagents and starting materials used for substrate synthesis were obtained from commercial suppliers, and used without further purification. Substrates **5** and **8** were obtained from Sigma–Aldrich in the highest purity available. Syntheses of substrates **6**[11] and **7**,[12] as well as other substrates screened, and their precursors,[13] were done according to literature procedures and can be found in the Supporting Information. Thin layer chromatography was carried out with precoated aluminium silica gel 60 F_{254} plates, column chromatography with Merck Silica Gel 60 (0.040–0.063 mm). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III with autosampler (^{1}H NMR 300.36 MHz, ^{13}C NMR 75.53 MHz). Chemical shifts for ^{1}H NMR are reported in ppm relative to Me_4Si as internal standard. Screening reactions were run on an Eppendorf comfort thermomixer. Absorption measurements were done on a Fluostar plate reader from BMG lab tech at 340 nm and HPLC-MS analysis on an Agilent 1200 series using a phenomenex Gemini-NX 3 C18 110 A (150×2.0 mm) column and 20 mM ammonium acetate in water and acetonitrile as eluents.

Preparation of Wild-Type and Mutant NRed for Screening Reactions

Strains, vectors and growth conditions: *Geobacillus kaustophilus* HTA426 was grown on LB agar medium. The pEamTA vector[14] was used for recombinant expression. *E. coli* K12 Top10F' (Invitrogen, Carlsbad, CA, USA) was used to amplify and maintain engineered constructs and *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) for protein expression. LB medium and LB agar supplemented with ampicillin when appropriate (100 mg L^{-1}) was used for cell cultivation.

Vector construction and preparation of mutants: The *Geobacillus kaustophilus* nitrile reductase (*Gk*NRed) was amplified from genomic *G. kaustophilus* DNA employing the primers *Gk*Nred TA f: 5′-*atg g*ca gga aga aaa g-3′; *Gk*Nred TA r: 5′-*gaa ttc cta* gcg gtt gtc gac-3′ and Phusion DNA polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. All oligonucleotides used were manufactured by Integrated DNA Technologies, San Jose, CA, USA. The amplified PCR product was purified using a preparative gel. The DNA fragment was recovered from the gel using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany). The fragment (31 μL) was A-tailed by incubation with 5 μL of dNTPs (2 mM, each), 8 μL of magnesium chloride (25 mM), 5 μL of *Taq* buffer and 1 μL *of Taq* Polymerase (5 U μL^{-1}, Fermentas, Burlington, Canada) at 72 °C for 30 min. Subsequent purification with the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) yielded the A-tailed insert in approx. 5 ng mL^{-1} concentration. The linear pEamTA vector was prepared by digestion

of 44 μL of plasmid DNA (isolated with the http://www.fermentas.com/en/products/all/nucleic-acid-purification/kits/k050-genejet-plasmid-miniprep from Fermentas, Burlington, Canada) supplemented with 5 μL Eam1105I buffer (10X) and 1 μL of Eam1105I (Fermentas) at 37 °C for 4 h. The DNA fragment was recovered from the gel using the QIAquick® Gel Extraction Kit (Qiagen) yielded the linear vector in approx. 40 ng mL^{-1} concentration. The insert (110 ng, 22 μL) was ligated with the vector (80 ng, 2 μL) in an overnight reaction at 16 °C using 3 μL T4 DNA ligase buffer and 3 μL T4 ligase (Promega, Madison, WI, USA). After heat inactivation (65 °C, 20 min) and desalting on ultradialysis membranes (Millipore, 0.025 μm), the entire ligation mixtures were transformed into 180 μL of electrocompetent *E. coli* K12 Top10F' cells (Invitrogen) and selected on LB-ampicillin agar plates. Colonies with correctly integrated inserts were confirmed by digestions of plasmid DNA (isolated with the http://www.fermentas.com/en/products/all/nucleic-acid-purification/kits/k050-genejet-plasmid-miniprep from Fermentas) with *Nde*I and *Hind*III fast digest restriction enzymes (Fermentas) and verified by sequencing (LCG Genomics, Berlin, Germany) using the primers pEam f1 (5′-ttgtgagcggataacaatttc-3′) or pEam r1 (5′-tactgccgccaggcaaattct-3′). Retransformation of 1 μL of plasmid into chemically competent *E. coli* BL21 (DE3) (Invitrogen) resulted in the *Gk*NRedWT expression strain.

