Kinetic Analysis and Probing with Substrate Analogues of the Reaction Pathway of the Nitrile Reductase QueF from *Escherichia coli*†

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The enzyme QueF catalyzes a four-electron reduction of a nitrile group into an amine, the only reaction of this kind known in biology. In nature, QueF converts 7-cyano-7-deazaguanine (preQ0) into 7-aminomethyl-7-deazaguanine (preQ1) for the biosynthesis of the tRNA-inserted nucleoside queuosine. The proposed QueF mechanism involves a covalent thioimide adduct between preQ0 and a cysteine nucleophile in the enzyme, and this adduct is subsequently converted into preQ1 in two NADPH-dependent reduction steps. Here, we show that the *Escherichia coli* QueF binds preQ0 in a strongly exothermic process (ΔH = −80.3 kJ/mol; −TΔS = 37.9 kJ/mol, Kd = 39 nm) whereby the thioimide adduct is formed with half-of-the-sites reactivity in the homodimeric enzyme. Both steps of preQ0 reduction involve transfer of the 4-pro-R-hydrogen from NADPH. They proceed about 4–7-fold more slowly than trapping of the enzyme-bound preQ0 as covalent thioimide (1.63 s⁻¹) and are thus mainly rate-limiting for the enzyme’s *kcat* (0.12 s⁻¹). Kinetic studies combined with simulation reveal a large primary deuterium kinetic isotope effect of 3.3 on the covalent thioimide reduction and a smaller kinetic isotope effect of 1.8 on the imine reduction to preQ1. 7-Formyl-7-deazaguanine, a carbonyl analogue of the imine intermediate, was synthesized chemically and is shown to be recognized by QueF as weak ligand for binding (ΔH = −2.3 kJ/mol; −TΔS = −19.5 kJ/mol) but not as substrate for reduction or oxidation. A model of QueF substrate recognition and a catalytic pathway for the enzyme are proposed based on these data.

Reduction of a nitrile group to a primary amine is a transformation well known and important to synthetic chemistry (1–4). Its unique biological equivalent is the reaction catalyzed by QueF enzymes (5). QueF was discovered from the biosynthetic pathway of the tRNA-modified nucleoside queuosine (Q)² where it was shown to utilize NADPH for the conversion of 7-cyano-7-deazaguanine (preQ0) into 7-aminomethyl-7-deazaguanine (preQ1) (Fig. 1). Until today, QueF remains the only enzyme known to promote the intricate nitrile-to-amine chemistry. The catalytic mechanism of QueF is therefore of significant fundamental interest. In addition, it also has potential application relevance. Q is a nucleoside from the wobble position of the tRNAs for Asn, Asp, His, and Tyr, and it is thus required for translation efficiency and fidelity (6). Despite its ubiquitous occurrence in nature, Q is synthesized *de novo* only in bacteria (7–9). The enzymes of the Q pathway, QueF in particular, due to its apparent uniqueness, therefore represent promising drug targets to combat bacterial infections selectively. Moreover, due to the hazardous conditions required in the chemical reaction (1–4), “greener” routes of nitrile reduction are highly demanded. A biocatalytic route going by the QueF mechanism presents an interesting option.

QueF enzymes have been characterized structurally from unimodular and bimodular classes (10, 11), and the herein studied QueF from *Escherichia coli* is a bimodular protein. The bimodular QueF is a functional homodimer, whereas the unimodular QueF folds into a homodecamer (a dimer of pentamers) (5, 10, 11). In both types of QueF, the active site is positioned at a structural interface, created from different subunits in the unimodular QueF and from the tandem tunneling-fold domains of the same subunit in the bimodular QueF. The arrangement of catalytic groups is highly conserved in both enzymes (10–12).

The basic QueF mechanism was delineated in biochemical and structural studies of the unimodular enzyme from *Bacillus subtilis* (Fig. 1) (11, 13). The binding of preQ0 involves a large induced fit, resulting in the functional active site to be formed and to become completely secluded from solvent. Cys655 then attacks the nitrile group to build a covalent thioimide, where it was possible to trap both in the crystal and in solution. QM/MM calculations on the bimodular QueF from *Vibrio cholerae* suggested a role for Asp201 (Asp62 in *B. subtilis* QueF)

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‡‡†§ These abbreviations used are: Q, queuosine; preQ0, 7-cyano-7-deazaguanine; preQ1, 7-aminomethyl-7-deazaguanine; QM/MM, quantum mechanics/molecular mechanics; 7-formyl-preQ0, 7-formyl-7-deazaguanine; ecQueF, *E. coli* QueF; 2-deamino-preQ0, 7-cyano-2-deamino-7-deazaguanine; 6-deoxy-preQ0, 7-cyano-6-deoxy-7-deazaguanine; LTQ, linear ion trap; FT, Fourier transform; KIE, kinetic isotope effect; ITC, isothermal titration calorimetry.
as a proton shuttle during the thioimide formation (14) (Fig. 1). Hydride reduction of the thioimide by NADPH, also under protonic assistance by Asp, subsequently gives a covalent hemithioaminal. NADP$^+$ is exchanged by NADPH and the C–S bond cleaved to yield a non-covalent imine intermediate, which is finally reduced to preQ$_1$. The QM/MM calculations suggested that Asp$^{201}$ protonates the Cys$^{194}$ in *V. cholerae* QueF on hemithioaminal breakdown and provides electrostatic stabilization during the subsequent hydride transfer (14). Free energy barrier analysis indicated that the imine reduction is slowest among the chemical steps of the computed reaction pathway for the *V. cholerae* QueF. In *B. subtilis* QueF, the $k_{cat}$ (0.011 s$^{-1}$) (5, 13) is much smaller than the rate constant of thioimide formation (2.78 s$^{-1}$) (11). Therefore, this also locates the rate-determining step in the sequence of steps involved in the NADPH-dependent reductions.

Despite these preceding insights into QueF structure and function, fundamental issues of the enzymatic mechanism remain. Which interactions between QueF and preQ$_0$ are required in the induced-fit binding of the substrate or in the formation of a competent thioimide intermediate? How does NADPH bind and which of its diastereotopic hydrogens at the nicotinamide C4 is used for hydride transfer in each reduction step? Is the very small $k_{cat}$ of QueF really limited by one of the hydride transfer steps, as QM/MM calculations have suggested (14), or are rather the associated physical steps (which were not analyzed computationally) slow? To address these questions, we prepared tailored analogues of preQ$_0$, in which specific recognition sites for QueF binding were removed or altered by targeted organic synthesis (Fig. 2). Together with the native preQ$_0$, we used these analogues to characterize the kinetics and thermodynamics of substrate binding by the QueF enzyme from *E. coli* (ecQueF). We applied transient and steady-state kinetic analysis in combination with kinetic simulations to characterize and to determine rate constants for the individual steps of the enzymatic reaction pathway. We also developed a synthesis of 7-formyl-7-deazaguanine (Fig. 2) as a carbonyl surrogate of the imine intermediate of the QueF reaction. We characterized the binding of this analogue to ecQueF and examined its reactivity as substrate for enzymatic reduction or oxidation. A model of substrate binding recognition by ecQueF is suggested based on the evidence presented. We show that each reduction step by the enzyme involves transfer of the pro-4-$R$-hydrogen of NADPH. We also show that the hydride transfer steps are rate-determining in the overall reaction.

