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9, 842Interplay of nucleophilic catalysis with proton
transfer in the nitrile reductase QueF from
Escherichia coli†Jihye Jung,^{ab} Jan Braun,^a Tibor Czabany^{ab} and Bernd Nidetzky ^{*ab}

Enzymatic transformations of the nitrile group are important in biology as well as in synthetic chemistry. The enzyme QueF catalyses the conversion of 7-cyano-7-deazaguanine (preQ₀) to 7-aminomethyl-7-deazaguanine (preQ₁), a unique approach towards biological four-electron reduction of a nitrile to an amine. The catalytic reaction involves a QueF–preQ₀ thioimide adduct that is converted to preQ₁ in two NADPH dependent reduction steps via an imine intermediate. The QueF active site comprises a cysteine nucleophile flanked by an aspartic acid and additionally contains a histidine. Here, we used mutagenesis of *E. coli* QueF (C190A, C190S, D197A, D197H, and H229A) to study the functional interplay between these enzyme residues in covalent catalysis. Substitution of Cys190 or Asp197 annihilates preQ₀ covalent binding and largely disrupts the nitrile-to-amine reductase activity. The H229A variant readily forms the thioimide adduct and is 24-fold less active for preQ₀ reduction than wild-type ecQueF ($k_{\text{cat}} = 7.2 \text{ min}^{-1}$). Using isothermal titration calorimetry, we show that the non-covalent step of preQ₀ binding involves proton uptake mediated by Asp197 with His229 as the likely protonated group. Catalytic proton transfer from the Cys190 thiol via Asp197 to the nitrile nitrogen promotes the covalent intermediate. We suggest that protonated (charged) His229 facilitates the polarization of the substrate nitrile for nucleophilic attack on carbon by Cys190, and through proton relay via Asp197, it could provide the proton for re-protonating Cys190 during the formation of the imine intermediate.

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Introduction

Enzymatic transformations of the nitrile group are important biologically, for example, in secondary and xenobiotic metabolisms of plants and microorganisms.^{1–3} They also have significant applications in synthetic chemistry wherein nitriles often represent important intermediates.^{4,5} The nitrile group is converted by hydrolytic enzymes to an amide or to a carboxylic acid^{6–9} and by reductive enzymes to an amine.^{10–13} Despite the different reactivities, nitrile-converting enzymes share covalent catalysis from an active-site nucleophile as a common feature of their mechanisms. Nitrilase^{8,9,14–16} and nitrile reductase^{17–20} both utilize a cysteine to form a thioimide adduct between the enzyme and nitrile substrate (Scheme 1A). The covalent catalysis in each enzyme requires assistance from catalytic proton transfer. The enzyme nucleophile is activated by deprotonation. Conversion of nitrile to

the covalent intermediate and further on to the product necessitates acid–base catalysis, as shown in Scheme 1A. Nitrile reductase and nitrilase both have a candidate acid–base residue (Asp or Glu) positioned close to their cysteine nucleophile^{8,9,11,16,18,21} but the precise role of the Asp/Glu remains to be elucidated. Moreover, the critical interplay between nucleophilic and general acid–base catalysis is not well understood in both enzymes. In this study, therefore, we sought to clarify the involvement of catalytic proton transfer in the build-up and degradation of the covalent thioimide intermediate during enzymatic nitrile reduction.

The biological principle of nitrile reduction is embodied in the enzyme QueF.^{12,22–26} The natural reaction of QueF is conversion of 7-cyano-7-deazaguanine (preQ₀) to 7-aminomethyl-7-deazaguanine (preQ₁) by NADPH (Scheme 1B).^{17–19,21,27,28} QueF is a bacterial enzyme from the biosynthetic pathway for the modified nucleoside queuosine (Q).^{12,22,29} Inserted into tRNA as preQ₁ and further converted to Q, queuosine modulates the codon–anticodon binding efficiency for decoding NAC/U codons to Asn, Asp, His, and Tyr.^{22,29,30} The proposed catalytic mechanism of QueF is summarized in Scheme 1B. The core characteristics of this mechanism are well supported by enzyme crystal structures and biochemical evidence.^{17,18,21,27,28} Therefore, the