Site specific mutagenesis: pEamTA *Gk*NRedWT, was employed as the template for a two stage mutagenesis reaction protocol. Briefly, 5 μL of *PfuUltra* HF reaction buffer (10X), the template (1 μL, 10 ng), 1 μL dNTP mix (10 mM), 1 μL of forward OR reverse primer (5 mM) and 1 μL of *PfuUltra* High Fidelity DNA polymerase (2.5 U μL^{-1} from Stratagene (La Jolla, CA, USA) were added to 41 μL of doubly distilled water (Fresenius, Graz, Austria). The two-stage PCR was conducted on a Gene Amp System 2400 thermo cycler (Applied Biosystems, Foster City, CA) under the following conditions: 95 °C for 1 min, 4 cycles of 50 s at 95 °C, 50 s at 60 °C and 5 min at 68 °C and then 7 min at 68 °C for the final elongation step. After these initial 4 cycles of primer extension, 25 μL of the reaction containing the forward primer were combined with 25 μL of the reaction containing the reverse primer. Additional 18 cycles were run under the above conditions. Subsequently, dpnI (1 μL, Fermentas) was added to digest the template at 37 °C for 1 h. 2 μL of the mixture were transformed into 60 μL of electrocompetent *E. coli* K12 Top10F' cells (Invitrogen) and selected on LB-ampicillin agar plates. The mutations were confirmed by sequencing (LCG Genomics). Plasmids from mutants having the correct sequence were replicated and transformed into *E. coli* BL21 (DE3). PAGE purified primers used for site specific mutagenesis: *Gk*NRedC55Af 5′-c cgg agt tta cga cgt tgg **cgc** caa aaa ccg gac aac cg-3′; *Gk*NRedC55Ar 5′-cgg ttg tcc ggt ttt tgg **cgc** caa cgt cgt aaa ctc cgg-3' *Gk*NRedF95Af 5′-c ttc cgc aat cat ggc gac **gcg** cac gaa gac tgc gtc aac a-3′; *Gk*NRedF95Ar 5′-t gtt gac gca gtc ttc gtg **cgc** gtc gcc atg att gcg gaa g-3′; *Gk*NRedH96Af 5′-gc ttc cgc aat cat ggc gac ttt **gc**c gaa gac tgc gt-3′; *Gk*NRedH96Ar 5′-ac gca gtc ttc g**gc** aaa gtc gcc atg att gcg gaa gc-3′; *Gk*NRedH96Ff 5′-gc ttc cgc aat cat ggc gac ttt **tt**c gaa gac tgc gt-3′; *Gk*NRedH96Fr 5′-ac gca gtc ttc g**aa** aaa gtc gcc atg att gcg gaa gc-3′; *Gk*NRedE97Af 5′-cat ggc gac ttt cac g**c**a gac tgc gtc aac atc-3′; *Gk*NRedE97Ar 5′-gat gtt gac gca gtc t**g**c gtg aaa gtc gcc atg-3′; *Gk*NRedE97Sf 5′-gc aat cat ggc gac ttt cac **tc**a gac tgc gtc aac atc att a-3′; *Gk*NRedE97Sr 5′-t aat gat gtt gac gca gtc t**ga** gtg aaa gtc gcc atg att gc-3′.

Protein expression and purification: The wild-type *Gk*NRedWT and the mutants were cultivated as follows: overnight cultures (20 mL LB/Amp, inoculated with a single colony and grown at 37 °C in an orbital shaker) were used to inoculate 500 mL LB/Amp medium in 2-L baffled Erlenmayer flasks. These main cultures were grown at 37 °C and 130 rpm to an OD of 0.7, induced with 0.5 mL of IPTG (1 M) and incubated for 24 h at 16 °C and 130 rpm. The cells were harvested by centrifugation (4,000 rpm, 4 °C, 10 min). The pellet was resuspended in reaction buffer [12.1 $g L^{-1}$ Tris, 3.73 $g L^{-1}$ KCl, 287 $mg L^{-1}$ tris(2-carboxyethyl)phosphine hydrochloride (TCEP); pH 7.5 adjusted with HCl 37%] and disrupted by ultrasonication. The cell debris was removed by ultracentrifugation at 40,000 rpm for 45 min at 4 °C. The supernatant (crude lysate) was purified by heat precipitation of host proteins at 70 °C for 10 min and then applied to a centrifugation step (4,000 rpm, 4 °C, 10 min). The supernatant contained fairly pure nitrile reductases and was stored at −20 °C.

