Evidence of a sequestered imine intermediate during reduction of nitrile to amine by the nitrile reductase QueF from *Escherichia coli*

Jihye Jung\(^{1,2}\) and Bernd Nidetzky\(^{1,2,*}\)

From the \(^{1}\)Austrian Centre of Industrial Biotechnology, Petersgasse 14, and the \(^{2}\)Institute of Biotechnology and Biochemical Engineering, NAWI Graz, Graz University of Technology, Petersgasse 12/1, A-8010 Graz, Austria

**Running title:** Imine intermediate during nitrile reduction by QueF

* To whom correspondence should be addressed. Tel.: +43-316-873-8400; Fax: +43-316-873-8434; E-mail: bernd.nidetzky@tugraz.at

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**Abstract**

In the biosynthesis of the tRNA-inserted nucleoside queuosine, the nitrile reductase QueF catalyzes conversion of 7-cyano-7-deazaguanine (preQ\(_0\)) to 7-aminomethyl-7-deazaguanine (preQ\(_1\)), a biologically unique four-electron reduction of a nitrile to an amine. The QueF mechanism involves a covalent thioimide adduct between the enzyme and preQ\(_0\) which undergoes reduction to preQ\(_1\) in two NADPH-dependent steps, presumably via an imine intermediate. Protecting a labile imine from interception by water is fundamental to QueF catalysis for proper enzyme function. In the QueF from *E. coli*, the conserved Glu\(^{89}\) and Phe\(^{228}\) residues together with a mobile structural element comprising the catalytic Cys\(^{190}\) form a substrate-binding pocket that secludes the bound preQ\(_0\) completely from solvent. We show here that residue substitutions (E89A, E89L, F228A) targeted at opening up the binding pocket weakened preQ\(_0\) binding at the preadduct stage, by up to +10 kJ/mol, and profoundly affected on catalysis. Unlike wild type enzyme, the QueF variants, including L191A and I192A, were no longer selective for preQ\(_1\) formation. The E89A, E89L and F228A variants performed primarily (≥ 90%) a two-electron reduction of preQ\(_0\), releasing hydrolyzed imine (7-formyl-7-deazaguanine) as the product. The preQ\(_0\) reduction by L191A and I192A gave preQ\(_1\) and 7-formyl-7-deazaguanine at a 4:1 and 1:1 ratio, respectively. The proportion of 7-formyl-7-deazaguanine in total product increased with increasing substrate concentration, suggesting a role for preQ\(_0\) in a competitor-induced release of the imine intermediate. Collectively, these results provide direct evidence for the intermediacy of an imine in the QueF-catalyzed reaction. They reveal determinants of QueF structure required for imine sequestration and hence for a complete nitrile-to-amine conversion by this class of enzymes.

**Introduction**

Sequestration of reactive intermediates, to avoid their release into solvent where they might decompose, is a hallmark of enzyme catalysis (1, 2). Mobile elements of the protein structure are often used to close down over the substrate while it undergoes the catalytic transformation into product. The nitrile reductase QueF deals with a notably difficult problem of intermediate sequestration (3–7). QueF catalyzes a four-electron reduction of a nitrile to an amine in two NADPH-dependent steps, presumably via an imine intermediate. However, imines are extremely unstable molecular groups in water and will hydrolyze to aldehyde immediately. Therefore, to ensure that every substrate "makes it through" to the amine, QueF must do both, keep a firm grip on the labile imine during the catalytic act and exclude water from the binding site. This study was performed to identify elements of the QueF catalytic apparatus that make nitrile reductases the high-fidelity enzymes they are by nature, converting nitrile to amine without detectable loss of the supposed imine to off-pathway reactions (3–7).

QueF was described from the biosynthetic pathway of the nucleoside queuosine (Q) where
the enzyme functions in turning 7-cyano-7-deazaguanine (preQ₀) into 7-aminomethyl-7-deazaguanine (preQ₁) (8, 9). The preQ₁ is inserted into tRNA and subsequently converted to Q. Bound at the wobble position of tRNAs decoding NAC/U codons, Q plays a role in modulating codon-anticodon binding efficiency (8, 10, 11). De novo biosynthesis of Q is exclusively found in bacteria (11–13). Although Q is not essential for bacterial life (14), knock out of its biosynthesis can reduce, or eliminate, pathogenicity (15, 16). QueF among all other enzymes of the Q pathway, therefore, presents a potential target for anti-infective strategies directed selectively against bacteria. QueF also has attracted considerable mechanistic interest (3, 5–7, 17) for the reaction catalyzed by it is apparently unique to biology.

Two-fold enzymatic reduction of preQ₀ is proposed to proceed in three catalytic steps, as shown in Scheme 1 (3–7). A covalent thioimide adduct is formed between the enzyme’s catalytic nucleophile (cysteine) and preQ₀ (3, 5, 7). Its subsequent reduction by NADPH yields a covalent thiohemiaminal intermediate. After exchange of coenzyme and breakdown of the covalent thiohemiaminal to imine, a second reduction by NADPH gives the amine product. The QueF mechanism necessitates that the different chemical steps of the enzymatic reaction are coordinated exactly with the intermediate physical steps of coenzyme binding and release, and these in turn must be precisely timed with the disengagement of free imine from the covalent thiohemiaminal. The way of how QueF orchestrates these multiple steps (4–6) immediately suggests strategies possibly utilized for intermediate sequestration.

QueF enzymes are oligomeric proteins built from subunits that possess a small, so-called tunneling-fold (T-fold) domain (18). The core T-fold domain consists of a ββαββ arrangement of secondary structure (4, 5, 9). The known QueF enzymes are divided into unimodular (e.g., QueF from Bacillus subtilis; bsQueF) (3, 5, 19, 20) and bimodular groups (e.g., QueF from Escherichia coli; ecQueF) (4, 7, 21–24), depending on whether they comprise subunits containing only a single T-fold domain or two T-fold domains in tandem repeat, respectively. Both types of QueF have their active site residues, and their structural arrangement into a functional catalytic center, are highly conserved in both QueF types (4, 5, 21). Besides the catalytic cysteine (Cys₁⁹⁰ in ecQueF), there is an invariant pair of acidic residues of which the aspartic acid (Asp¹⁹⁷ in ecQueF) plays a role in proton transfer, as shown in Scheme 1, and the glutamic acid (Glu⁸⁹ in ecQueF) is involved in binding recognition of preQ₀.

Crystallographic studies of bsQueF (5, 20) and the bimodular QueF from Vibrio cholerae (vcQueF; (4)) show that preQ₀ binding involves an induced-fit conformational change in protein structure, with the consequence that the substrate binding site, which is quite open in the apo-enzyme, is closed up completely in the holoenzyme. The bound preQ₀ becomes sequestered completely from the solvent as a result (Fig 1).

In vcQueF, for which crystal structures of apo-enzyme (PDB: 3RJ4), noncovalent complex of a C194A variant with preQ₀ (PDB: 3RZP, 3UXV) and covalent complexes of H233A (PDB: 4GHM) and R262L (PDB: 3S19, 3UXJ) variants with preQ₀ were determined, the induced-fit binding of preQ₀ comprises three main elements in particular. A characteristic device, generally referred to as the "QueF motif", is embedded structurally in a α-helix flanking the active site (5, 9). At its N-terminal end, this helix has a glutamic acid (Glu⁸⁹ in ecQueF), which forms a hydrogen bond with preQ₀ (2.8 Å) in the enzyme-substrate complex, as shown in Fig. 1. The glutamic acid is positioned opposite to an adaptable element of the QueF structure (Fig. 1 B,C) contributing the catalytic cysteine to the active site. This "Cys element", which is completely conserved within bimodular QueF enzymes, additionally comprises Leu¹⁹¹ and Ile¹⁹² (ecQueF numbering). The Cys element rearranges to cover the active site in the preQ₀-enzyme complex structure (PDB: 4GHM; Fig. 1C). Finally, a conserved phenylalanine (Phe²²₈ in ecQueF), which adopts a position to open up the preQ₀ binding pocket in the apo-enzyme, closes down over the bound substrate, by forming pi-stacking interactions with it (3.7 Å), in the holoenzyme (Fig. 1C). Two key residues of preQ₀ binding, Glu⁸⁹ and Phe²²₈, and the catalytic Cys₁⁹⁰ thus get “locked” onto the bound substrate at the end of the structural rearrangement. These features of vcQueF structure important for enzymatic function appear to be completely conserved in...
EcQueF. Overall, the sequence identity between the two enzymes is 65%.