**Results**

**ITC Study of Substrate and Coenzyme Binding to ecQueF**—ITC experiments were conducted to determine the thermodynamic characteristics of substrate and NADPH binding to
ecQueF (25 °C, pH 7.5; 100 mM sodium phosphate buffer). The obtained thermodynamic parameters are summarized in Table 1. A-D, preQ0 (0.25 mM, A), 2-deamino-preQ0 (5 mM, B), 7-formyl-preQ0 (3 mM, C), and NADPH (2.9 mM, D) were titrated to wild type ecQueF solution (10 μM for preQ0, 200 μM for 2-deamino-preQ0, and NADPH, and 150 μM for 7-formyl-preQ0). The DMSO concentration of the enzyme solutions varied as follows: 1.2% for preQ0, 2.5% for 2-deamino, and 7-formyl-preQ0, and no DMSO for NADPH. The c values (c = [protein]/Kd) obtained from the experiments were 259 for preQ0, and 12 for NADPH. Because of the relatively low c values for 2-deamino-preQ0 and 7-formyl-preQ0 (0.9 and 1.1, respectively), thermodynamic parameters for the two ligands were calculated under a fixed molar stoichiometry (ligand bound/ecQueF homodimer) of 1 that was obtained from the preQ0 binding measurement. E and F, preQ0 (0.8 mM, E) and NADPH (1.2 mM, F) were titrated to a solution of the C190A variant (60 μM, E; 92 μM, F). The thermodynamic characteristics of NADPH binding to the C190A variant were not affected by the presence of DMSO. G, solution of the preQ0-C190A complex (104 μM) was titrated into the NADPH solution (11 μM). The DMSO concentration in both ligand and substrate solution was 2% for preQ0 and 1.4% for NADPH. The c values were 11 for preQ0 and 5 (C190A variant) or 4 (the preQ0-C190A complex) for NADPH. H, dependence of the apparent binding enthalpies (ΔHapp) of preQ0 binding to the wild type (closed circles) and C190A variant (open circles) as a function of buffer ionization enthalpy (ΔHion) is shown. The slope is +0.76 for the wild type enzyme (R² = 0.996) and +0.51 for C190A variant (R² = 0.921). The thermodynamic parameters are summarized in Tables 1 and 2.

The results are shown in Fig. 3 and the parameters calculated from the data are summarized in Table 1. The binding of preQ0 was a strongly exergonic process (ΔG <0) in which a highly favorable enthalpy term (ΔH) compensated the nonfavorable contribution from the binding entropy (−TΔS). The 39 mM dissociation constant (Kd) thus determined was in good agreement with the Kd estimate of 36 mM obtained from a gel filtration analysis of the unimodular QueF from B. subtilis (11). The number of preQ0-binding sites occupied in the ecQueF homodimer was determined from the ITC data to be unity (±5%). Binding models comprising two equivalent or distinct binding sites/enzyme homodimer were also examined but were inconsistent with the experimental results. The half-of-the-sites reactivity of ecQueF implied by these findings was in line with the structural suggestion that the minimal catalytic unit of V. cholerae QueF should be the protein homodimer (10).
The 2-deamino deactivation of preQ\(_0\) resulted in a drastic weakening of the substrate binding, reflected in a 21.5 kJ/mol loss in the \(\Delta G\) stabilizing the ecQueF-bound state, comparing 2-deamino-preQ\(_0\) to preQ\(_0\). This is equivalent to a \(6 \times 10^{3}\)-fold increase in the \(Kd\) of the 2-deaminated substrate. The effect in \(\Delta G\) was due to a strongly lowered enthalpy contribution to the binding of 2-deamino-preQ\(_0\) as compared with preQ\(_0\) (\(\Delta H = +51.4\) kJ/mol). The entropy term was, however, less unfavorable for the binding of 2-deamino-preQ\(_0\) than it was for the binding of preQ\(_0\). It thus compensated to some degree for the very large spontaneous process characterized by a negative \(\Delta G\). Despite the relatively small difference in \(\Delta G\) of binding (\(\Delta\Delta G = -4\) kJ/mol), the thermodynamic signature of NADPH binding to the C190A-preQ\(_0\) complex was quite distinct from that of NADPH binding to the wild type enzyme. The binding stoichiometry was 1 NADPH/enzyme dimer. The results are summarized in Table 1. As in the wild type enzyme, the \(\Delta H\) of preQ\(_0\) binding to the C190A variant was strongly dependent on the buffer used (phosphate, HEPES, Tris). The \(\Delta G\) of binding was however hardly affected by the buffer (Table 2). We determined from the data that 0.46 \pm 0.27 protons were taken up by the C190A variant on binding of preQ\(_0\) (pH 7.5; Table 1).

The binding of NADPH by QueF is generally not well understood. We show here (Fig. 3; Table 1) that NADPH was bound by the free forms of wild type ecQueF and the C190A variant thereof. With both enzymes the binding occurred in an enthalpy-driven fashion. The overall binding was highly exergonic (\(\Delta H = -25.4\) kJ/mol) and hence turned from being non-favorable to favorable for the wild type enzyme. This reflected a drastically lowered enthalpy contribution to the binding (\(\Delta H = +35.6\) kJ/mol) in the enzyme variant as compared with wild type ecQueF. The entropy term of preQ\(_0\) binding to the C190A variant was positive (\(\Delta S = +14.6\) kJ/mol). The results are summarized in Table 1. As in the wild type enzyme, the \(\Delta H\) of preQ\(_0\) binding to the C190A variant was strongly dependent on the buffer used (phosphate, HEPES, Tris). The \(\Delta G\) of binding was however hardly affected by the buffer (Table 2). We determined from the data that 0.46 \pm 0.27 protons were taken up by the C190A variant on binding of preQ\(_0\) (pH 7.5; Table 1).

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The preQ₀ complex lacked a contribution from entropy (\(-T \Delta S = +0.07 \text{ J/mol}\)), and the enthalpy contribution was lowered (\(\Delta H = +16.2 \text{ J/mol}\)) in comparison with NADPH binding to the free enzyme. Note: NADPH binding to the covalent adduct between wild type enzyme and preQ₀ does not give a stable complex because catalytic reduction takes place. It was therefore not examined with ITC.

Substrate Binding and Covalent Thioimide Formation—As in \(B.\) \(s\)ubtilis QueF (11), the substrate binding in ecQueF was traceable by both quenching and blue shifting of the protein’s Trp fluorescence (Fig. 4, A and B). Trp²⁵⁷ and Trp²⁹⁸ in the substrate-binding pocket (Fig. 5) are the likely reporter groups. On addition of preQ₀, the ecQueF fluorescence (excitation, 280 nm) was quenched, and a slight blue shift in the maximum wavelength of emission (\(\lambda_{max} = 334 \rightarrow 330 \text{ nm}\)) was observed. The quenching yield increased dependent on the molar ratio of preQ₀ and the enzyme homodimer used. It reached a maximum of about 0.7 when this ratio was approximately unity (Fig. 4A).

The titration of ecQueF with 2-deamino-preQ₀ and 6-deoxo-preQ₀ also produced fluorescence quenching, but the corresponding yields were lower, 0.21 and 0.36, respectively (Fig. 4B), than for preQ₀. The 7-formyl-preQ₀ behaved differently. Its incubation with ecQueF caused a slight enhancement of the protein fluorescence (10%), and the \(\lambda_{max}\) was up-shifted (334 → 336 nm), as shown in Fig. 4B. Binding of preQ₀ to the C190A variant of ecQueF caused only a small amount of fluorescence quenching (15%).

Formation of the covalent thioimide between ecQueF and preQ₀ was detectable by appearance of an absorbance band with maximum absorption at 380 nm (Fig. 4C). A molar extinction coefficient of 10.02 \((\text{M}^{-1} \text{cm}^{-1})\) was determined. With this, we could show that the preQ₀ was linked to each
ecQueF dimer in a 1:1 stoichiometry. Results of protein mass analysis (Fig. 4D) were in good agreement with these findings, showing that the sample from incubation of ecQueF with preQ₀ was composed of about equal amounts of the monomeric enzyme-preQ₀ adduct (43%) and also the monomeric apoenzyme (57%). Reactivity of half-of-the-sites in the ecQueF dimer was thus strongly supported. Time-resolved titration analysis in stopped-flow experiments also revealed that a molar preQ₀ concentration equivalent to that of the ecQueF dimer was sufficient for complete covalent conversion of the available active sites (Fig. 6).