Enzyme Characterization and Determination of Kinetic Parameters

Cultivation: *E. coli* BL21 *Gk*NR was grown in 1000-mL baffled shaken flasks containing 200 mL of LB media supplemented with 115 mg/L ampicillin. Flasks were shaken at 120 rpm and 37 °C in a Certomat® BS-1 incubator from Sartorius. Recombinant protein production used a standard procedure in which cultures were cooled to 25 °C when an optical density of 0.6 (±10%) was reached. Isopropylthio-β-D-galactoside (IPTG) was added in a concentration of 1.0 mM, and the cultivation time after induction was 18 h. Cells were harvested by centrifugation, washed with physiological NaCl solution, re-centrifuged and diluted into 100 mM Tris buffer, pH 7.5, supplemented with 50 mM KCl and 1 mM TCEP. Cells were disrupted by two passages through a French press (American Instrument Company, Silver Springs, Maryland, USA) operated at an internal cell pressure of 25,000 psi. The crude cell extract obtained was clarified by centrifugation (16,000 *g*, 45 min, 4 °C) prior to enzyme purification.

Enzyme purification: *E. coli* protein was separated from the cell-free extract by incubation at 75 °C for 10 min, thermo-precipitated protein was centrifuged (16,000 *g*, 10 min).

Recombinant enzyme was further purified by ion exchange chromatography using a QFF from GE Healthcare (bed volume 55 mL). The column was equilibrated with 100 mM Tris buffer, 50 mM KCl, pH 7.5. Adsorbed protein was eluted at a flow rate of 4 $mL min^{-1}$ with a step gradient of 0.1 M and 1 M KCl. Fractions of 5 mL were collected and the 0.1 M KCl peak containing most of the *Gk*NR, as judged by activity measurements and SDS-PAGE, was pooled. The pooled fractions were concentrated in Vivaspin 20-mL Concentrator tubes with 10,000 molecular weight cut off to a volume of less than 0.5 mL and re-diluted into 100 mM Tris, 50 mM KCl and 1 mM TCEP.

Reductase activity measurement: Specific reductase activity was assayed spectrophotometrically by monitoring the

reduction of NADPH at 340 nm. Typically, rates of 0.05–0.10 ΔA/min were measured over a time period of 5 min. One unit of enzyme activity refers to 1 μmol of NADPH consumed per minute. All measurements were performed with a Beckman DU-800 spectrophotometer thermostated at 55 °C. The assay contained 100 μM $preQ_0$ and 250 μM NADPH. $preQ_0$ was dissolved in DMSO prior to dilution into buffer to give a final DMSO concentration of 1% (v/v). Unless otherwise stated, Tris/HCl buffer, pH 7.5 supplemented with 50 mM KCl was used. Reactions were always started by the addition of coenzyme. Activity measurements for pH studies were performed in either 100 mM Tris buffer (pH values 5.5; 6,0; 6.5; 7.0) or Bis-Tris buffer (pH values 7.0; 7.5; 8.0; 8.5; 9.0; 10.0) supplemented with 100 mM KCl. Measured rates were corrected for appropriate blank readings accounting for non-specific decomposition of NADPH.

Enzyme stability: Purified enzyme was diluted to 11 mg mL^{-1} in 100 mM Tris buffer, pH 7.5, containing 100 mM KCl with or without 1 mM TCEP [tris(2-carboxyethyl)phosphine]. Experiments were carried out in Eppendorf tubes incubated at 55 °C and 500 rpm in a Thermomixer comfort from Eppendorf. Samples were withdrawn every hour and specific activities were determined as described above.

Steady-state enzyme kinetics: All experiments were carried out in 100 mM Tris buffer, pH 7.5, supplemented with 50 mM KCl, and, unless otherwise stated, at 55 °C. Measurements of the initial rates of substrate reduction were performed with a Beckman DU-800 monitoring the consumption of NADPH over a time period of 5 min. Initial rate data were measured under conditions where substrate (or coenzyme) was held at a constant saturating concentration and the coenzyme (or substrate) was varied in the range $0.4 < K_m < 4$- to 10-fold. Typical reaction mixtures contained 80 μM *Gk*NRed wild-type. Kinetic parameters were obtained from unweighted non-linear least-square fits of experimental data to Eq. (1) using the program Sigmaplot 2004 (for Windows, version 9.0). In Eq. (1),
v is the initial rate, [E] is the molar concentration of the enzyme subunit (38.8 kDa), [A] is the substrate or coenzyme concentration, k_{cat} is the turnover number (min^{-1}) and $K_{m\,A}$ is an apparent Michaelis–Menten constant. Two NADPH

$$v = k_{cat}[E][A]/(K_{m\,A} + [A]) \quad (1)$$

consumed account for one turnover. All rates were corrected for the appropriate blank readings accounting for the non-specific decomposition of NADPH. Unless otherwise stated, estimates of kinetic parameters had standard errors of $< 20\%$.