Kinetic studies of ecQueF indicate that NADPH binds to QueF already at the non-covalent complex with preQ₀ (7). The presence of NADPH enhances about 4-fold the thioimide formation rate compared to reaction of free enzyme and preQ₀. The substrate-binding pocket has a narrow aperture, made up from Glu⁸⁹ and the Cys element, Ile¹⁹² in particular (Fig. 1), that appears to be used for threading the nicotinamide moiety of NADPH into the active site (21). Structurally, therefore, substrate cannot be released before the coenzyme has dissociated. Accordingly, an important feature of QueF efficiency in intermediate sequestration, suggested from kinetic analysis, is the enzyme's extremely tight binding of NADPH at the stage of the thiohemiaminal/imine (7).

The evidence just summarized suggests that the residues Glu⁸⁹ and Phe²²⁸ and also the Cys element are significant for intermediate sequestration in ecQueF. Anchoring the proposed imine through interactions from these QueF structural elements might be crucially important. In this study, therefore, we performed mutagenesis of ecQueF, replacing the "anchoring" Glu⁸⁹ and Phe²²⁸ by residues (Ala or Leu) unable to fulfill the analogous function. We additionally replaced Leu¹⁹¹ and Ile¹⁹² by Ala with the aim of perturbing the active site-closing movement of the Cys element. Overall, we considered that due to a substrate-binding site not properly closable any more, the ecQueF variants might no longer behave as high-fidelity nitrile reductases. The putative imine intermediate might become intercepted by water able to enter an opened-up active site in the variant enzymes. Detailed characterization of these ecQueF variants reveals two-electron reduction of nitrile to imine for the first time in a nitrile reductase. The imine intermediate might become intercepted by water able to enter an opened-up active site in the variant enzymes.

**Results**

*Isothermal titration calorimetry study of preQ₀ binding to ecQueF variants* - Purified ecQueF variants were shown to bind preQ₀, and the corresponding thermodynamic characteristics were determined, in ITC experiments. Binding of preQ₀ by ecQueF is a two-step process in which an initially formed noncovalent enzyme-preQ₀ complex is converted to a covalent thioimide adduct (Scheme 2; ref. 7). Note that with ITC, it was not possible to distinguish between the two steps of preQ₀ binding and the data report on the binding process as a whole. The results are shown in Fig. 2 and the parameters calculated from the data are summarized in Table 1.

Strong heat release on titrating enzyme solution with preQ₀ solution (Fig. 2), similarly as it occurred with the wildtype enzyme (7), was good evidence that ecQueF variants had retained the ability to bind preQ₀. Substitution of Glu⁸⁹ caused substantial weakening of the preQ₀ binding as compared to wildtype enzyme, reflected in a smaller negative value of ΔG<sub>binding</sub> (ΔΔG<sub>binding</sub> = +14 kJ/mol) and increased overall apparent dissociation constant Kₐ (292-fold). The degree of disruptive effect was similar in both enzyme variants. The F228A variant also showed weakened preQ₀ binding, reflected in a smaller negative value of ΔG<sub>binding</sub> (ΔΔG<sub>binding</sub> = +10.8 kJ/mol) and 90-fold increased Kₐ. In terms of ΔG<sub>binding</sub> and Kₐ, preQ₀ binding was unaffected by the replacement of Leu¹⁹¹ or Ile¹⁹² with Ala. Interestingly, the ΔG<sub>binding</sub> of the E89A, E89L, and F228A variants was comparable to the ΔG<sub>binding</sub> of the previously reported C190A variant which can bind preQ₀ just noncovalently (7). However, contrasting their similarity overall, the ΔG<sub>binding</sub> values of these ecQueF variants involved distinct relative contributions from enthalpy (ΔH<sub>binding</sub>) and entropy (ΔS<sub>binding</sub>). The ΔH<sub>binding</sub> became more negative (favorable of binding) and the ΔS<sub>binding</sub> more positive (nonfavorable of binding) in going from C190A via the variants. A covalent thioimide formation more pronounced in E89L compared to the E89A and F228A variants and lacking completely in C190A variant could explain these trends in ΔH<sub>binding</sub> and ΔS<sub>binding</sub>.

In comparison to the wildtype enzyme, the ΔΔG<sub>binding</sub> of the E89A variant was primarily due to effect on ΔH<sub>binding</sub>. In the E89L and F228A variants, however, the ΔΔG<sub>binding</sub> involved effects, of a similar degree, on ΔH<sub>binding</sub> and ΔS<sub>binding</sub>. The ΔG<sub>binding</sub> of L191A variant involved a ΔH<sub>binding</sub> lower and a ΔS<sub>binding</sub> higher than the corresponding preQ₀ binding parameters of the
wildtype enzyme. However, the $\Delta H_{\text{binding}}$ and -$T\Delta S_{\text{binding}}$ terms compensated each other in an overall unchanged $\Delta G_{\text{binding}}$. A -$T\Delta S_{\text{binding}}$ term substantially higher in the ecQueF variants (E89L, L191A) is therefore worth noting. In the I192A variant, the $\Delta H_{\text{binding}}$ and -$T\Delta S_{\text{binding}}$ terms of preQ0 binding were comparable to as they were in the wildtype enzyme.

**Kinetic analysis of covalent thioimide adduct formation in ecQueF variants** - Absorbance at 380 nm ($\varepsilon = 10.02 \pm 0.14 \text{mM}^{-1}\text{cm}^{-1}$) indicates the covalent thioimide intermediate of wildtype ecQueF (7). Titration of the enzyme variants with preQ0 also gave rise to a "thioimide" absorbance band, as shown in Fig. 3. Maximum absorption was however shifted to a slightly higher wavelength (< 5 nm) in the Glu89 variants. In wildtype ecQueF based on half-of-the-sites reactivity of the enzyme dimer, the molar equivalent of preQ0 was sufficient to convert all enzyme present into the covalent adduct (7). In ecQueF variants, especially the ones having Glu89 replaced, excess preQ0 (~10-fold) was required for full conversion of the enzyme (Fig. 3A).

In wildtype ecQueF as shown recently (7), the thioimide formation involves a non-covalent enzyme-preQ0 complex in rapid equilibrium, which reacts only relatively slowly to the covalent adduct (Scheme 2). Absorbance time courses from preQ0 binding experiments with the E89A, L191A, I192A and F228A variant were best fit with single exponentials. The overall fit was not improved when double exponentials were used (data not shown). The corresponding rate constants and amplitudes (data not shown) were hyperbolically dependent on the preQ0 concentration, as expected if the ecQueF variants reacted in two distinct steps, as shown in Scheme 2, analogous to the wildtype enzyme (7). A global fit of the reaction time courses was therefore performed using the kinetic mechanism in Scheme 2. Experimental data are superimposed in Fig. 3 with the fitting results. Parameters of preQ0 noncovalent binding ($K_{d_{\text{kinetic}}} = k_2/k_1$) and thioimide formation ($k_3$, $k_4$) by the ecQueF variants are summarized in Table 2 along with the corresponding parameters of the wildtype enzyme.