Incubation of ecQueF in the presence of 2-deamino-preQ₀ also gave rise to a new absorbance band, but compared with the preQ₀ thioimide adduct, its maximum absorption was at a lower wavelength of around 340 nm (Fig. 4C). A molar extinction coefficient of 10.50 ± 0.04 mM⁻¹ cm⁻¹ was determined. Contrary to preQ₀, a substrate/enzyme molar ratio of about 13 was required so that covalent conversion of ecQueF with 2-deamino-preQ₀ was complete (Fig. 4E). Protein mass analysis confirmed the presence of the covalent adduct and furthermore revealed that the portion of total ecQueF covalently modified (maximum, ~40–50% of dimer) was strongly dependent on the amount of 2-deamino-preQ₀ offered (Fig. 4D). A $K_d$ of 155 μM was determined for 2-deamino-preQ₀ binding to ecQueF (Fig. 4F), in useful accordance with the results of the ITC experiments (Table 1). No evidence of covalent linkage formation between ecQueF and 6-deoxo-preQ₀ or ecQueF and 7-formyl-preQ₀ was found in spectrophotometric titration analysis. Likewise, the C190A variant did not show evidence of covalent binding of preQ₀, as expected.

Addition of preQ₀ to a pre-incubated mixture of ecQueF with any of the three preQ₀ analogues reinstated in each case the effect on fluorescence quenching and “thioimide absorbance band” formation that preQ₀ alone had. Therefore, this showed effective competition of preQ₀ with the other ligands for binding to ecQueF and also demonstrated the reversibility of thioimide covalent linkage formation by 2-deamino-preQ₀ (Fig. 4C).
Stereospecificity of ecQueF for Hydrogen Transfer from NADPH—This central characteristic of the QueF mechanism has not been elucidated in previous biochemical and structural studies of the enzyme. We used NADPH, (4R)-[2H]NADPH, or (4S)-[2H]NADPH for the reduction of preQ0 and analyzed with 1H NMR the products formed, NADP+ and preQ1. Results are shown in Fig. 7. In all reactions, the conversion of preQ0 into preQ1 was traced by monitoring the singlet signal of the deaza-guanine C8 hydrogen, which appears at 7.42 ppm in substrate and at 6.76 ppm in product. When NADPH was used, the proton spectra of the product exhibited a singlet peak at 4.08 ppm, which is assigned to the hydrogens of the aminomethyl carbon of preQ1 (Fig. 7A). This peak was absent from the spectra of the preQ1 released in the reaction with (4R)-[2H]NADPH (Fig. 7B). Besides, the product of the oxidation of (4R)-[2H]NADPH was NADP+ and not 4-[2H]NADP+. The hydrogen at position C4 on the nicotinamide ring was identified unequivocally from the doublet signal at 8.67–8.68 ppm (Fig. 7B). When the alternatively deuterium-labeled (4S)-[2H]NADPH was used, only the hydrogen was transferred in the enzymatic reaction of preQ0, and authentic preQ1 was obtained together with 4-[2H]NADP+ (Fig. 7D). These results are clear in showing that ecQueF is specific for transferring the 4-pro-R-hydrogen from NADPH, and it does so in both hydride reduction steps of the catalytic conversion of preQ0 to preQ1.

Steady-state Kinetic Study of ecQueF—Based on the measurement of both NADPH consumption and amine product formation (Fig. 8), we showed that besides preQ0, the 2-deamino derivative was also a workable substrate of ecQueF (see below). The 6-deoxy-preQ0 was inactive within the detection limits of the assay used. The 7-formyl-preQ0 (100 μM) was not reduced by ecQueF in the presence of NADPH (150 or 300 μM) nor was it oxidized in the presence of NADP+ (300 μM). Absence of activity with this substrate was shown at different pH values in the range 5.5–7.5 (100 mM ammonium acetate buffer, pH 5.5 or pH 6.7; 100 mM Tris buffer, pH 7.5). Considering the poor solubility of 7-formyl-preQ0 in water, we added 7-formyl-preQ0 (dissolved in DMSO) directly to the enzyme solution contain-
ing NADPH so that enzymatic reduction could precede substrate precipitation. However, no reaction was observed, despite the use of ecQueF concentrations (7.6–30.0 μM) that would have allowed detection of just 0.1 enzyme turnover. Just to note, oxidation by NADP+ might have involved the 7-formyl group in native or hydrated form, requiring nucleophilic catalysis from active-site cysteine in the former but not the latter (15–18). Enzymatic conversion of 7-formyl-preQ0 was also analyzed in the presence of (NH4)2SO4 or NH4Cl (each at 50 mM) and NADPH (3 mM). No reaction was observed, indicating the absence of an oxidation of preQ1 under the conditions used.

We furthermore examined the reverse reaction of ecQueF, measuring reduction of NADP+ (100 μM), and product formation associated with it, in the presence of preQ1 (40 μM). Using the standard pH of 7.5 but also elevated pH values of up to 9.0 in order to facilitate deprotonation of the 7-amino group, there was no conversion of preQ1 above the detection limit, which was roughly 0.1 turnover of the molar enzyme concentration used (20–80 μM). To prevent the product attached to the enzyme from escaping detection, we denatured the protein with methanol and repeated the HPLC analysis, thereby confirming the absence of an oxidation of preQ1 under the conditions used.

Initial rate analysis showed that the Km value for NADPH was not affected by variation of the preQ0 concentration in the range 5–100 μM. The lower end limit of the substrate concentration was defined by the time resolution and sensitivity of the spectrophotometric assay. Considering the 39 nm Kd value for preQ0, only a negligible portion of free enzyme could have existed at steady state under these conditions. Therefore, this explains a coenzyme Km apparently independent of the substrate concentration. Results are summarized in Table 1. In terms of kcat, 2-deamino-preQ0 was an equally good nitrile substrate as was preQ0. The NADPH Km was, however, elevated about 4-fold in the reaction with 2-deamino-preQ0 (Table 1). A huge difference existed in the substrate Km, consistent with results of the ITC binding study of preQ0 and 2-deamino-preQ0.

**Transient Kinetic Study and Kinetic Simulation**—To characterize individual steps of the ecQueF reaction, a transient kinetic analysis in stopped-flow experiments was performed. Fig. 6 shows representative time courses of thioimide formation upon mixing the enzyme with preQ0, in the absence of NADPH. The absorbance increased exponentially with an amplitude determined by the limiting concentration of ecQueF dimer or preQ0. The rate constants kobs (1.35 ± 0.15 s–1) were independent of the initial concentrations of enzyme and preQ0. Using global fitting with COPASI, a two-step binding-reaction model, E + S ⇌ ES ⇌ E-S, was useful to describe the data recorded at varied enzyme and substrate concentrations (Fig. 6, A and B). It involved a relatively fast binding of preQ0 with a dissociation constant (Kd kinetic) of 2.63 (±0.01) μM that was followed by a slower and, within the limit of accuracy of the method used, effectively irreversible transformation of the non-covalent ES into the covalent complex E-S with a rate constant (k3) of 1.63 (±0.01) s–1 (Table 3). The obtained k3 was in a good agreement with the experimentally determined kobs. The relationship between the overall 39 nm dissociation constant determined by ITC and the kinetically determined parameters, i.e. Kd overall = kcat/kinetic/(1 + k3/Kd), can be used to calculate an approximate value of 0.024 s–1 for the thioimide release rate constant (k3).