Substrate Screening

Photometric assays for substrate screening were carried out as described for $preQ_0$. Activities were normalized to $preQ_0$ reductase activity. Assays for inhibitor screening contained 10 μM $preQ_0$ and the compounds listed in the Supporting Information, Table S2. Residual activities refer to reductase activities obtained with 10 μM $preQ_0$. Measured rates were corrected for appropriate blank readings accounting for non-specific decomposition of NADPH.

Substrate Synthesis

2-Chloro-3-oxopropanenitrile: NaH (1.0 g, 50% in mineral oil) was washed twice with cyclohexane and once with THF and was then suspended in 20 mL THF. The suspension was cooled to 0 °C and methyl formate (0.90 mL, 14 mmol) was added. Chloroacetonitrile (0.83 mL, 13 mmol) was added dropwise to the stirred solution and the solution was stirred for additional 3.5 h. Afterwards, 20 mL deionized water were added and the mixture was extracted twice with ethyl acetate. The layers were separated and the pH value of the aqueous layer was adjusted to pH 4 with 5 M HCl. The aqueous layer was then extracted three times with diethyl ether. The combined organic phases from the second extraction were dried over Na_2SO_4 and concentrated under reduced pressure to afford a brown oil which was used for the next synthetic step without further purification; yield: 1.142 g (85%).

2-Amino-5-cyano-7H-pyrrolo[2,3-d]pyrimidin-4-one (preQ$_0$, 1):[15] Sodium acetate trihydrate (2.693 g, 19.79 mmol) was dissolved in 40 mL deionized water and 4-diamino-6-hydroxypyrimidine (1.225 g, 9.32 mmol) was added. The suspension was heated to reflux. 2-Chloro-3-oxopropanenitrile (1.142 g, 11.03 mmol) was dissolved in 20 mL deionized water and added dropwise to the reaction mixture. The reaction mixture was stirred at reflux overnight. On cooling to room temperature the beige product precipitated from the solution. The product was filtered off, washed with copious amounts of water and acetone. After drying, the product was obtained; yield: 840 mg (51%). ^{1}H NMR (DMSO-d_6): $\delta = 6.39$ (s, 2H, NH_2), 7.62 (s, 1H, H-6), 10.72 (s, 1H, NH-7), 11.99 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6): $\delta = 85.53$ (C-5), 98.73 (C-4a), 115.87 (CN), 127.77 (C-6), 151.66 (C-7a), 153.72 (C-2), 157.56 (C-4).

4-Amino-5-cyano-7H-pyrrolo[2,3-d]pyrimidine (4): 4-Amino-5-cyano-7*H*-pyrrolo[2,3-*d*]pyrimidine was prepared analogous to a literature procedure.[16] 4-Amino-6-bromo-5-cyano-7*H*-pyrrolo[2,3-*d*]pyrimidine (1.60 g, 6.72 mmol) was dissolved in 300 mL ethanol and 300 mL 33% aqueous ammonium hydroxide solution. 10% palladium on charcoal catalyst was added and the reaction mixture was stirred in a hydrogen atmosphere (hydrogen balloon) for 12 h. The catalyst was removed by filtration over celite, and washed with hot ethanol/ammonium hydroxide solution. The filtrate was concentrated under vacuum to about one half of the original volume. On cooling a white solid precipitated from the solution. The product was isolated by filtration. After drying the product was obtained; yield: 686.5 mg (64%). ^{1}H NMR (DMSO-d_6) δ 6.75 (bs, 2H, NH_2), 8.16 (s, 1H, H-6), 8.20 (s, 1H, H-2), 12.60 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6): $\delta =$ 82.02 (C-5), 100.89 (C-4a), 115.96 (CN), 132.33 (C-6), 150.88 (C-7a), 153.52 (C-2), 156.89 (C-4).

5-Cyano-7H-pyrrolo[2,3-d]pyrimidin-4-one (2):[17] Starting from 6-bromo-5-cyano-7*H*-pyrrolo[2,3-*d*] pyrimidin-4-one (836 mg, 3.50 mmol), the product was obtained; yield: 200 mg (36%). Anal. calcd. for $C_7H_4N_4O$: C 58.33, H 2.80, N 38.87; found: C 58.23, H 2.88, N 38.90; ^{1}H NMR (DMSO-d_6): $\delta = 12.14$ (bs, 1H, NH-7), 8.02 (s, 1H, H-2), 8.01 (s, 1H, H-6); ^{13}C NMR (DMSO-d_6): $\delta = 85.98$ (C-5), 107.13 (C-4a),

115.24 (-CN), 130.82 (C-6), 145.85 (C-2), 148.56 (C-7a), 157.07 (C-4).