Compared to the wildtype enzyme, the $K_{d_{\text{kinetic}}}$ was increased in all ecQueF variants (F228A: 59-fold; E89A: 41-fold; L191A: 7.1-fold), except for the I192A variant that exhibited the same $K_{d_{\text{kinetic}}}$ as the wildtype enzyme. The $k_3$ was decreased in all enzyme variants, most strongly so in the F228A variant (10-fold). The ratio $k_3/k_4$ describes the tightening of preQ0 binding in consequence of covalent thioimide formation. The effect was more pronounced in the L191A variant ($k_3/k_4 = 53$) than in the E89A ($k_3/k_4 = 53$), I192A ($k_3/k_4 = 26$), and F228A variant ($k_3/k_4 = 40$). Including the wildtype enzyme in this comparison is difficult because only a rough lower limit of 53 could be given for $k_3/k_4$. Since in kinetic experiments the $k_4$ was not different from zero (7), the relationship $K_{d_{\text{overall}}} = K_{d_{\text{kinetic}}}/(1+k_3/k_4)$ was used to calculate the upper bound of $k_4$ from the wildtype enzyme's apparent $K_{d_{\text{overall}}}$ determined in ITC experiments. The values of $K_{d_{\text{overall}}}$ determined kinetically and obtained from ITC data agreed for the L191A and F228A variant. They however differed by as much as 5-fold for the E89A and I192A variant. We explain this difference by the fact that in the ITC measurement, both the covalent and the noncovalent complex contribute to the recorded heat signal whereas absorbance is completely specific for detecting the thioimide adduct. Therefore, only absorbance measurements allow for a clear-cut determination of $K_{d_{\text{overall}}}$.

**Reduction of preQ0 by the ecQueF variants** - Direct analysis of the reaction mixtures by 1H NMR (Fig. 4) and HPLC (Fig. 5) was used to examine preQ0 reduction by the ecQueF variants. Initial evidence was that while F228A and both Glu89 variants showed conversion of preQ0 at a slow rate, they hardly produced preQ1. We considered that any imine intermediate exposed to water after the first reduction would be detected as 7-formyl-7-deazaguanine. Using authentic reference material from chemical synthesis (7), we identified 7-formyl-7-deazaguanine as the main product of preQ0 reduction by E89A, E89L and F228A variants, as shown in Fig. 4 and 5. Proton signals characteristic of preQ1 were below detection in these reactions. Conversely, 7-formyl-7-deazaguanine was completely absent from, and preQ1 the only detectable product in, reactions of the wildtype enzyme. Reaction of the L191A variant gave a mixture of products comprising mainly preQ1 (80 - 90%), but also 7-formyl-7-deazaguanine that accounted for the rest of preQ0 converted. The preQ0 reduction by the I192A variant also proceeded to form both preQ1 and 7-formyl-7-deazaguanine, roughly at a ratio of 1:1.
LC and LC-MS analysis confirmed the ecQueF variants to exhibit the particular change in product formation from preQ_0 (Fig. 5). When the preQ_0 reduction with the variants were done in Tris buffer, LC traces revealed a putative product peak of unknown identity, appearing always together with the expected product peak for 7-formyl-7-deazaguanine (Fig. 5B). The unknown peak exhibited absorbance at 262 nm and 340 nm, just like 7-formyl-7-deazaguanine but contrary to preQ_0 and preQ_1, which both show absorbance only at 262 nm. When the same enzymatic reactions were done in phosphate buffer, the second peak was missing and only the peak for 7-formyl-7-deazaguanine was found (Fig. 5C, D). This together with the characteristic mass of the unknown peak (H^+, 282) suggested an imine adduct between 7-formyl-7-deazaguanine and Tris, as shown in Fig. 5, that may have formed in solution already during the enzymatic reaction or during sample preparation.

Ability of the LC and LC-MS analytic method to quantify even small amounts of the secondary reaction product (e.g., 5% preQ_1 in preQ_0 conversion by the E89A variant) made it possible to determine whether the NADPH or the preQ_0 concentration had an influence on the product distribution in the enzymatic conversions. Whereas high concentrations of NADPH might prevent the loss of imine intermediate from the enzyme and so pull the enzymatic reaction towards preQ_1 formation, the opposite effect, namely preference for formation of aldehyde, could be expected at high preQ_0 concentration (Scheme 3). In each enzyme including wildtype ecQueF, there was no effect from variation in [NADPH] between 1 and 10 mM. Note that relatively high NADPH concentrations were used in the experiment to maximize the possible "pull" towards preQ_1. The case of the L191A and I192A variants, in which on average 1 imine was lost in every 5 (L191A) and 2 (I192A) preQ_0 conversion events, clearly shows that exchange between NADP^+ and NADPH for complete two-fold reduction of preQ_0 was still somewhat functional after the replacements of Leu_191 and Ile_192. This result immediately suggests the possibility that imine was intercepted by water in, or escaped into solution from, the L191A and I192A variants before the new NADPH had the chance to bind, implying in turn an early disengagement of the imine from the covalent thiohemiaminal. The alternative possibility is that imine was intercepted by water in, or was released from, the ternary complexes of L191A and I192A variants with NADPH.

Contrary to NADPH, the preQ_0 substrate, on variation of its concentration, affected strongly the product distribution from nitrile reduction by the different ecQueF variants. In each enzyme as shown in Fig. 6, the percent imine in the total reaction product released increased upon increase in the molar ratio [preQ_0]/[enzyme dimer] used in the experiment. The effect was most pronounced in the E89A and F228A variants, which switched from forming primarily preQ_1 (80%) when preQ_0 was used at the same concentration as the enzyme dimer to being almost completely selective for imine formation (≥ 95%) at higher [preQ_0]/[enzyme dimer] ratios. The E89L variant gave predominantly imine at all conditions used. In the I192A variant, the percent imine in total product increased steadily dependent on the [preQ_0]/[enzyme] ratio and reached a limiting value of about 50% when preQ_0 was added in excess. The L191A variant formed imine as the minor product (≤ 20%). In all enzyme variants except for F228A, the percent imine in total product typically exhibited a roughly hyperbolic dependence on the [preQ_0]/[enzyme] ratio used (Fig. 6A-D), from which the maximum imine formation at saturating preQ_0 was determined. In the F228A variant, maximum imine formation was established from the data, but the underlying dependence of product selectivity on [preQ_0]/[enzyme] ratio appeared complex (Fig. 6E).

Kinetic analysis and KIE studies – To analyze the enzyme kinetics in more detail, and determine primary isotope effects (KIE) from the use of (4R)-[^H]-NADPH as reductant, we monitored the preQ_0 conversion by wildtype and variant enzymes by in situ proton NMR and also by LC. Kinetic data obtained from time-course analysis are summarized in Table 3. Material balances were fully consistent with the chemical reaction, preQ_0 + NADPH + H_3O^+ → 7-formyl-7-deazaguanine + NADP^+ + NH_3. At long reaction times (≥ 24 h), however, decomposition of NADPH became significant as a spontaneous side reaction. The reaction of wildtype ecQueF was confirmed as preQ_0 + 2 NADPH + 2H^+ → preQ_1 + 2 NADP^+.