Fig. 9A shows absorbance time traces at 340 and 380 nm for a reaction under conditions of a single turnover of the enzyme present. Time traces for reactions under multiple turnover conditions in which a limiting enzyme concentration (5 μM) was used are shown additionally in Fig. 9, B–D. The single-turnover time traces were characterized by a fast initial increase in absor-

### TABLE 3

| Kinetic constants | Kinetic constants |
|------------------|------------------|
| Kd               | (2.63 ± 0.01) × 10^-6 M |
| kcat             | 1.63 ± 0.01 s^-1 (0.024 s^-1) |
| k3               | (20.4 ± 2.0) × 10^-5 M |
| k4               | (3.23 ± 0.02) × 10^-6 M |
| k5               | 5.2 ± 0.2 s^-1 (0.56 s^-1) |
| k6               | 0.234 ± 0.002 s^-1 (3.3 ± 0.1) |
| k7               | ≤0.1 × 10^-6 M |
| k8               | 0.47 ± 0.002 s^-1 (1.8 ± 0.1) |
| k9               | 0.15 ± 0.001 s^-1 (2.8 ± 0.2) |
| k10              | (23.7 ± 2.0) × 10^-5 M |

- k5 was obtained from the relationship kcat = kcat/kinetic/[1 + k3/Kd]. The kcat/kinetic was 39 nm. k3 was not included in the global fitting in COPASI. If it was included, its value was close to zero. Therefore, the kcat value shown probably gives an upper bound for the dissociation rate constant.

- k4 was obtained from the relationship kcat = kcat/kinetic/[1 + k3/Kd]. Note that k3 was not included in the global fitting in COPASI. If it was included, its value was close to zero. The kcat value shown probably gives an upper bound for the dissociation rate constant.

- KEs were obtained by comparing rate constants of preQ0 reduction by NADPH, as shown in the table, and 4Δ'H,NADPH (k1 = 0.069 ± 0.001 s^-1, k10 = 0.25 ± 0.01 s^-1, k11 = 0.053 ± 0.001 s^-1).
bance, at 380 nm in particular, which reflected formation of the covalent intermediate (Fig. 9A). The subsequent decrease in absorbance was due to NADPH consumption mainly but also included the effect of the concomitant decay of the intermediate, especially when single-turnover conditions were used. Fitting the initial increase in absorbance with an exponential function (Fig. 9D), the corresponding $k_{obs}$ showed a hyperbolic dependence on the NADPH concentration, with a $K_d$ of 3.0 ($\pm$ 1.6) $\mu$M and a maximum value of 7.32 ($\pm$ 0.46) s$^{-1}$ (Fig. 9E). The presence of NADPH therefore resulted in a 4.5-fold speeding up of the formation of the thioimide intermediate. The decrease in absorbance at both 340 and 380 nm was best fit with a single exponential (single-turnover reactions) or a straight line (multiple-turnover reactions). The rate constants calculated from these fits were hyperbolically dependent on the NADPH concentration, with a $K_d$ of 10 ($\pm$ 2) $\mu$M and a maximum value of 0.15 ($\pm$ 0.01) s$^{-1}$, which is similar to the $k_{cat}$ (Fig. 9F).

The kinetic model in Scheme 1 was used to fit the data from all experiments, comprising a large set of averaged stopped-flow time traces from 28 independent reaction conditions, involving variation in NADPH concentration between 10 and 150 $\mu$M. Fig. 9, A–C, compares the experimental progress curves to the corresponding fitting results, which reveals useful agreement between the two. Table 3 summarizes the rate and binding constants thus determined. The kinetic steps of thioimide and imine reduction were the slowest in the enzymatic pathway, and their corresponding rate constants were similar. Note that based on a decrease in NADPH absorbance (at 340 nm) alone, it would not have been possible to distinguish the two steps. However, because the thioimide reduction also involves concomitant decrease in absorbance at 380 nm, the first hydride transfer step has a spectral signature different from the second. This could be used to determine the rate constant associated with each step. The rate constants of the microscopic reaction steps (Table 3) were consistent with the $k_{cat}$ measured at steady state (Table 1) and also with the rate constants from stopped-flow experiments. Fig. 9G shows the distribution of different enzyme forms in a single-turnover stopped-flow reaction. The enzyme complexes with preQ$_0$ ($E_{preQ_0}$), present as imine intermediate ($E_{imine}$), and bound to NADPH ($E_{NADPH}$) were negligible and are therefore not shown. The gray shaded area in G indicates the phase of covalent thioimide formation also shown in A. H, simulated curves of NADPH consumption and of formation of NADP$^+$ and preQ$_0$ are shown. The disappearance of free preQ$_0$ was too fast to be represented here ($\leq$ 1 s).
**Reaction Pathway of the Nitrile Reductase QueF from E. coli**

\[ E + \text{preQ}_0 + \text{NADPH} \xrightarrow{k_1} E_{\text{preQ}_0} + \text{NADPH} \xrightarrow{k_3} E_{\text{preQ}_0} + \text{NADPH} \]

\[ \xrightarrow{k_4} \text{E}_{\text{min}} + \text{NADP}^+ + \text{NADPH} \]

*SCHEME 1. Proposed kinetic mechanism of preQ₀ reduction by ecQueF is shown. E is the free ecQueF, and the enzyme-bound preQ₀ and NADPH are indicated by subscript and superscript, respectively. An asterisk on E indicates the covalent thioimide linkage between preQ₀ and enzyme. E_{min} indicates enzyme with bound imine intermediate. The steps included in kinetic simulations are indicated in light gray. NADPH binding to E was excluded from the kinetic simulation and is indicated in light gray. The kinetic constants from simulation and fitting of reaction time courses are shown in Table 3. The following rate constants were obtained only as ratios to give the corresponding dissociation constants in rapid equilibrium: \( K_{\text{p}} = k_{-1}/k_1 \) (green); \( K_{\text{d}} = k_6/k_5 \) (light blue); \( K_{\text{s}} = k_{10}/k_7 \) (blue); and \( K_{\text{d}} = k_{11}/k_{13} \) (purple). The chemical and isotope-sensitive steps are thioimide reduction (\( k_{11} \)) and imine reduction (\( k_{13} \)). These steps were assumed to be effectively irreversible under the reaction conditions used. Likewise, formation of the thioimide intermediate (\( k_3 \) and \( k_9 \)) was assumed to be irreversible in the fitting because the rate constants of the reverse reaction (\( k_4 \) and \( k_6 \)) are very small in comparison.

Complex and NADPH dissociation from the covalent adduct was not allowed, failed to describe the experimental time courses properly. Second, the extremely small dissociation constant for NADPH binding to the hemithioaminal/imine intermediate is worth noting. It suggests that NADPH was bound almost irreversibly by ecQueF at this step. Unless having this small value of \( K_{\text{p}} \), the dependence of the reaction on the NADPH concentration could not be properly explained. Third, the covalent ternary complex showed a much lower absorbance both at 340 nm (~54%) and 380 nm (~60%) than expected from the sum of the corresponding individual absorbances of NADPH and thioimide adduct. Note that the absorbance of NADPH was not affected by binding to ecQueF. The result is interesting as it suggests that the thioimide adduct and NADPH perturb each other electronically in the ternary complex. More interestingly, even the extent of this electronic perturbation was attenuated strongly when the ternary complex was formed from (4R)-\[^{2}H\]NADPH instead of NADPH. The electronic effect and its dependence on the isotopic substitution in NADPH might be explained by a “near-attack” ground-state conformer that involves close alignment, and perhaps even partial bonding, between the thioimide carbon and the C4 nicotinamide hydrogen of NADPH. Electronic preorganization of the ternary complex for efficient hydride reduction is therefore suggested.

**KIE Study**—Substitution of the reactive 4-pro-R-hydrogen of NADPH by deuterium caused a slowdown of the enzymatic preQ₀ reduction. The effect was expressed in a substantial primary KIE on both the maximum rate (\( \Delta V_{\text{max}} = 2.40 \pm 0.12 \)) and the catalytic efficiency for coenzyme (\( \Delta V_{\text{max}}/K_M = 2.68 \pm 0.41 \)).