^a Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, NAWI Graz, Petersgasse 12/1, A-8010 Graz, Austria.E-mail: bernd.nidetzky@tugraz.at^b Austrian Centre of Industrial Biotechnology, Petersgasse 14, A-8010 Graz, Austria

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Scheme 1 Mechanistic analogies of nitrile reductase and nitrilase (A) and the proposed mechanism of nitrile reduction by ecQueF (B). (A) Catalytic conversion of the nitrile substrate via a common covalent thioimide enzyme intermediate is shown. Base-catalyzed activation of the cysteine nucleophile is water-mediated in the nitrilase. The water is shown in blue. Reduction and hydrolysis of the thioimide both involve general acid-base catalysis. (B) A detailed mechanism of preQ₀ reduction is shown. Interception of the imine intermediate by water results in the formation of 7-formyl-7-deazaguanine. This off-reaction is not significant in wildtype ecQueF but occurs in certain variants of the enzyme.²⁷

thioimide adduct is formed from a non-covalent QueF-preQ₀ complex that secludes the nitrile substrate completely from the solvent (Schemes 1B and 2).^{17–20} The covalent intermediate is converted to preQ₁ in two NADPH dependent reduction steps *via* an imine. The imine is sequestered effectively in the QueF active site to prevent its decomposition.^{17,27} NADPH binds very tightly to the enzyme-imine complex.¹⁷ The enzyme thus ensures that every preQ₀ converted is reduced fully to preQ₁.

QueF structures reveal that the cysteine catalytic nucleophile is flanked by an aspartic acid. These two residues form the immediate catalytic center (Fig. 1A and B).^{18,21,31} A histidine residue is additionally present. In the enzyme from *E. coli* studied here (ecQueF), the relevant residues are Cys190, Asp197 and His229 and this amino acid numbering is used throughout.^{11,12,17,27} QM/MM computational

analysis of the reaction path of QueF (from *Vibrio cholerae*, vcQueF) suggested involvement of Asp197 in each catalytic step (Scheme 1B).²⁸ In thioimide formation, Asp197 would deprotonate Cys190 to subsequently protonate the nitrile nitrogen atom of the preQ₀ substrate. In the first and second steps of reduction by NADPH, respectively, Asp197 would stabilize positive charge development on the reactive nitrogen of the protonated thioimide and imine intermediate. Additionally, upon decomposition of covalent thiohemiaminal to non-covalently bound imine, Asp197 would re-protonate Cys190. In the proposed mechanism,²⁸ His229 had no immediate role and was excluded from involvement in proton transfer. However, the computational study started from the non-covalent complex between the enzyme and preQ₀, not from the free enzyme, and it did not include macromolecular dynamics in the calculations. It also did not evaluate whether variation in the protonation states of the active-site residues affects the catalytic reaction. Crystal structures of thioimide enzyme-preQ₀ adducts of vcQueF and QueF from *Bacillus subtilis* (bsQueF) both reveal a hydrogen bond network connecting the thioimide N10 *via* Asp197 Oδ2 and an intermediary water to His229 Nδ1, as shown in

Scheme 2 Two-step mechanism of preQ₀ binding to QueF. QueF-preQ₀, noncovalent complex; QueF-preQ₀, covalent thioimide adduct. In wildtype ecQueF, the conversion of QueF-preQ₀ to QueF-preQ₀ is irreversible within limits of detection.¹⁷

Fig. 1 Crystallographic evidence for a possible proton relay involving the conserved Asp and His residues in QueF active sites. (A and B) The covalent thioimide adducts between preQ₀ and the enzymes are shown for vcQueF (A, R262L variant, PDB code: 3S19) and bsQueF (B, wildtype, PDB code: 4F8B). Water molecules are shown as red balls. The full proton relay involving the substrate, Asp, water and His is established. (C) The apo-enzyme of vcQueF (C194A variant, PDB code: 3RJ4) is shown. (D and E) Noncovalent complexes of vcQueF with guanine (D, C194A variant, PDB code: 3BP1) and bsQueF with preQ₀ (E, C55A variant, PDB code: 4FGC) are shown. In panel E, a proton relay involving Asp, water and His is suggested. (F) The covalent preQ₀-vcQueF complex with NADPH (R262L variant, PDB code: 3UXJ) is shown. The full proton relay may be functional. In panels A–F, active-site residues are indicated and numbered. Note: the ecQueF residues corresponding to the vcQueF residues are Glu89, Cys190, Asp197, Phe228, His229, and Glu230. Elements of the secondary structure are indicated. In bsQueF (panels B and E) the prime is used to indicate secondary structure elements from another protein subunit. Crystal structures of vcQueF and bsQueF are from ref. 18 and 21 and from unpublished data in the database.