Utilization of NADPH for preQ_0 reduction by the
L191A and I192A variants was fully consistent with the preQ1 and 7-formyl-7-deazaguanine obtained. Product selectivity of the enzyme variants was confirmed and shown to be constant over the whole time course prevailing steady state conditions ([preQ0] > 15 [enzyme dimer]). The specific rate of preQ0 conversion was slowed in the enzyme variants compared to wildtype ecQueF, most strongly in E89L (700-fold), then E89A (160-fold), F228A (140-fold), L191A (24-fold) and I192A (2.6-fold).

Using (4R)-[2H]-NADPH instead of NADPH, we showed that the deuterium was transferred from coenzyme to form 7-formyl-7-deazaguanine (all enzyme variants) and preQ1 (L191A and I192A variants), indicating the same pro-R stereoselectivity for NADPH conversion in the variants as in wildtype enzyme (7).

The KIE determined from a direct comparison of reaction rates with NADPH and (4R)-[2H]-NADPH of a similar magnitude as the first-step KIE, i.e., the KIE of 2.3 (±0.2) for the E89L variant, 2.8 (±0.4) for the F228A variant. The "reference KIE" for the reaction of the wildtype enzyme was 1.8 (±0.5). The shown KIEs are means (± S.D.) from 5 independent determinations and represent the averaged isotope effects on the rates of substrate consumption (preQ0, NADPH) and product formation (preQ1 or 7-formyl-7-deazaguanine, NADP+). The L191A and I192A variants differed from the wildtype enzyme and the Glu89 and Phe228 variants in that their reaction rates based on isotopic substitution in coenzyme, the isotope fraction of imine intermediate reacting to 7-formyl-7-deazaguanine is defined from Scheme 3.

Because only the imine reduction is sensitive to deuterium, the KIEs determined from a direct comparison of reaction rates with NADPH and (4R)-[2H]-NADPH (ratio: 0.48) instead of NADPH was used (ratio: 2.6). The KIE on preQ0 consumption was 4.1 (±0.3), which was higher than the KIE of 1.5 (±0.3) on formation of 7-formyl-7-deazaguanine, but lower than the KIE of 8.9 (±0.3) on preQ1 formation. The KIE on conversion of NADPH to NADP+ was 5.0 (±0.6).

There is only one isotope-sensitive step involved in 7-formyl-7-deazaguanine formation, that of reduction of preQ0 to imine. The KIEs for the E89A, E89L and F228A variants therefore reflect the slowing down of the enzymatic reactions at just this step. The individual KIEs associated with reductions of the preQ0 substrate and the imine intermediate by wildtype ecQueF were determined previously as 3.3 and 1.8, respectively (7). Global fitting of reaction time courses of preQ0 conversion were used in our earlier study (7) to obtain these KIEs. The KIEs on conversion of preQ0 to 7-formyl-7-deazaguanine by the Glu89 variants and the F228A variant were of a similar magnitude as the first-step KIE, i.e., preQ0 reduction to imine, for the reaction of wildtype ecQueF.

In the L191A and the I192A variant, partitioning of the imine intermediate between reaction to preQ1 and reaction to 7-formyl-7-deazaguanine constitutes the point of departure for analyzing the different KIEs in these two enzymes. The molar ratio (R) of preQ1 and 7-formyl-7-deazaguanine reflects the rate constant ratio of imine reduction/imine hydrolysis (k8/k7), as shown in the kinetic mechanism depicted in Scheme 3. Because only the imine reduction is sensitive to isotopic substitution in coenzyme, the isotope effect on the product ratio (4/1.8 = 2.22 for L191A; 2.6/0.48 = 5.4 for I192A) is also the KIE on k8. A KIE on preQ0 conversion smaller than on preQ1 formation arises because partitioning of the imine intermediate is also subject to an isotope effect. The fraction of imine intermediate reacting to 7-formyl-7-deazaguanine is defined from Scheme 3 as F = I/(1 + R). F has a value of 0.2 and 0.36 in L191A reactions with NADPH (F_D) and NADPD (F_D), respectively. The corresponding F values for the I192A variant are 0.28 (F_D) and 0.67 (F_D). The relative flux from preQ0 to 7-formyl-7-deazaguanine, therefore, increases 1.8-fold (L191A variant) and 2.4-fold (I192A variant) due to deuteration of the coenzyme. Considerations of flux balance reveal the set of KIE data for the L191A and the I192A variant to be internally consistent.
With $F_H$ and $F_D$ known, the KIE on preQ_0 consumption obtains, according to Scheme 3, from the KIE on preQ_1 formation as $\text{KIE}_{\text{preQ}_0} = \text{KIE}_{\text{preQ}_1}(1 - F_D)/(1 - F_H)$. The calculated value of $\text{KIE}_{\text{preQ}_0}$ is therefore 4.08 for the L191A variant. The same value is obtained for the I192A variant. The KIE on formation of 7-formyl-7-deaza-guanine obtains from the relationship, $\text{KIE}_{\text{preQ}_0} F_W/F_D$, as 2.27 and 1.70 for the L191A and the I192A variant, respectively. Finally, the KIE on NADPH consumption, which is the same as the KIE on NADP⁺ formation, is calculated with the relationship, $\text{KIE}_{\text{preQ}_0}(2 - F_W)/(2 - F_D)$, as 4.48 and 5.28 for the L191A and the I192A variant, respectively. These calculated KIE data agree well with the experimentally obtained values.

Evidence summarized in Table 3, that in preQ_1 formation the complete reaction of the L191A or the I192A variant was affected by a substantially larger KIE than the partial reaction of imine reduction ($k_a$), identifies nitrile reduction to imine as the rate-determining step of the overall preQ_0 conversion by these two ecQueF variants. Linear time courses of NADPH consumption by the enzyme, with no sign of a presteady state burst, were consistent with this notion (data not shown).

**Discussion**

Conversion of preQ_0 to 7-formyl-7-deaza-guanine represents the first reaction of this kind catalyzed by an enzyme. Chemically, it consists in a single hydride transfer reduction of a nitrile group from NADPH followed by addition of water to the imine thus obtained. Catalysis from the enzyme is required only for nitrile reduction. Imine hydrolysis is a spontaneous reaction, well established from other enzymatic transformations, like those of amino acid dehydrogenases for instance (25–27). Mechanistically, the "interrupted" (two-electron) reduction of preQ_0 is significant for it provides first-time direct evidence on the occurrence of an imine intermediate in the reaction pathway of QueF nitrile reductases. Besides reinforcing the current mechanistic proposal for QueF (Scheme 1) (3, 5–7), these results advance comprehension of the enzyme's mode of action, demonstrating how critically important is imine intermediate sequestration for complete two-fold reduction of preQ_0 to preQ_1. The finding that site-directed substitutions interfering most significantly with intermediate sequestration (e.g., Glu^{89}, Phe^{228}) were also strongly disruptive on the hydride transfer revealed that the major physical steps of the enzymatic process proceeded tightly interconnected with the catalytic act(s). It furthermore indicated that ecQueF made parsimonious use of its active-site structural devices to accomplish the different tasks in the overall multistep catalytic reaction. The thermodynamic signatures of preQ_0 binding by the different ecQueF variants appear consistent with the enzyme-substrate interactions revealed in the crystallographic evidence on vcQueF (Fig. 1).

The hydride transfers from NADPH are the slowest steps in the overall conversion of preQ_0 to preQ_1 by ecQueF (7). Evidence that catalytic rates were lowered by up to ~700-fold in the ecQueF variants, while their associated KIEs due to deuteration of the coenzyme were not decreased as compared to the wildtype KIEs, strongly supports the suggestion that catalysis was also rate limiting in the enzyme variants. The crucial importance of substrate positioning at the stage of imine reduction was most vividly evident from the fact that substitutions of Leu^{191} and Ile^{192}, and even more so those of Glu^{89} and Phe^{228}, caused loss of imine from the enzyme during the reaction. The fraction of preQ_0, which on conversion by the ecQueF variants didn't make it through to the fully reduced amine preQ_1, increased dependent on the substrate amount present. A simple model, having preQ_0 compete with imine intermediate for binding to the enzyme in rapid equilibrium, fails to explain the experimental product distributions, for it predicts that at "saturating" [preQ_0] no preQ_1 will be formed. This is most evidently refuted from the behavior of the L191A and the I192A variants.