Stopped-flow progress curves of preQ₀ reduction by (4R)-\[^{2}H\]NADPH under multiple-turnover reaction conditions are shown in Fig. 10. The corresponding single-turnover reactions are also presented there. Exponential fit of the initial absorbance increase revealed that the deuterium of NADPH did not cause slowdown of the formation of the thioimide intermediate (\( k_{\text{obs}} = 6.4 \pm 0.3 \text{ s}^{-1} \)), as expected. The dependence of \( k_{\text{obs}} \) on the NADPH concentration was also unaffected (\( K_s = 3.0 \pm 1.5 \mu M \)), as shown in Fig. 10, D and E. Rate constants determined from fits of the absorbance decrease in individual stopped-flow traces are shown on Fig. 10F. From the hyperbolic dependence of \( k_{\text{obs}} \) on the NADPH concentration, a maximum rate constant of 0.055 (± 0.003) s⁻¹ was obtained, and the corresponding \( K_d \) was 14 (± 3) \( \mu M \). Therefore, the deuteration of NADPH affected strongly the enzymatic rate, although the apparent binding was hardly influenced.

Results of a global fit of Scheme 1 to the data are shown in the figures, and the reaction constants determined are summarized in Table 3. Deuteration of the coenzyme caused slowdown of both reduction steps, but the effect was by far more pronounced on the thioimide conversion. The corresponding KIEs were determined as 3.3 ± 0.1 (thioimide reduction) and 1.8 ± 0.1 (imine reduction). The value of \( \Delta V_{\text{max}} \) can be explained from these two KIEs (Table 3).

**Discussion**

Groups of preQ₀ Required for Binding Recognition and Cova lent Adduct Formation by ecQueF—Structural and sequence-based comparisons reveal the bimodal QueFs (from *V. cholerae* and *E. coli*) to exhibit substrate binding pockets highly similar to that of their unimodular counterpart (*B. subtilis*) for which a preQ₀ complex structure has been determined (10–12). Evidence characterizing the substrate binding in ecQueF is therefore interpreted on the basis of a high degree of functional residue conservation in these enzymes. Comparing the changes in protein fluorescence, the heat released, and the amount of covalent thioimide adduct formed in titrations of ecQueF with preQ₀ (or analogues thereof), the overall substrate binding is found to be composed of three main elements. The initial accommodation of the substrate in the binding pocket lacks a spectroscopic signature in absorbance and fluorescence, but by analyzing the binding of the non-reactive substrate analogue 7-formyl-preQ₀, it becomes traceable with ITC. Results show that this first step in the binding is driven exclusively by loss in entropy, which probably reflects the desolvation of the substrate, the binding site in ecQueF, or both. The complete lack of an enthalpic contribution to the binding of 7-formyl-preQ₀ (Table 1) suggests that specific interactions are not developed between this ligand and the enzyme. The second step involves a protein conformational rearrangement, detectable mainly by ITC but also by fluorescence, which, in analogy to the induced-fit binding of preQ₀ in the *B. subtilis* enzyme (11), is assumed to preorganize and close up the ecQueF active site. Trp₁⁰⁹ of ecQueF (like the corresponding Trp₁¹⁹ of *B. subtilis* QueF) would thus be placed in a more hydrophobic microenvironment, with consequent effects on their fluorescence properties. The preQ₀ complex structure of the C55A variant of *B. subtilis* QueF adopts the “closed” conformation just as the
Covalent thioimide formation in multiple-turnover reaction is shown. Data, and the kinetic pathway that differ from preQ0 only in the chemical nature of preQ0 binding to ecQueF is strongly shaped by the energetics of covalent bond formation, which almost completely masks the effects of the non-covalent interactions.

On binding of preQ0 at pH 7.5, each ecQueF dimer was shown by ITC to take up 0.78 ± 0.09 protons from the bulk solvent. Preliminary evidence from time-resolved experiments performed in the stopped-flow apparatus and measuring proton uptake with a pH indicator further reveals that the proton uptake occurred concurrently with formation of the thioimide intermediate. A proton is required during conversion of the nitrile to the thioimide group. In the case that the observable proton uptake by ecQueF was an immediate consequence of this reaction, binding of preQ0 by an enzyme variant that is incapable of thioimide adduct formation would accordingly not involve proton uptake. Clear evidence of proton uptake also by the C190A variant therefore eliminated this mechanistic possibility. An alternative mode of protonation, occurring in consequence of the induced-fit protein conformational change in preQ0 binding, is therefore suggested. Identification of the groups involved in the protonation requires further study.

**Kinetic Pathway of ecQueF Involves Thioimide Reduction as the Rate-limiting Step**—Because preQ0 and NADPH both bind to free ecQueF, the kinetic mechanism appears to be random in principle. However, in the global simulation-fitting analysis with COPASI, binding of NADPH to the free enzyme appeared to be not kinetically significant, at least under the conditions used. In addition, although preQ0 forms a catalytically compe-
tent thioimide adduct with ecQueF even in the absence of NADPH, the preferred reaction path when neither preQ₀ nor NADPH is limiting appears to be through the non-covalent ternary complex (Scheme 1). Formation of the thioimide adduct is accelerated about 3.6-fold when NADPH is present, and it is relatively slow compared with the actual substrate binding steps (Table 3). The preQ₀ substrate binds in a stoichiometry of 1 per ecQueF dimer. The possibility of half-of-the-sites reactivity was also hinted at by the structure of V. cholerae QueF (10), suggesting that only a single NADPH would be bound in two possible orientations to load one of the two active sites. Based on the structural model of ecQueF, Wilding et al. (12) arrived at the same conclusion, suggesting however that only one of two catalytic centers in the protein dimer was accessible, whereas the other was buried deeply inside the protein. Evidence from the study of the inactive C190A variant of ecQueF showed that 1 NADPH was bound to each non-covalent complex between enzyme dimer and preQ₀.

According to Scheme 1, the observable kinetic parameters from initial rate measurements are related to rate constants as shown in Equations 1 and 2 (19).

\[
E/V_{\text{max}} = 1/k_9 + 1/k_{11} + 1/k_{14} \quad \text{(Eq. 1)}
\]

\[
K_{M} (\text{NADPH}) = K_{1} + K_{2} + K_{3} = (k_6 + k_{11})/k_5 + (k_9 + k_6)/k_7 + (k_{13} + k_{14})/k_{12} \quad \text{(Eq. 2)}
\]

Because of the slow breakage of the covalent thioimide linkage (Table 3), the preQ₀/K₁₄ value tends to be far too low to be experimentally measurable. The results in Table 3 show that \(V_{\text{max}}\) was limited, to a similar degree, by the rate constants of thioimide and imine reduction. Rate limitation solely by imine reduction, as suggested by Ribeiro et al. (14) from their computational study of the reaction of V. cholerae QueF, was ruled out for ecQueF based on the kinetic evidence. The stopped-flow progress curves were not consistent with a kinetic mechanism in which the first hydride transfer was faster than the second. The \(K_{M}(\text{NADPH})\) mainly reflects the binding of NADPH to the covalent adduct of ecQueF and preQ₀ (\(K_{1}\)). A reaction pathway in which the enzyme “loosens its grip” on NADPH while moving from the non-covalent to the covalent complex with the preQ₀ substrate requires comment; however, we believe there might be biological significance to it. Under the premise of ecQueF being present intracellularly in a molar concentration comparable with that of preQ₀, thioimide adduct formation from an enzyme-preQ₀-NADPH complex, in which the NADPH is bound tightly (\(K_{2}\)), might serve to ensure that the available preQ₀ is trapped fast and completely in a covalently enzyme-bound form. Availability of NADPH in relation to \(K_{1}\) would determine the portion of total enzyme adduct able to undergo conversion into preQ₁. The remaining portion, despite being unreactive in the absence of bound NADPH, might still be useful, for it could temporarily “store” part of the preQ₀ until reduction occurs. Because flux through the “normal” reaction pathway (\(k_{\text{cat}} = 0.15 \text{ s}^{-1}\), Table 3) exceeds the “off-pathway” thioimide cleavage, loss of adduct to dissociation could thus be prevented effectively. The very small \(K_{3}\) for the binding of NADPH for imine reduction would help to ensure that any preQ₀ that enters the reduction pathway is taken completely to the preQ₁ product. Considering that the preQ₀-derived imine might immediately hydrolyze to the corresponding aldehyde on being exposed to water, an important task of the enzyme should be to avoid any partial reduction of the nitrile substrate.