Fig. 1A and B.^{18,21} This suggests the possibility of a proton relay involving His229. We have shown in preliminary experiments that preQ₀ binding by ecQueF was accompanied by net proton uptake from solution.¹⁷ This finding carries immediate implications for the enzymatic mechanism, but is unaccounted for in the computational reaction path of QueF. In this study, therefore, we used mutagenesis of active-site residues in ecQueF (C190A, C190S, D197A, D197H, and H229A) to study their functional interplay in covalent catalysis for nitrile reduction. We present evidence from kinetic and ligand binding studies that suggests a refined QueF mechanism. In particular, we show that the non-covalent step of preQ₀ binding involves proton uptake from His229 *via* Asp197. Catalytic proton transfer from the Cys190 thiol *via* Asp197 to the nitrile nitrogen drives the formation of the covalent intermediate. We also show that His229 has an auxiliary role in ecQueF catalysis. The positive charge on His229 could facilitate the polarization of the substrate nitrile for nucleophilic attack on carbon by Cys190. Through proton relay *via* Asp197, His229 could provide the proton for re-protonation of the cysteine during the formation of the imine intermediate.

Experimental

Chemicals

NADPH (purity >98%) and NADP⁺ (purity >97%) were from Carl Roth (Karlsruhe, Germany). Materials were of the highest purity available from Carl Roth and Sigma-Aldrich (St. Louis, MO, USA). preQ₀ and 7-formyl-7-deazaguanine were synthesized as described previously.^{10,17}

Site-directed mutagenesis

Mutagenesis leading to site-directed substitution of Cys190 by Ser (C190S) and Asp197 by Ala (D197A) or His (D197H) was performed according to a standard two-stage PCR protocol.^{27,32} A pEHISTEV vector including the ecQueF gene (pEHISTEV:EcNRedWT) was used as the template. The oligonucleotide primers are shown with the mismatched bases underlined.

C190S forward

5'-CTGCTGAAATCAAACAGCCTGATCACCCATCAACC-3'

C190S reverse

5'-GGTTGATGGGTGATCAGGCTGTTTGTATTCAGC-3'

D197A forward

5'-CCTGATCACCCATCAACCAGCGTGGGGTTCGCTCC-3'

D197A reverse

5'-GGAGCGAACCCACGCTGGTTGATGGGTGATCAGG-3'

D197H forward

5'-CCTGATCACCCATCAACCACATTGGGGTTCGCTCC-3'

D197H reverse

5'-GGAGCGAACCCCAATGTGGTTGATGGGTGATCAGG-3'

Mutagenesis to substitute Cys190 by Ala (C190A) and His229 by Ala (H229A) was reported in an earlier study.^{11,17} All mutations were verified by gene sequencing.

Enzyme preparation

The ecQueF variants were obtained as N-terminally His-tagged proteins using expression in *E. coli* BL21-DE3 as described previously.^{17,27} All the enzymes were purified by immobilized metal ion affinity chromatography and gel filtration. The enzyme purity ($\geq 99\%$) was confirmed by SDS-PAGE. The HisTrap affinity column (GE Healthcare, Buckinghamshire, UK) was regenerated fully after each use. The PD10-desalting columns (GE Healthcare) were always freshly used. Contamination with the protein carried over from previous purification runs was thus ruled out rigorously. The protein concentration was measured with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Germering, Germany). Enzyme stock solutions (0.4–0.8 mM) were stored at $-20\text{ }^{\circ}\text{C}$ and used up within 3 weeks.