Formally, a general model of molecular complex dissociation, induced by a competing ligand as shown in literature (28), would be consistent with the effect of [preQ_0] on the product distribution in the ecQueF variants (Scheme 4). The model involves ligand dissociation effectively from a pre-associated complex, which enables rapid rebinding of the ligand to give the actual biological form of the complex. The model predicts that competitor molecules will accelerate the breakdown of enzyme-ligand complexes by occluding the rapid rebinding of enzyme and ligand from the level of their pre-associated complex. It is therefore worth noting that the model does not imply the formation of a ternary
complex between enzyme, ligand and competitor. The ligand dissociation rate \((k_{\text{off}})\), enhanced in the presence of competitor \((C)\), is thus given by the relationship, derived from Scheme 4, \(k_{\text{off}} \cdot (C) = k_a \times (k_{\text{esc}} + k_{\text{on}}C) \div (k_a + k_{\text{esc}} + k_{\text{on}}C)\), where \(k_a\) and \(k_{\text{on}}\) are first-order association and dissociation rate constants for (rapid) formation of the fully associated complex, \(k_{\text{esc}}\) is the first-order rate constant of ligand release into solution, and \(k_{\text{on}}\) is the rate constant for competitor binding (28). Note that the \(k_{\text{esc}}\) step involves hydrolysis of imine. Scheme 4 does not distinguish mechanistically between whether the hydrolysis occurs in solution or, more likely, with imine still bound to the enzyme.

Results in Fig. 6 suggest that the ecQueF variants differed in the relative contribution of \(k_{\text{esc}}\) and \(k_{\text{on}}\) to \(k_{\text{off}}\). Whereas in the E89L variant the escape of imine appeared to be largely controlled by \(k_{\text{esc}}\) (since \(k_{\text{off}}\) was hardly dependent on the \(\text{preQ}_0\) concentration), the product formation by the L191A and I192A variants reflected a prominent effect from \(k_{\text{on}}\). The E89A variant adopted an intermediate position between the two extremes, with both \(k_{\text{esc}}\) and \(k_{\text{on}}\) affecting the product distribution dependent on the competitor \(\text{preQ}_0\). A molecular account of the proposed kinetic scenario for ecQueF is not easy to conceive, and the model is essentially independent of such details. However, a preassociated enzyme-imine complex in a somewhat open conformation might make possible the kinetically implied displacement of the imine by \(\text{preQ}_0\).

In conclusion, identification of residues in the catalytic apparatus of ecQueF essential (Glu\(^{89}\), Phe\(^{228}\)) or auxiliary (Leu\(^{191}\), Ile\(^{192}\)) for complete twofold reduction of nitrile to amine has led to uncover the imine intermediate, and the necessity of its efficient sequestration in the active site, as central features of the enzymatic mechanism. Structure-based interpretation of the effect of site-directed substitutions on the reaction selectivity of ecQueF is that due to a substrate-binding site (Figure 1 B-C) not fully closable any longer, the enzyme variants lose the imine intermediate to hydrolysis, completely in E89A, E89L and F228A and partly in L191A and I192A. Although not proven by the evidence shown, a likely scenario is that the imine is captured by water now able to enter the partly opened-up active site in the variant enzymes. The hydrolyzed imine, 7-formyl-7-deazaguanine, is not recognized by ecQueF as a substrate of NADPH-dependent reduction, as demonstrated previously (7). It would therefore be released into solvent.

While in terms of reaction catalyzed, QueF nitrile reductases share commonalities with other four-electron reductases, such as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (29–31) and the non-ribosomal peptide synthetase module myxalamin reductase (32, 33), that use NAD(P)H to convert thioester-activated forms of carboxylic acids into primary alcohols via an aldehyde intermediate, they are individuated strongly from these other enzymes in having to deal with a highly decomposition-prone intermediate. The central role of intermediate sequestration in the QueF mechanism seems to be reflected in the fact that structural devices used to keep a firm grip on the imine intermediate are likewise involved directly in the catalytic events of the enzymatic reaction.

**Experimental Procedures**

**Chemicals and materials used** — NADPH (purity >98%) and NADP\(^+\) (purity >97%) were from Carl Roth (Karlsruhe, Germany). 2-Propanol-d\(_8\) (99.5 atom % D) was from Sigma (St. Louis, Missouri, USA). All other materials were of the highest purity available from Carl Roth and Sigma. The \(\text{preQ}_0\) and 7-formyl-7-deazaguanine were synthesized as described previously (7, 19). A pET-28a(+) expression vector encoding *Thermoanaerobium brockii* alcohol dehydrogenase as an N-terminally His-tagged protein was ordered from GenScript (Piscataway, New Jersey, USA). Standard expression in *Escherichia coli* BL21-DE3 and His-tag purification were used to prepare the enzyme.

**Site-directed mutagenesis** — Mutagenesis leading to site-directed substitution of Leu\(^{191}\) by Ala (L191A) was performed according to a standard two-stage PCR protocol (34, 35). A pEHISTEV expression vector comprising the ecQueF gene was used as the template. The oligonucleotide primers used are shown with the mismatched bases underlined.

L191A forward: 5'GCTGAAATCAGAAGCGCGATCCCACCATCAACC3'
L191A reverse: 5'-GGTTGATGTTGATCCGGATGTCTCACGTCG-3'

Mutagenesis to substitute Glu\(^{89}\) by Ala or Leu was reported in an earlier study (21). Replacement of
Ile<sup>192</sup> or Phe<sup>228</sup> by Ala was ordered from Genscript. All mutations were verified by gene sequencing.

**Enzyme preparation**—The ecQueF wildtype and site-directed variants thereof (E89A, E89L, L191A, I192A and F228A) were obtained as N-terminally His-tagged proteins using expression in *E. coli* BL21-DE3 as described previously (7, 21). All enzymes were purified by a reported two-step procedure, consisting of immobilized metal ion affinity chromatography and gel filtration (7). Enzyme purity was confirmed by SDS-PAGE. The background of the *E. coli* expression host did not contain QueF activity in amounts detectable with the assays used. Contamination of ecQueF variants with endogenous wildtype activity could thus be ruled out firmly. The protein concentration was measured with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Germering, Germany). A DMSO solution (1.8 mM) using illustra NAP 5 columns (GE Healthcare, Buckinghamshire, UK). A DMSO concentration of maximally 2.0% (v/v) was used in all experiments.

**ITC and spectrophotometric analysis of preQ<sub>0</sub> binding in ecQueF variants**—A VP-ITC Micro Calorimeter from Microcal (Malvern Instruments Ltd., Malvern, UK) was used at 25 °C. The enzyme was gel-filtered twice to phosphate buffer (100 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5, 50 mM KCl) using illustra NAP 5 columns (GE Healthcare, Buckinghamshire, UK). A DMSO concentration of maximally 2.0% (v/v) was used in both the enzyme and the preQ<sub>0</sub> solution. Experiments were conducted and data evaluation done as described previously (7). The enzyme molar concentration was based on the protein concentration assuming a functional ecQueF homodimer with a molecular mass of 71,772 Da (wildtype enzyme), 71,656 Da (E89A variant), 71,740 Da (E89L variant), 71,688 Da (L191A or I192A variant), or 71,620 Da (F228A variant).