Evidence that in each step of the NADPH-dependent reduction by ecQueF the hydride transfer took place by 4-pro-R stereoselectivity provided the basis for employing primary deuterium KIEs as selective probes of the enzymatic reaction. There are two isotope-sensitive steps in the enzymatic mechanism (Scheme 1). Results of stopped-flow kinetic analysis suggested a much larger KIE on the thioimide reduction than on imine reduction. The directly measured KIE on \(V_{\text{max}}\) is explicable as the combined result of the two individual KIEs. According to Scheme 1, the \(V_{\text{max}}/K_{M}\) for NADPH includes in principle all steps from the binding of NADPH up to the product release after the imine reduction. However, because \(K_{3}\) is much smaller than \(K_{1}\), the actual \(V_{\text{max}}/K_{M}\) value for ecQueF reflects the thioimide reduction, and also the KIE is therefore primarily from this step. Note that the \(V_{\text{max}}/K_{M}\) value determined at a saturating preQ₀ concentration implies reaction via binding of NADPH to the covalent thioimide adduct. The \(V_{\text{max}}/K_{M}\) contained a relatively high standard error, but its value of 2.68 (± 0.41) appears largely consistent with the calculated KIE of 3.3 on the thioimide reduction step.

An interesting feature of the preQ₀ conversion by ecQueF was that a reverse reaction of preQ₁ and NADP⁺ was not experimentally detectable, despite the use of a broad range of pH conditions to evaluate the reactivity of preQ₁ protonated and unprotonated. Note: the \(pK_a\) value of the 7'-amino group of preQ₁ was estimated to be around 8.6. One possibility is that the oxidation of preQ₁ was disfavored thermodynamically to an extent that prevented products (e.g. NADPH) to accumulate above the detection limit; another possibility is that incorrect protonation of preQ₁, enzyme, or both caused the reaction rate to decrease below a level appreciable with the assays used.

The complete lack of activity of ecQueF toward reducing 7-formyl-preQ₀ was explicable from the evidence of binding studies that this compound did not elicit a proper substrate-binding recognition in the enzyme. Exclusion already at the level of binding therefore superseded the possible use of 7-formyl-preQ₀ as a probe of the imine reduction step in the enzymatic mechanism.

In conclusion, the multistep catalytic reaction pathway of ecQueF was characterized using kinetic analysis and probing with substrate analogues. The key role of the nitrile group for substrate binding recognition was elucidated, and an energetic fingerprint for the different steps of substrate binding was obtained. Hydride reduction of the covalent thioimide intermediate is shown to be the slowest reaction step overall. NADPH binds extremely tightly to the hemithioaminal/imine intermediate compared with its binding to the thioimide intermediate. Therefore, this property of ecQueF may be relevant physiologically, as it ensures that nitrile reduction is always taken completely to the amine when escape of the imine is prevented effectively.
Experimental Procedures

**Chemicals**—NADPH (purity >98%) and NADP⁺ (purity >97%) were from Carl Roth (Karlsruhe, Germany). 2-Propanol-d₆ (99.5 atom % D) and alcohol dehydrogenase from *Thermoanaerobium brockii* were from Sigma. Materials were of the highest purity available from Carl Roth and Sigma. The preQ₀ and its analogues, 7-cyano-2-deamino-7-deazaguanine (2-deamino-preQ₀) and 7-cyano-6-deoxo-7-deazaguanine (6-deoxo-preQ₀), were synthesized as described previously (20).

**Synthesis of 7-Formyl-7-deazaguanine (7-Formyl-preQ₀)—** A new synthesis of 7-formyl-preQ₀ (systematic name, 2-amino-5-formylpyrrolo[2,3-d]pyrimidin-4-one) that, contrary to literature-known procedures (21–23), does not require protecting group chemistry was developed. PreQ₀ (808 mg, 4.61 mmol) and sodium hypophosphate dihydrate (1.44 g, 13.6 mmol) were dissolved in the solvent mixture (15 ml of pyridine/acetic acid/deionized water in a ratio of 2:1:1) and stirred under inert atmosphere. Raney nickel catalyst (slurry in water, ~500 mg) was added to the stirred reaction mixture. Safety precaution: dry Raney nickel is pyrophoric and should always be handled under inert atmosphere. The resulting reaction mixture was heated to 50 °C for 5 h. Subsequently, the reaction mixture was cooled to 0 °C and neutralized by addition of concentrated aqueous HCl. The remaining solids were filtered off. The filtrate was cooled to 0 °C and precipitated from the solution and was isolated by filtration and washed with copious amounts of ice water and acetone (brown solid, 770 mg, 94%). ¹H NMR (DMSO-d₆) δ 6.35 (bs, 2H, NH₂), 7.49 (s, 1H, H-8), 10.04 (s, 1H, CHO), 10.72 (bs, 1H, H-9), 11.96 (bs, 1H, H-1); ¹³C NMR (DMSO-d₆) δ 98.36 (C-7), 120.34 (C-8), 124.50 (C-5), 153.40 (C-2), 153.83 (C-4), 159.02 (C-6), 185.47 (CHO). NMR data were recorded on a Bruker AVANCE III with autosampler (¹H NMR 300.36 MHz, ¹³C NMR 75.53 MHz).

**Enzyme Preparation**—Wild type ecQueF and its C190A variant were obtained as N-terminally His-tagged proteins using expression in *E. coli* BL21-DE3 (12). Cells induced with isopropyl β-D-1-thiogalactopyranoside (1 mM; 25 °C, 20 h) were suspended in storage buffer (100 mM Tris, 50 mM KCl, 1 mM tris (2-carboxyethyl)phosphine, and 1% glycerol, pH 7.5) and sonicated. The enzymes were purified from cell extract on a HiTrap™ FF column (GE Healthcare, Buckinghamshire, UK) using a linear gradient of imidazole (10–500 mM) in 50 mM Tris buffer (pH 7.4, 100 mM NaCl, 1 mM DTT, 3 mM EDTA). Fractions containing the enzyme were gel-filtered on a HiPrep™ 26/10 column or PD-10 desalting columns (GE Healthcare) equilibrated with storage buffer. The purified enzyme was concentrated with Amicon Ultra-15 centrifugal filters (Merck Millipore) to about 75–80 mg/ml. The protein concentration was measured with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Germering, Germany). Protein stock solutions were stored at −20 °C and used up within 3 weeks due to enzyme stability.

**ITC**—A VP-ITC micro-calorimeter from Microcal (Malvern Instruments Ltd., Malvern, UK) was used at 25 °C. The enzyme was gel-filtered twice to sodium phosphate buffer (100 mM NaH₂PO₄-NaHPO₄, pH 7.5, 50 mM KCl) using illustra NAP 5 columns (GE Healthcare). Each experiment consisted of an initial 2 μl injection of ligand solution into enzyme solution, followed by 25–29 injections of 6–10 μl of this solution, leaving 300–330 s between each injection. To avoid heat changes due to the DMSO added from the ligand solution, DMSO was also added to the enzyme solution (∼2.5%, v/v). Data were normalized and evaluated using ORIGIN with a single binding site model as described previously (24). The enzyme molar concentration was based on the protein concentration assuming a functional ecQueF homodimer with a molecular mass of 71,772 Da (wild type enzyme) or 71,708 Da (C190A variant). Note that 3-deoxo-preQ₀ was not used for ITC measurement because of its prohibitively low water solubility.