2-Amino-4-chloro-5-cyano-7H-pyrrolo[2,3-d]pyrimidine: 2-Amino-4-chloro-5-cyano-7*H*-pyrrolo[2,3-*d*]pyrimidine was prepared according to a modified literature procedure.[18] $PreQ_0$, **1**, (2.00 g, 11.42 mmol) was suspended in 12 mL acetonitrile. Triethylamine (3.2 mL, 23 mmol) was added and the suspension was heated to 95 °C. Phosphoryl chloride (2.2 mL, 24 mmol) was slowly added to the warm reaction mixture. The reaction mixture was allowed to stir at 95 °C overnight. The reaction mixture was then allowed to cool to 65 °C and an additional 10 mL acetonitrile, phosphoryl chloride (2.2 mL, 24 mmol), and triethylamine (3.2 mL, 23 mmol) were added. The reaction was allowed to stir at 65 °C for additional 48 h at which time HPLC-MS analysis showed full conversion. The reaction mixture was cooled to room temperature and ice was slowly added. The mixture was then heated to 75 °C for 15 min and subsequently the precipitate was filtered off. The pH value of the filtrate was adjusted to pH 2 using aqueous ammonia solution. The filtrate was then cooled to 0 °C. The product precipitated from the solution and was isolated by vacuum filtration. After washing with water and drying the product was isolated as a brown solid; yield: 807.7 mg (37%). ^{1}H NMR (DMSO-d_6): $\delta = 6.96$ (s, 2H, NH_2), 8.12 (s, 1H, H-6); ^{13}C NMR (DMSO-d_6): $\delta = 83.05$ (C-5), 106.10 (C-4a), 115.05 (CN), 134.16 (C-6), 151.09 (C-7a), 154.49 (C-4), 160.38 (C-2).

2-Amino-5-cyano-7*H*-pyrrolo[2,3-*d*]pyrimidine (3): 2-Amino-4-chloro-5-cyano-7*H*-pyrrolo[2,3-*d*]pyrimidine (650 mg, 3.36 mmol) was suspended in 80 mL ethanol. 10% palladium on charcoal catalyst (65 mg) and sodium bicarbonate (33.8 mg, 4.02 mmol) were added. The suspension was stirred at 500 rpm at 90 °C and 80 bar of hydrogen pressure in a steel autoclave for 40 h. The catalyst was removed by filtration over celite and washed with ethanol. The filtrate ws concentrated under vacuum until dryness. 2-Amino-5-cyano-7*H*-pyrrolo[2,3-*d*]pyrimidine was obtained as brown solid; yield: 440 mg (82%). Anal. calcd. for $C_7H_5N_5$ C 52.83, H 3.17, N 44.01: found: C 54.30, H 4.23, N 41.47; ^{1}H NMR (DMSO-d_6): $\delta = 6.55$ (s, 2H, NH_2), 8.02 (s, 1H, H-6), 8.63 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6): $\delta = 82.83$ (C-5), 108.96 (C-4a), 115.45 (CN), 133.01 (C-6), 149.93 (C-4), 153.55 (C-7a), 161.13 (C-2).

Screening Reactions

Screening reactions were run in 96-deep well plates using the following conditions and concentrations: nitrile reductase (55 μM, purified by heat precipitation, purity between 60% and 85%) in buffer (100 mM Tris, 50 mM KCl, 1.15 mM TCEP, pH 7.5 adjusted with conc. HCl), substrate in DMSO (2 mM, 10% v/v DMSO), NADPH (0.25 mM in buffer), total volume 200 μL. Blank reactions contained buffer, substrate in DMSO (2 mM, 10% v/v DMSO), and NADPH (0.25 mM in buffer). Reactions were started by addition of NADPH, and NADPH depletion was monitored at 30 °C on a plate reader for 14 h. Subsequently, additional 4 mM of NADPH were added to each well and the screening reactions were placed on a thermomixer at 30 °C and 500 rpm for 24 h to allow full conversion. Samples were then analyzed with HPLC-MS to observe possible product formation. In the case of the natural substrate $preQ_0$, additionally, a synthetic reference material was used to verify the HPLC-MS results. For all screening reactions, including blank reactions, multiple parallel determinations were run.

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