Study of preQ₀ binding by ecQueF variants

For isothermal titration calorimetry (ITC), a VP-ITC micro calorimeter from Microcal (Malvern Instruments Ltd., Malvern, UK) was used at $25\text{ }^{\circ}\text{C}$. The enzyme was gel-filtered twice to phosphate buffer (100 mM $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$, pH 7.5, additionally containing 50 mM KCl) using illustra NAP 5 columns (GE Healthcare). A DMSO concentration of maximally 2% (v/v) was used in both the enzyme and the preQ₀ solution. Experiments were conducted and data evaluation done as described previously.^{17,27} The enzyme molar concentration was based on the protein concentration assuming a functional ecQueF homodimer with a molecular mass of 71 740 Da (C190S variant), 71 684 Da (D197A variant), 71 816 Da (D197H variant), or 71 640 Da (H229A variant).

Proton uptake or release in conjunction with ligand binding was determined by ITC experiments done in different buffers featuring different ionization enthalpies (ΔH_{ion}). The principle of the method is that the change in ligand binding enthalpy (ΔH) is determined in dependence of ΔH_{ion} . The slope of the linear relationship between ΔH and ΔH_{ion} indicates the number of protons involved. A positive slope indicates proton uptake; a negative slope means proton release. Besides the phosphate buffer described above, HEPES and Tris buffers (each 100 mM, pH 7.5, additionally containing 50 mM KCl) were used, as reported previously.¹⁷ The number of protons taken up or released was calculated using the equation, $n_{\text{H}^+} = (\Delta H_{\text{buffer 1}} - \Delta H_{\text{buffer 2}}) / (\Delta H_{\text{ion, buffer 1}} - \Delta H_{\text{ion, buffer 2}})$, where n_{H^+} is the number of protons involved in the binding.^{33,34} The ΔH_{ion} of phos-

phate, HEPES, and Tris at pH 7.5 and $25\text{ }^{\circ}\text{C}$ was obtained from the literature as 3.60, 20.40, and 47.45 kJ mol^{-1} , respectively.³⁵ The values are averages from three independent sets of experiments.

The covalent thioimide adduct of ecQueF was detected from its characteristic absorbance with maximum absorption at around 380 nm.¹⁷ Absorbance titrations were carried out with a Beckman DU 800 spectrophotometer (Beckman Coulter, Pasadena, CA, USA) as described previously.^{17,27} preQ₀ was titrated to the enzyme solution in the absence of NADPH.

Quenching of the intrinsic Trp fluorescence is a useful reporter of preQ₀ binding, as shown previously for bsQueF and ecQueF.^{17,18} Fluorescence titrations 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^{-1} with an excitation wavelength of 280 nm. preQ₀ was titrated to the enzyme solution in the absence of NADPH. The quenching yield was determined as the ratio $(F_0 - F_s)/F_0$, where F_0 and F_s are the protein fluorescence intensities in the absence and presence of the substrate recorded at the same wavelength of emission.

Protein mass analysis

Samples were prepared in Tris-HCl buffer (100 mM, pH 7.5), additionally containing 50 mM KCl and 1.15 mM tris(2-carboxyethyl)phosphine, as described previously.¹⁷ The enzyme and preQ₀ concentration was 130 μM and 500 μM , respectively. After the desalting process using Amicon Ultra 0.5 mL centrifugal filters (Merck Millipore, Burlington, MA, USA), a final protein concentration of 30 $\mu\text{mol L}^{-1}$ was obtained in water containing 5% acetonitrile and 0.1% trifluoroacetic acid. The samples were separated on a capillary HPLC system (Dionex Ultimate 3000, Thermo Fisher Scientific) and analysed in a maXis II electron transfer dissociation mass spectrometer (Bruker, Bremen, Germany) as described in our earlier study of wildtype ecQueF.¹⁷ The capillary spray source in positive mode with a mass range of 250–3000 m/z was used. The obtained protein mass spectra (e.g., D197H variant after incubation with preQ₀, see Fig. S1 in the ESI†) were deconvoluted by data analysis software, using the MaxEnt2 algorithm.