ITC measurements were also performed in HEPES and Tris buffer, each 100 mM, at pH 7.5 and 25 °C. The buffers additionally contained 50 mM KCl. Data were acquired and processed as described above. It was shown previously that a comparison of the binding enthalpies (ΔH) in HEPES and Tris is applicable to determine the proton uptake or release in conjunction with ligand binding (25, 26). Equation 3 was used, where n₁H⁺ is the number of protons involved in the binding, and ΔH⁺ is the ionization enthalpy of the buffer.

\[
\Delta \text{H}_\text{ion} = (\Delta \text{H}_{\text{buffer } 1} - \Delta \text{H}_{\text{buffer } 2}) / (\Delta \text{H}_{\text{ion, buffer } 1} - \Delta \text{H}_{\text{ion, buffer } 2})
\]

(Eq. 3)

The ΔH⁺ of phosphate, HEPES, and Tris at pH 7.5 and 25 °C was obtained from the literature as 3.60, 20.40, and 47.45 kJ/mol, respectively (27). The number of protons calculated from three independent sets of experiments was averaged.

The preQ₀-C190A complex was prepared by mixing enzyme (105 μM) with preQ₀ (548 μM) and incubating for 40 min at 25 °C. The solution of the enzyme complex was then used for titration into the NADPH solution. Using the 5.5 μM K_s from Table 1, the final concentration of preQ₀-C190A complex was calculated as 104 μM.

**Spectrofluorometric and Spectrophotometric Analysis of Substrate Binding in ecQueF**—Quenching of the intrinsic Trp fluorescence was shown for *B. subtilis* QueF to serve as reporter of the formation of the non-covalent complex between enzyme and preQ₀ (11). Fluorescence titrations to study with ecQueF in the binding of preQ₀ and analogues thereof were performed using a fluorescence spectrophotometer F-4500 (Hitachi, Ltd., Tokyo, Japan). Emission spectra were recorded in the range 300–500 nm at 1200 nm/min with the excitation wavelength at 280 nm. The quenching yield was determined as the ratio (F₀ – F) / F₀, where F₀ and F are the protein fluorescence intensities in the absence and presence of substrate recorded at the same wavelength of emission.

Formation of the thioimide intermediate is traceable in *B. subtilis* QueF by the appearance of a new absorbance band at 370 nm (11, 13). Using ecQueF, we therefore analyzed substrate binding also by absorbance measurements. A Beckman DU 800 spectrophotometer (Beckman Coulter, Inc.) was used. Before and after each addition, a wavelength scan in the range 300–800 nm was performed. The total volume change due to multi-
ple substrate additions was less than 5%, and the final DMSO concentration in the enzyme solution did not exceed 2%. The substrate concentration range used was dependent on the binding affinity (see under "Results"). In both fluorescent and spectrophotometric measurements, Tris buffer (100 mM, pH 7.5) containing 50 mM KCl and 1.15 mM tris(2-carboxyethyl)phosphine was used. The fluorescence and spectrophotometric data were averaged in triplicate measurements. It was confirmed that the unbound ligands, in the concentrations used, did not interfere with the fluorescence measurements. Also, all ligands had negligible absorbance in the wavelength range of the experiment.

**Protein Mass Analysis**—The ecQueF solution (130 μM, 0.6 ml) containing preQ0 (130 μM) or 2-deamino-preQ0 (0.13, 0.52, and 2.6 mM) was incubated at room temperature in 1 h and then it was desalted using Amicon Ultra 0.5 ml Centrifugal filters (Merck Millipore). A final protein concentration of 30 pmol/μl was obtained in water containing 5% acetonitrile and 0.1% trifluoroacetic acid. The samples with preQ0 were separated on a capillary HPLC system (1200 Agilent, Santa Clara, CA) equipped with a PepSwift RP monolithic column (500 μm × 50 mm, Thermo Fisher Scientific) at a flow rate of 20 μl/min using a gradient prepared from solvent A (0.05% trifluoroacetic acid in water) and solvent B (0.05% trifluoroacetic acid in acetonitrile), 0–5 min 10% B, 5–55 min 10–100% B, 55–56 min 100–10% B, and 56–71 min 10% B. The injection volume was 5 μl. The column temperature was 60 °C. Mass analysis was performed in a Thermo LTQ-FT mass spectrometer (Thermo Fisher Scientific) operated with an ESI source in positive mode with mass range of 300–2000 m/z. The protein mass spectra were deconvoluted (Protein Deconvolution 2.0 software, Thermo Fisher Scientific), using the Xtract algorithm. Enzyme samples incubated with 2-deamino-preQ0 were not detectable on a Thermo LTQ-FT mass spectrometer. These samples were therefore separated on a capillary HPLC system ( Dionex Ultimate 3000, Thermo Fisher Scientific) with the protocol described above. The flow rate was 15 μl/min. Solvent A was 0.3% trifluoroacetic acid in water, and solvent B was 0.3% trifluoroacetic acid in acetonitrile. The sample was analyzed in maXis II electron transfer dissociation mass spectrometer (Bruker, Bremen, Germany) operated with the captive spray source in positive mode with a mass range of 250–3000 m/z. The obtained protein mass spectra were deconvoluted by data analysis software, using the MaxEnt2 algorithm.

**1H NMR Measurement of the Stereochemical Course of Hydrogen Transfer from NADPH—NADPH and (4R)-[2H]NADPH were prepared by using T. brockii alcohol dehydrogenase to reduce NADP+ (2.9 mM) from 2-propanol and 2-propanol-d6 (each 80 mM), respectively (28). Tris buffer (25 mM, pH 9.0; pD = pH meter reading + 0.4) in D2O (99.8% D) was used. The reaction was stopped after 30 min when about 2 mM NADP+ had been reduced. Enzyme was filtered off (Amicon Ultra-15 centrifugal filter), and 2-propanol and acetone were evaporated at 40 °C and about 20 mbars (Laborota 4000 efficient, Heidolph, Schwabach, Germany). Enzyme and 2-propanol were added fresh to the mixture to reduce all of the remaining NADP+. The NADPH was recovered as described. (4S)-[2H]NADPH was obtained via reduction of NADP+ (3 mm) by 1-[2H]-d-glucose (3 mm) and catalyzed by glucose dehydrogenase from Bacillus megaterium (0.017 mg/ml) (29) in 25 mM Tris buffer, pH 8.5, containing 125 mM KCl. The pH was controlled at pH 8.5 during the reaction (30 °C, 600 rpm in a Thermomixer Comfort, Eppendorf, Hamburg, Germany). The deuterium content of (4R)-[2H]NADPH and (4S)-[2H]NADPH thus obtained was determined by 1H NMR and LC-MS to be 98% or greater. The coenzyme preparations were used for reduction of preQ0 with ecQueF. Enzyme was removed by precipitation with methanol (10%, by volume) and the sample analyzed with 1H NMR spectroscopy (Fig. 7).

Spectra were recorded at 499.98 MHz and 30 °C using a Varian INOVA 500 MHz spectrometer (Agilent Technologies). VNMRJ 2.2D software was used to process the spectra. The spectra of coenzymes, preQ0, and preQ1, were compared with the literature-known spectra of these compounds (20, 30, 31). Considering the possibility of deuterium labeling at the C4 of NADP+, reported in the literature to occur in reactions of the T. brockii alcohol dehydrogenase under the conditions used here (32, 33), we checked carefully with LC-MS the (4R)-[2H]NADPH preparation used. Relevant masses for doubly deuterated forms of NADPH (1H+, 746; 2H+, 373; and K+, 784) were not detected.