The covalent thioimide adduct between preQ<sub>0</sub> and ecQueF in wildtype or variant form was detected from its characteristic absorbance with maximum absorption at around 380 nm. Absorbance titrations were carried out with a Beckman DU 800 spectrophotometer (Beckman Coulter, Pasadena, CA, USA) as described previously (7). Kinetic study of the thioimide formation was done with a SX.18 MV Stopped flow spectrophotometer (Applied Photophysics; Leatherhead, UK). The experimental progress curves of absorbance (380 nm) increase recorded at different protein and preQ<sub>0</sub> concentrations were analyzed by a global fitting procedure, as previously described (7), that employed the program COPASI (version 4.11_build 65) and used the mechanism in Scheme 2. A least squares fitting routine implemented in COPASI was used to determine the kinetic rate constants and their standard deviations. In the fitting, <i>k</i><sub>1</sub> and <i>k</i><sub>2</sub> were not determined individually, but only their ratio <i>K</i><sub>d_kinetic</sub> (=<i>k</i><sub>2</sub>/<i>k</i><sub>1</sub>) was obtained. We showed that the fitting always converged to a unique and well-defined solution that was independent of the parameter start values used. All fitted parameters (<i>K</i><sub>d_kinetic</sub>, <i>k</i><sub>3</sub>, <i>k</i><sub>4</sub>) were well determined as regards their associated standard deviation. An overall binding constant (<i>K</i><sub>d_overall</sub>) was calculated from the results, using the relationship<br>

\[
K_{d_{\text{overall}}} = K_{d_{\text{kinetic}}}/(1+<i>k</i><sub>3</sub>/<i>k</i><sub>4</sub>)
\]

Note that <i>K</i><sub>d_overall</sub> thus includes the covalent thioimide formation.

Binding constants were transformed into binding energies using the relationship

\[
\Delta G = -RT \ln K_d
\]

A 1 M standard state for all reactants was assumed. R is the gas constant (8.314 J/mol·K) and <i>T</i> is the temperature (298.15 K).

**Enzymatic reduction of preQ<sub>0</sub>**—Experiments were performed to characterize the preQ<sub>0</sub> conversion by the different ecQueF enzymes. Besides determining the specific rate of substrate consumption, determination of the product(s) formed by preQ<sub>0</sub> reduction was of main interest. Reactions were carried out by incubating buffered solutions of enzyme, preQ<sub>0</sub> and NADPH on a Thermomixer Comfort (Eppendorf, Hamburg, Germany) at 25 °C with agitation at 700 rpm for about 24 h. The DMSO concentration used was below 2.5%. The concentrations of preQ<sub>0</sub> and NADPH were varied as indicated. The enzyme concentration was 50 µM (E89A, E89L, I192A variant), 10 µM (L191A variant) and 1.8 µM (wildtype enzyme). Reactions were stopped by precipitating the enzyme with methanol (10%, by volume) at 70 °C for 10 min (1000 rpm in a Thermomixer Comfort) (7). Samples were applied to <sup>1</sup>H NMR, HPLC, and LC-MS from comprehensive analysis.

**HPLC analysis**—The samples were analyzed using an Agilent 1200 HPLC system (Santa Clara, CA, USA) equipped with a 5 µm SeQuant ZIC-HILIC column (200 Å, 250 × 2.1 mm; Merck, Billerica, MA, USA), the corresponding guard column (20 × 2.1 mm; Merck) and a UV detector.
Imine intermediate during nitrile reduction by QueF.

A 20 mM ammonium acetate buffer (pH 6.67) was used. A decreasing gradient in acetonitrile was applied for compound separation over a 20-min run time. Specifically, the acetonitrile concentration was decreased only slightly from 80% to 78% over 5 min, and a steeper ramp from 78% to 60% was then used from 5 to 20 min. The column was washed with 60% and 80% acetonitrile for 5 min after each analysis. The flow rate was 0.5 ml/min. The column temperature was 30 °C.

Optionally, a mass detector (Agilent 6200 Quadrupole) was coupled to the HPLC system, which in this case was equipped with a 2.7 µm Poroshell 120 EC-C18 column (120 Å, 100 × 3 mm; Agilent) and a UV detector (λ = 210, 262, and 340 nm). The masses of the products from the enzymatic reactions were scanned in a mass range of 100-850 using a positive mode. The masses of preQ0 (1H+, 176), preQ1 (1H+, 180), and 7-formyl-7-deazaguanine (1H+, 179) were also analyzed in SIM mode. A linear gradient of acetonitrile was used in water containing 0.01% formic acid. The gradient was from 0% to 25% acetonitrile over 5 min. The column was washed with 90% acetonitrile for 1 min and water for 0.5 min. The post time was 1 min. The flow rate was 0.7 ml/min. The column temperature was 30 °C.

KIE studies—NADPH and (4R)-[²H]-NADPH were prepared by reducing NADP⁺ in the oxidation of 2-propanol and 2-propanol-d₈ with T. brockii alcohol dehydrogenase. A reported protocol was used for their synthesis (7, 38). Primary KIEs on preQ₀ reduction were obtained by comparing initial rates measured with NADPH and (4R)-[²H]-NADPH, both synthesized as described above. Enzymatic reactions were monitored by HPLC and for certain enzymes (wildtype, E89A, E89L, L191A) additionally by in situ 1H-NMR. Initial rates were obtained from linear fits to HPLC or spectral data. Note: since all substrates and products of the reaction were measured in the analysis, a total of four or five reaction rates was obtained: preQ₀ and NADPH consumption and preQ₁, 7-formyl-7-deazaguanine and NADP⁺ formation. The enzyme concentrations used in the reactions were: wildtype, 1.5 µM; E89A and E89L, 25 µM; L191A, 8 µM; I192A, 10 µM; and F228A, 20 µM. The preQ₀ concentration was saturating, that is, 500 µM using the wildtype enzyme, the L191A, and the I192A variant and 800 µM using the E89A, the E89L, and the F228A variant. The NADPH concentration was 1.5 mM. The temperature was 30 °C. The DMSO concentration was always at or below 2 %. Phosphate buffer (100 mM Na₂HPO₄-NaH₂PO₄, pH 7.5) containing 50 mM KCl was used.

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Author’s Contributions - J.J. and B.N. designed the research and wrote the paper. J.J. conducted experiments and analyzed data together with B.N. All authors agreed on the final version of the paper.

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**FOOTNOTES**

preQ$_0$, 7-cyano-7-deazaguanine; preQ$_1$, 7-aminomethyl-7-deazaguanine; Q, queuosine; T-fold, tunneling-fold; bsQueF, *Bacillus subtilis* QueF; ecQueF, *E.coli* QueF; ITC, isothermal titration calorimetry; KIE, kinetic isotope effect.
### Table 1. Thermodynamic parameters of preQ₀ binding by ecQueF wildtype and variants are shown.

|                  | Apparent $K_d$ (µM) | $\Delta H_{binding}$ (kJ/mol) | $-T\Delta S_{binding}$ (kJ/mol) | $\Delta G_{binding}$ (kJ/mol) | Ref.  |
|------------------|----------------------|-------------------------------|---------------------------------|------------------------------|------|
| wildtype         | 0.039 ± 0.004        | -80.3 ± 0.5                   | 37.9                            | -42.4 ± 0.5                  | (7)  |
| C190A            | 5.5 ± 0.1            | -44.7 ± 0.2                   | 14.6                            | -30.1 ± 0.2                  | (7)  |
| E89A             | 11.4 ± 0.4           | -63.3 ± 0.7                   | 34.9                            | -28.4 ± 0.7                  | This study |
| E89L             | 10.3 ± 1.1           | -73.3 ± 3.7                   | 44.7                            | -28.6 ± 3.3                  | This study |
| L191A            | 0.040 ± 0.004        | -97.5 ± 0.6                   | 55.1                            | -42.4 ± 0.6                  | This study |
| I192A            | 0.023 ± 0.011        | -82.9 ± 4.0                   | 39.0                            | -43.8 ± 1.1                  | This study |
| F228A            | 3.3 ± 0.5            | -61.7 ± 1.4                   | 30.1                            | -31.6 ± 0.5                  | This study |

*a The $K_d$ is denoted apparent because the binding process analyzed involves noncovalent binding of preQ₀ and covalent thioimide formation with the enzyme.