Enzymatic reactions of ecQueF variants

preQ₀ conversion. Reactions for preQ₀ reduction were carried out at $25\text{ }^{\circ}\text{C}$ using agitation at 500 rpm in a Thermomixer Comfort (Eppendorf, Hamburg, Germany). The enzyme concentration was 10 μM (wildtype) or 50 μM (Cys190 and Asp197 variants). The H229A variant was used at 10, 20 and 50 μM . The preQ₀ concentration was 250 μM . For the H229A variant, several preQ₀ concentrations were used: 50, 100, 150, and 200 μM . The NADPH concentration was 500 μM . Tris-HCl and sodium phosphate buffer (100 mM, pH 7.5), additionally containing 50 mM KCl and 1.15 mM tris(2-carboxyethyl)phosphine, were used. The corresponding

buffers of pH 6.0 and pH 9.0 were also used for the conversion of preQ₀ by the D197A variant. Samples were taken at certain times up to 96 h and analysed by HPLC with UV/vis and/or MS detection. All the compounds known to be involved in the reaction according to Scheme 1B (preQ₀, preQ₁, 7-formyl-7-deazaguanine, NADPH, NADP⁺) were analysed with the method used.

Absence of nitrile reductase activity in *E. coli* strain background. The cell-free extract of *E. coli* BL21-DE3 harboring the pEHISTEV vector was used. It was prepared using procedures exactly identical to those used for preparing the enzymes. The cell-free extract from BL21-DE3 harboring the pEHISTEV:EcNRedD197A vector for expression of the D197A variant was used as a positive control. The conditions used were as described above, with preQ₀ and NADPH concentrations of 250 μM and 500 μM, respectively. The protein concentration in the reaction was 3.6 mg mL⁻¹. For the positive control this would correspond to an estimated concentration of the D197A variant of 50 μM. Samples were analysed by HPLC after 24 h. No preQ₁ was formed in the negative control above a detection limit of 1 μM preQ₁ formed or 5 μM preQ₀ consumed. The positive control showed preQ₁ formation (3 μM) as expected.

Proton consumption during preQ₀ reduction. This was measured at 25 °C using the pH indicator phenol red using reported protocols.^{36,37} Immediately prior to the reaction the wildtype enzyme was gel-filtered twice to a 0.5 mM Tris-HCl buffer (pH 7.51) containing 34 μM phenol red and 150 mM KCl. Reaction mixtures contained 3.6 μM enzyme, 100 μM NADPH and 20–40 μM preQ₀. The DMSO concentration was 1% (v/v). Proton consumption was determined by the absorbance increase at 556 nm. The calibration was done with KOH. NADPH conversion was monitored at 340 nm.

HPLC analytics

The products of preQ₀ reduction were analysed at 30 °C 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) and the corresponding guard column (20 × 2.1 mm; Merck), and a UV detector (λ = 254, 262 and 340 nm), as described previously.²⁷

Results and discussion

Covalent thioimide formation

The thioimide adduct of ecQueF with preQ₀ is detectable by absorbance with maximum absorption at 380 nm (ε = 10.02 ± 0.14 mM⁻¹ cm⁻¹).¹⁷ In assessing ecQueF variants (D197A, D197H, and H229A) for thioimide formation, the incompetent C190A variant served as a negative control.^{11,17} In absorbance titrations wherein preQ₀ was used in up to 5-fold molar excess over the enzyme and the wavelength range 300–500 nm was scanned for the absorbance change, no thioimide adduct was measured for the D197A and D197H variants. The limit of thioimide detection was ~0.2% of the enzyme concentration used (50 μM). The

H229A variant formed the thioimide adduct similar to the wildtype enzyme. The C190S variant could potentially form a covalent imide involving serine as the enzyme nucleophile. From absorbance titrations, no evidence for such an intermediate was obtained.

Protein mass analysis confirmed the absorbance data. In wildtype ecQueF, the covalent adduct (35 906.0 ± 1.7 Da) was detected besides the unliganded enzyme (35 732.1 ± 0.5 Da).¹⁷ Only the unliganded enzyme was detected in samples of C190A (35 700 ± 2 Da), C190S (35 716 ± 2 Da) and D197A variants (35 689 ± 2 Da) incubated with preQ₀ in 4-fold molar excess over the enzyme subunit present. For the D197H variant, as shown in Fig. 2, mass peaks corresponding to unliganded (35 754 ± 1 Da) and preQ₀-bound enzymes (35 929 ± 1 Da) were detected. In the absence of the thioimide adduct as demonstrated in absorbance titrations, the observable enzyme complex likely involved preQ₀ tightly but noncovalently bound.