**Kinetic Studies at Steady State**—Experiments were performed in 100 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl and 1.15 mM tris(2-carboxyethyl)phosphine. BSA (0.2 g/liter) was added to stabilize ecQueF (0.5 μM). DMSO (1–2%, by volume) was used to increase substrate solubility in all reactions. The assay volume was 0.5 ml, and reactions were started by adding enzyme (10 μl) to the substrate (490 μl). Initial rates were determined from the NADPH consumed in the enzymatic reaction at 25 °C, by absorbance at 340 nm (εNADPH = 6.22 mmol cm−1). It was confirmed by HPLC that the nitrile substrate was converted into the amine product (Fig. 8). Using cuvettes with a 1 cm light path in the Beckman DU 800 spectrophotometer, the lowest substrate or NADPH concentration usable in the assay was about 2 μM. The averaged initial rates from triplicate determinations were used. Kinetic parameters were determined from non-linear fits of the Michaelis-Menten equation to the data. When the preQ0 concentration was constant (5.0, 10.0, 20.0, 50.0, and 100 μM), the NADPH concentration was varied between 2.0 and 330 μM. When the NADPH concentration was constant (30.0, 50.0, 100, 200, or 340 μM), the preQ0 concentration was varied (1.10–200 μM). Using 2-deamino-preQ0, the substrate concentration (10.0–1000 μM) was varied at different constant NADPH concentrations (30.0, 50.0, 100, 200, 300, or 380 μM). Under the conditions used, the decrease in absorbance was linear over 1–10 min.

For the enzymatic conversion of 7-formyl-preQ0, Tris buffer (25 mM, pH 7.5) containing 125 mM KCl was used. The 7-formyl-preQ0 (0.5 mM) was mixed with (NH4)2SO4 or NH4Cl (each at 50 mM) prior to attempted reduction (NADPH, 300 μM or 1 mM) or oxidation (NADP+, 1 mM) in the presence of ecQueF (30 μM) at 30 °C for 16 h.

For the oxidation of preQ1 with ecQueF, preQ1 was prepared by enzymatic reduction of preQ0, and then purified from the reaction mixture by HPLC. After removing acetonitrile and ammonium acetate buffer by vacuum evaporation, the preQ1
was dissolved in DMSO (≥98% purity). Reactions were started with NADP⁺ and lasted for 90 min. Tris buffer (pH 7.5, 8.0, 8.5, or 9.0) was used.

KIEs on enzymatic preQ₀ reduction due to deuteration of the coenzyme were obtained by comparing initial rates measured with NADPH and (4R)-[2H]NADPH, both synthesized as described above. The coenzyme concentration was varied (2.0–36 μM) at a constant saturating concentration of preQ₀ (10 μM). KIEs were calculated by fitting Equation 4 to the data.

\[ V = V_{\text{max}} \left( \frac{[S]}{[K_{M} + F \cdot E_{\nu}] + [S]} \right) + \left( \frac{[1 + F \cdot E_{\nu}]}{[1 + E_{\nu}] \cdot K_{M}} \right) \]  

\( E_{\nu}/K \) and \( E_{\nu} \) are the KIEs –1 on \( V_{\text{max}}/K_{M} \) and \( V_{\text{max}} \) respectively. \( F \) is the deuterium fraction in \( S \). Superscript D is used to indicate a primary deuterium KIE (e.g. \( D \cdot V_{\text{max}} \)).

**Stopped-flow Kinetic Studies and Time Course Fitting and Simulation—**Rapid-mixing kinetic analysis was done at 25 °C using a SX.18 MV stopped-flow spectrophotometer from Applied Photophysics (Leatherhead, UK). Enzyme and substrate solutions were mixed in equal volumes. In a multiple-turnover reaction, the concentrations of enzyme (5 μM) and preQ₀ (30, 40, and 60 μM) were constant, whereas NADPH or (4R)-[2H]NADPH (10–150 μM) was varied. Reaction progress was monitored by absorbance at 340 and 380 nm recorded simultaneously. A multiple wavelength detector was used. The absorbance data were averaged in triplicate measurements. Time courses were simulated and reaction models fitted to them using the program COPASI (version 4.11_build 65) (34). To translate absorbances and molar concentrations into one another between experiment and simulation, partial overlap in the absorbance spectra of the reaction components had to be taken into account. The ε₃₄₀ nm values (mm⁻¹ cm⁻¹) used in analyzing the results were 5.7 (NADPH), 4.5 (thioimide adduct), 0.126 (preQ₀), 0.087 (NADP⁺), 0.63 (ecQueF), and 6.0 or 8.0 (thioimide adduct with NADPH or 4R-[2H]NADPH, respectively). The corresponding ε₃₈₀ nm values (mm⁻¹ cm⁻¹) were 1.4 (NADPH), 11 (thioimide adduct), 0.07 (preQ₀), 0.023 (NADP⁺), 0.33 (ecQueF), and 7.5 or 10 (thioimide adduct with NADPH or 4R-[2H]NADPH, respectively).

The enzymatic reaction model was developed based on the literature (10, 11, 13), and the evidence from this study is as described under "Results." Because the reaction steps of the enzymatic mechanism are all relatively slow, we assumed binding of preQ₀ and NADPH in rapid equilibrium. It was furthermore assumed that the dissociation steps are irreversible under the conditions used. Finally, reaction steps were also assumed to be irreversible. A similar approach of reducing the number of model parameters to facilitate convergence of the fitting, also using COPASI, has been applied to enzymatic pathway analysis before (35). Robust parameter estimates with standard deviation ≤5% were obtained.

Reaction rate constants and binding constants were determined by least squares model fitting that employed the evolutionary programming algorithm with a standard setting in COPASI. The units of concentration and time used in the model were micromolars and seconds, respectively. The least sum of squares as objective function was minimized, as described previously (34, 35). Models were evaluated by comparing the objective value of the estimation between the different models. The model described in Scheme 1 was obtained with the lowest objective value, and the best fitting to the experimental data was observed. Unconstrained fitting was used for all parameters. Start values of the parameters were those determined from direct fits of the data. For example, the \( K_{M} \) values for preQ₀ (3 μM) and NADPH (10 μM) were used, and the rate constant of thioimide formation in the absence and presence of NADPH was 1.4 and 7.3 s⁻¹, respectively. The hydride transfer rate constants were set to \( k_{\text{cat}} \). Using these start conditions, the fitting converged readily to a unique solution with stable and well-defined parameter estimates. We also showed that the fitting results were relatively insensitive to change in the initial parameter values by about 5-fold. From covariance analysis it was found that each rate constant was determined largely independently, i.e., statistical correlation with other rate constants was low. In addition, we used different algorithms (particle swarm and simulated annealing), but the rate constants shown in Table 3 were hardly changed.

**HPLC Analytics—**The samples were analyzed using an Agilent 1200 HPLC system equipped with a 5 μm SeQuant ZIC-HILIC column (200 Å, 250 × 4.6 mm; Merck) and a UV detector (λ = 262 and 340 nm). A linear gradient of 90 to 60% buffer B (acetonitrile) in buffer A (100 mM ammonium acetate, pH 6.67) over 15 min was used. The column was washed with 60% B for 5 min and 90% B for 7 min after each analysis. The flow rate was 2.0 ml/min. The column temperature was 30 °C. Optionally, a mass detector (Agilent 6120 Quadrupole) was coupled to the HPLC system equipped with a 2.7 μm Poroshell SB-C18 column (120 Å, 100 × 3 mm; Agilent) and a UV detector (λ = 210, 254, and 340 nm) to analyze the synthesized coenzyme. The mass of the synthesized coenzymes was scanned in a range of 50–1000 with negative mode. A linear gradient of 2–12% buffer B (acetonitrile) in buffer A (5 mM ammonium acetate, pH 6.0 and 7.0) over 12 min was used. The post time was 2 min. The flow rate was 0.7 ml/min. The column temperature was 30 °C.

**Author Contributions—**J. J. and B. N. designed the research. J. J. and T. C. performed biochemical experiments and analyzed data together with B. N. B. W. and N. K. synthesized the substrates used. J. J. and B. N. wrote the paper. All authors commented and agreed on the final version of the paper.

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