### Table 2. Kinetic constants of preQ₀ binding by ecQueF wildtype and variants are shown.

|                  | $K_{d_{kinetic}}$ ($k_2/k_1$) | $k_3$ | $k_4$ | $K_{d_{overall}}$ | Ref.  |
|------------------|-------------------------------|-------|-------|-------------------|------|
| wildtype         | (2.63 ± 0.01) × 10⁻⁶ M        | 1.63 ± 0.01 s⁻¹ | ≤ 0.024 s⁻¹ | ≤ 0.039 µM | (7)  |
| E89A             | (109 ± 10) × 10⁻⁶ M           | 0.32 ± 0.01 s⁻¹ | 0.006 ± 0.001 s⁻¹ | 2.0 µM | This study |
| L191A            | (18.7 ± 7.0) × 10⁻⁶ M         | 0.79 ± 0.02 s⁻¹ | 0.0018 ± 0.0003 s⁻¹ | 0.042 µM | This study |
| I192A            | (2.8 ± 1.0) × 10⁻⁶ M          | 0.36 ± 0.03 s⁻¹ | 0.014 ± 0.001 s⁻¹ | 0.10 µM | This study |
| F228A            | (155 ± 30) × 10⁻⁶ M          | 0.16 ± 0.02 s⁻¹ | 0.004 ± 0.001 s⁻¹ | 3.8 µM | This study |

*a $k_4$ was obtained from the relationship $K_{d_{overall}} = K_{d_{kinetic}}/(1+k_3/k_4)$ as described in Ref. (7).

*b As an upper bound of $K_{d_{overall}}$ for preQ₀ binding to wildtype ecQueF, the apparent $K_d$ from ITC measurement (Table 1) was taken.

### Table 3. Catalytic reaction rates and their associated KIEs are shown for ecQueF wildtype and variants thereof.

|                  | wildtype enzyme | E89A | E89L | L191A | I192A | F228A |
|------------------|-----------------|------|------|-------|-------|-------|
| preQ₀            | -4.1 ± 0.1      | -0.025 ± 0.001 | -0.006 ± 0.001 | -0.17 ± 0.03 | (3.7 ± 0.2) | -1.5 ± 0.3 | (4.1 ± 0.3) | -0.030 ± 0.006 |
| preQ₁            | 3.9 ± 0.3       | 0.16 ± 0.02 | (5.1 ± 0.2) | 0.8 ± 0.1 | (8.9 ± 0.3) | 0.3 ± 0.1 | (5.4 ± 0.3) | 0.035 ± 0.006 |
| 7-formyl-7-deazaguanine | 0.022 ± 0.001 | 0.006 ± 0.001 | 0.03 ± 0.01 | (2.3 ± 0.2) | 0.3 ± 0.1 | (1.5 ± 0.3) | 0.035 ± 0.006 |
| NADPH            | -8.4 ± 0.4      | -0.018 ± 0.007 | -0.005 ± 0.002 | -0.27 ± 0.02 | (4.4 ± 0.2) | -1.1 ± 0.1 | (5.0 ± 0.6) | -0.022 ± 0.005 |
| NADP⁺            | 7.6 ± 0.3       | 0.022 ± 0.001 | 0.009 ± 0.001 | 0.29 ± 0.02 | (4.4 ± 0.2) | 1.8 ± 0.3 | (5.3 ± 0.5) | 0.024 ± 0.005 |
| $\text{D}^\text{V}$ | 1.8 ± 0.5       | 2.9 ± 0.7 | 3.0 ± 0.6 | not applicable | not applicable | 2.8 ± 0.4 |

*a The catalytic reaction rates are given as µmol product released (or substrate converted)/(µmol enzyme × min). They were determined individually for each compound participating in the reaction. Reaction rates
of substrate (preQ₀, NADPH) consumption are negative, those of product (preQ₁, NADP⁺) formation are positive.

b $^D\nu$ is the primary KIE, due to deuteration of NADPH, on the catalytic rate. It is the average of the five individual KIEs determined from preQ₀, preQ₁, 7-formyl-7-deazaguanine, NADH and NADP⁺. Each individual KIE is the average of 5 independent reactions. Note that in reactions of the E89A, E89L and F228A variants, which perform a single-step reduction of preQ₀ to produce 7-formyl-7-deazaguanine from, the experimental $^D\nu$ equals $^Dk₅$ according to Scheme 3. In the wildtype enzyme, $^D\nu$ involves contribution from the KIEs on $k₅$ and $k₆$.

c In parenthesis is shown the primary KIE on the consumption rate or the formation rate of the compound indicated.

d The KIE on imine reduction ($k₆$ in Scheme 3) is shown in square brackets. As explained in text, this KIE is the same as the KIE on the experimental product ratio $R$, preQ₁/7-formyl-7-deazaguanine, formed in the reaction.

e Contrary to the wildtype enzyme and the E89A, E89L and F228A variants in which $^D\nu$ was not dependent on whether substrate consumption or product formation was analyzed and so an average value could be given, the L191A and I192A variants necessitated KIE evaluation separately for each compound involved in the reaction. The concept of an average KIE was therefore not applicable.
Figure Legends

Scheme 1. **The proposed mechanism of ecQueF reducing preQ₀ to preQ₁ is shown.** Residues involved in catalysis are indicated. Also shown are residues involved in closing up the binding site of preQ₀ and targeted by mutational analysis in this study. The amino acid residues are arranged to indicate their relative positions in the enzyme structure. Amino acid numbering of ecQueF is used. The dashed line indicates an electrostatic stabilization.

Scheme 2. **The two-step kinetic mechanism of preQ₀ binding is shown.** E is free ecQueF. E•preQ₀ indicates the noncovalent complex. E-preQ₀ indicates the covalent thioimide adduct.

Scheme 3. **The proposed kinetic mechanism of preQ₀ conversion by L191A and I192A variants of ecQueF via partitioning of the imine intermediate between reduction to preQ₁ and hydrolysis to 7-formyl-7-deazaguanine is shown.** Covalent (-) and noncovalent (•) binding of substrates/products and coenzymes to the enzyme are indicated. Rate constant numbering starts from Scheme 2 with the assumption that enzyme was saturated with NADPH. Nitrile reduction to imine (k₅) and imine reduction to preQ₁ (k₆) are isotope-sensitive steps. Coenzyme exchange in imine intermediate is assumed to be in rapid equilibrium.

Scheme 4. **A hypothetical kinetic mechanism of ecQueF-imine complex dissociation induced by preQ₀ is shown.** E is the free enzyme. The enzyme-bound preQ₀ and imine are indicated by subscript. E-imine indicates a preassociated complex of ecQueF with imine intermediate. The model is adapted from literature (28).