Noncovalent binding of preQ₀

This was analysed with fluorescence titration. Binding of preQ₀ is traceable by quenching of protein tryptophan fluorescence.^{17,18} We have shown in an earlier study comparing the wildtype and C190A enzyme that fluorescence quenching arises from the noncovalent step of preQ₀ binding (Scheme 2).¹⁷ Results for the different ecQueF variants are shown in Fig. 3. The degree of fluorescence quenching at saturating preQ₀ was similar in all the enzymes (85–90%). Dissociation constants (K_d) calculated from the data are summarized in Table 1. Among the enzymes not capable of thioimide formation, the D197H variant showed the highest affinity for preQ₀ binding. Its 0.55 μM K_d was on the same order of magnitude as the ~0.1 μM K_d of the H229A variant which binds preQ₀ covalently. The D197A variant showed a 25-fold loss of binding affinity as compared to the D197H variant. The K_d of the wildtype enzyme is extremely low (≤3 nM), reflecting nearly irreversible binding of preQ₀ as a covalent adduct with the enzyme.¹⁷

Fig. 2 Protein mass analysis for the D197H variant of ecQueF after incubation with preQ₀ is shown. Deconvoluted data was obtained from the protein mass spectrum (see Fig. S1 in the ESI†). The sample is shown to contain both the preQ₀-free monomeric protein (a, 35 754 ± 1 Da) and preQ₀-bound monomeric protein (b, 35 929 ± 1 Da).

Fig. 3 Fluorescence titration analysis of preQ₀ binding to wildtype ecQueF and variants thereof. The enzymes used are wildtype ecQueF (A), C190A (B), C190S (C), D197A (D), D197H (E), and H229A variant (F). The enzyme concentration was 3 μ M. Protein fluorescence was quenched upon addition of preQ₀ (top to bottom: no preQ₀; 1.5, 3, 7.5, 15, and 30 μ M preQ₀). The K_d values obtained from the data are summarized in Table 1.

Binding of preQ₀ analysed with isothermal titration calorimetry

preQ₀ binding to ecQueF is a strongly exergonic process that can be monitored well with ITC.^{17,27} Of note, a substantial amount (~50%) of the total heat released during preQ₀ binding is due to noncovalent complex formation (Scheme 2).¹⁷ ITC data of preQ₀ binding to the ecQueF variants in 100 mM phosphate buffer (pH 7.5) are shown in Fig. 4.

The corresponding thermodynamic parameters are summarized in Table 1. For the ecQueF variants unable to form the thioimide adduct with preQ₀, the Gibbs free energy of binding (ΔG) was consistently lower ($\Delta\Delta G = +8$ – 12 kJ mol^{−1}) than the corresponding ΔG for the wildtype enzyme. The $\Delta\Delta G$ for the

enzyme variants involved a large decrease in the enthalpy of binding ($\Delta\Delta H = +36$ – 41 kJ mol^{−1}), thus rendering preQ₀ binding less favorable. However, it also involved significant compensation from the entropy term ($-T\Delta S$), which decreased in the variants as compared to the wildtype enzyme. The H229A variant showed a ΔG of binding consistent with that for wildtype ecQueF. However, the ΔH was more negative and the $-T\Delta S$ more positive than in the case of the wildtype enzyme.

The kinetic study of wildtype ecQueF has shown that NADPH binds to both the covalent and the noncovalent complex of the enzyme with preQ₀.¹⁷ Binding of NADPH to the preQ₀ complex of the C190A variant was previously studied by ITC (Table 1).¹⁷ The 3.6 μ M K_d of NADPH binding to this complex was comparable to the corresponding K_d in the wildtype enzyme.¹⁷ Thermodynamic parameters of NADPH binding to the preQ₀ complex of the D197H variant were determined (Fig. 4E and Table 1). They are highly similar to the corresponding parameters of the C190A variant. Substitution of Asp197 with a histidine, therefore, does not interfere with binding of NADPH.

Proton uptake during preQ₀ reduction

The enzymatic reaction, preQ₀ + 2NADPH + 2H⁺ → preQ₁ + 2NADP⁺, implies the uptake of two protons for each nitrile substrate converted into the amine product. Assuming a protonated amine in preQ₁ at pH 7.5, a third proton would additionally be taken up in the reaction. Time-resolved analysis of proton consumption during preQ₀ reduction by wildtype QueF is shown in Fig. 5. The ratio of steady-state rates of proton uptake and NADPH oxidation was 1.5 (± 0.1), consistent with a proton/preQ₁ stoichiometry of 3.