Figure 1. **Sequestration of preQ₀ is revealed in crystal structures of vcQueF in apoform (C194A variant, slate, PDB: 3RJ4) and holoenzyme complex with preQ₀ (H233A variant, green, PDB: 4GHM).** (A) A superimposition of apoenzyme and holoenzyme structures of the vcQueF homodimer is shown. (B-C) A close-up view of the binding site for preQ₀ in apoenzyme (B) and holoenzyme (C) is shown. The orientation of NADPH binding is from the crystal structure of the ternary complex of enzyme, preQ₀ and NADPH (vcQueF R262L variant, PDB: 3UXJ). Despite missing density for the nicotinamide ring in the protein-bound NADPH (dark gray), the likely orientation of the nicotinamide moiety can be inferred from the binding positions of preQ₀ and the structurally resolved portion of NADPH. Binding of NADPH in ecQueF was also examined with structure modeling (21). The ecQueF residues corresponding to the vcQueF residues shown in panels A-C are: Glu89, Ser90, Cys190, Leu191, Ile192, Asp197, Phe228, His229 and Glu230. The preQ₀, NADPH, and amino acids in the binding pocket are indicated by element-based colors.

Figure 2. **ITC analysis of preQ₀ binding to ecQueF variants at 25 °C is shown.** E89A (A, 58 µM), E89L (B, 60 µM), L191A (C, 9.4 µM), I192A (D, 10 µM), and F228A (E, 37 µM) were used. The preQ₀ solution (1 mM for E89A and E89L, 0.25 mM for L191A and I192A, 0.8 mM for F228A) was titrated into the enzyme solution. The obtained thermodynamic parameters are summarized in Table 1. The c values (c = [dimeric protein]/Kₐ) obtained from the experiments were 5 for the E89A, 6 for the E89L, 232 for the L191A, 525 for the I192A and 14 for the F228A variant.

Figure 3. **Stopped-flow progress curves of thioimide formation by different ecQueF variants are shown together with fitting results.** The thioimide formation by the ecQueF variants was recorded by absorbance at 380 nm. E89A (A, 33 µM), L191A (B, 35 µM), I192A (C, 57 µM) or F228A (D, 53 µM) variant was mixed with varied concentration of preQ₀ (bottom to top; 15, 30, 45, 60, 120, and 300 µM). The DMSO concentration did not exceed 2%. Black dashed lines are the averaged data from triplicate measurements. Global fitting was performed using the two-step binding mechanism in Scheme 2. The fits are shown with orange lines. The obtained kinetic constants are summarized in Table 2.
Figure 4. *H NMR analysis of the products formed on preQ₀ conversion by ecQueF wildtype and variants thereof is shown.* (A) Enzymatic routes of conversion of preQ₀: two-fold reduction gives preQ₁ while single reduction results in an imine which on release to water decomposes to 7-formyl-7-deazaguanine. (B) *H NMR spectra of product mixtures obtained with the different enzymes are shown together with spectra of authentic reference material. Phosphate buffer (100 mM, Na₂HPO₄-NaH₂PO₄, pH 7.5, 99.8% D₂O; pD = pH meter reading + 0.4), additionally containing 50 mM KCl and 1.15 mM tris(2-carboxyethyl)phosphine, was used for the reactions of ecQueF wildtype and Glu⁸⁹ variants. NADPH (1.5 mM) and preQ₀ (0.5 mM) were added to the enzyme solution. The total reaction volume was 0.6 mL. The spectrum of 7-formyl-7-deazaguanine (3 mM) was obtained in the phosphate buffer. The preQ₀ reduction with L191A variant (6 μM) was conducted in Tris buffer (30 mM, pH 7.5) containing 50 mM KCl and 1.15 mM tris(2-carboxyethyl)phosphine. All peaks in the spectra of the ecQueF L191A reaction were slightly shifted by 0.03-0.06 ppm due to the presence of enzyme. The spectra of preQ₀, preQ₁, and coenzyme were compared with the previously reported spectra of these components (7, 36, 37).

Figure 5. HPLC-MS analysis of the products formed on preQ₀ conversion by ecQueF wildtype and variants thereof is shown. The enzyme concentration was varied: 1 μM for wildtype (A), 40 μM for E89A (B), and 20 μM for each I192A (C) and F228A (D). The preQ₀ concentration was 200 μM and the NADPH concentration was 500 μM. Incubation at 25 °C proceeded for 3-12 h. The total reaction volume was 1.5 (A, B) and 0.1 mL (C, D). (A-B) Tris buffer (100 mM, pH 7.5) containing 50 mM KCl and 1.15 mM tris(2-carboxyethyl)phosphine was used for wildtype enzyme and E89A variant. The Tris-imine complex (¹H⁺, 282) formed in solution between 7-formyl-7-deazaguanine and Tris was separated at 9.2 min. (C-D) Phosphate buffer (100 mM Na₂HPO₄-NaH₂PO₄, pH 7.5) containing 50 mM KCl was used for I192A and F228A variant. The preQ₁ was separated at 12.8 min (C) but it was not detectable in the reaction of the F228A variant (D).

Figure 6. Product distribution analysis for preQ₀ reduction by the ecQueF variants is shown. The relative proportion of preQ₁ and 7-formyl-7-deazaguanine in total product is shown in dependence of the molar ratio of preQ₀ and enzyme ([preQ₀]/[E]) used in the reaction. The panels show A, E89A; B, E89L; C, L191A; D, I192A; E, F228A. Phosphate buffer (100 mM Na₂HPO₄-NaH₂PO₄, pH 7.5), additionally containing 50 mM KCl and 1.15 mM tris(2-carboxyethyl)phosphine, was used. The enzyme solution (10, 50, and 100 μM) was mixed with preQ₀ (25 - 500 μM) and NADPH (0.6 or 1.5 mM). After incubation (30 °C, 2-24 hours), the samples were analyzed by LC-MS and HPLC. Open circles show preQ₁ and closed circles show 7-formyl-7-deazaguanine. The dotted line is a hyperbolic fit to the relative product proportion of 7-formyl-7-deazaguanine, and its maximum relative content is shown.
Imine intermediate during nitrile reduction by QueF

Scheme 1
Scheme 2

\[ E + \text{preQ}_0 \xrightarrow{k_1} E\text{-preQ}_0 \xrightarrow{k_2} \text{E-preQ}_0 \xrightarrow{k_3} \text{E-preQ}_0 \]
Imine intermediate during nitrile reduction by QueF

Scheme 3

\[ E \text{-preQ}_0 \cdot \text{NADPH} \xrightarrow{k_5} E \text{-imine} \cdot \text{NADP}^+ \quad \text{reverse reaction} \]

\[ E \text{-imine} \cdot \text{NADPH} \xrightarrow{k_6} E + \text{preQ}_1 + \text{NADP}^+ \]

\[ E \xrightarrow{k_7} H_2O \]

\[ E + 7\text{-formyl-7-deazaguanine} + \text{NADPH} \]
Scheme 4

\[ E_{\text{amine}} \overset{k_d}{\underset{k_a}{\rightleftharpoons}} E \sim \text{amine} \xrightarrow{H_2O \; \; k_{esc}} E + \text{7-formyl-7-deazaguanine} \]

\[ E_{\text{preQ0}} \xrightarrow{k_{on}} \text{preQ0} \]
Figure 2

Imine intermediate during nitrile reduction by QueF
Figure 3

Imine intermediate during nitrile reduction by QueF
Imine intermediate during nitrile reduction by QueF

Figure 4

A

B

wildtype enzyme

7-formyl-7-deazaguanine

E89A variant

E89L variant

L191A variant

Chemical shift (ppm)
Figure 5

Imine intermediate during nitrile reduction by QueF
Evidence of a sequestered imine intermediate during reduction of nitrile to amine by the nitrile reductase QueF from *Escherichia coli*

Jihye Jung and Bernd Nidetzky

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