Proton uptake during preQ₀ binding

The ITC study in buffers (phosphate, Tris, HEPES) differing in ionization enthalpy (ΔH_{ion}) was used to determine proton

Table 1 Thermodynamic parameters of preQ₀ binding to wildtype and variant forms of ecQueF

	K_d^a (μ M)		$K_{d \text{ app}}$ (μ M)	ΔH_{app} (kJ mol ^{−1})	$-T\Delta S_{\text{app}}$ (kJ mol ^{−1})	ΔG_{app} (kJ mol ^{−1})	n_{H^+}	Ref.
Wildtype	≤ 0.003	Phosphate	0.039	−80.3	37.9	−42.4	0.78 ± 0.09	17
		HEPES	0.034	−65.7	22.9	−42.8		
		Tris	0.054	−47.7	5.1	−41.6		
C190A	46.5	Phosphate	5.5 (3.6) ^b	−44.7 (−31.3) ^b	14.6 (0.07) ^b	−30.1 (−31.2) ^b	0.46 ± 0.27	17
		HEPES	4.26	−42.3	11.5	−30.8		
		Tris	4.05	−23.2	−7.7	−30.9		
C190S	15.3	Phosphate	5.3	−38.9	8.7	−30.2	0.31 ± 0.16	This study
		HEPES	4.6	−36.4	5.8	−30.6		
		Tris	3.7	−23.8	−7.3	−31.1		
D197A	13.7	Phosphate	3.5	−44.3	13.0	−31.3	≈ 0	This study
		HEPES	3.8	−50	18.9	−31.1		
		Tris	3	−42.7	11	−31.7		
D197H	0.55	Phosphate	1.3 (1.0) ^c	−43.9 (−27.0) ^c	10.1 (−7.5) ^c	−33.8 (−34.4) ^c	0.61 ± 0.05	This study
		HEPES	1.6	−33.1	−0.1	−33.2		
		Tris	1.9	−17.5	−15.3	−32.8		
H229A	≤ 0.1	Phosphate	0.054	−99.0	57.4	−41.6	1.40 ± 0.37	This study
		HEPES	0.127	−68.9	29.4	−39.5		
		Tris	0.159	−40.2	1.2	−39.0		

^a Dissociation constants from fluorescence titrations (Fig. 3). ^b NADPH binding to the noncovalent complex of C190A with preQ₀. ^c NADPH binding to the noncovalent complex D197H with preQ₀.

Fig. 4 ITC analysis of preQ₀ and NADPH binding to ecQueF variants at 25 °C is shown. Phosphate buffer (100 mM Na₂HPO₄-NaH₂PO₄, pH 7.5) containing 50 mM KCl was used. (A–D) C190S (A, 39 μM), D197A (B, 60 μM), D197H (C, 39 μM), and H229A (D, 8 μM) were used. The preQ₀ solution (800 μM for C190S, D197A, and D197H; 250 μM for H229A) was titrated into the enzyme solution. The DMSO concentration did not exceed 2% in both the enzyme and the substrate solution. The *c* values (*c* = [dimeric protein]/*K_d*) obtained from the experiments were 7 for C190S, 17 for D197A, 30 for D197H, and 148 for H229A. (E) NADPH (1 mM) was titrated to the noncovalent preQ₀-D197H complex (59 μM). The *c* value obtained from the experiment was 59. Results of fits of the data are summarised in Table 1.

uptake/release during preQ₀ binding.¹⁷ The change of binding enthalpy ΔH upon variation in ΔH_{ion} is measured and the

Fig. 5 Proton uptake (dashed lines) and NADP⁺ formation (solid lines) during preQ₀ reduction by wildtype ecQueF are shown. Reaction conditions: 3.6 μM enzyme; 100 μM NADPH; 20, 30 and 40 μM preQ₀ (bottom to top, purple, green, and black). A Tris buffer (0.5 mM, pH 7.51) additionally containing 150 mM KCl and 34 μM phenol red was used.

number of protons exchanged (n_{H^+}) is determined from the slope (positive: proton uptake; negative: proton release) of the linear dependence of ΔH on ΔH_{ion} .^{33,34} Results for ecQueF variants are summarized in Table 1 along with previously reported data for the wildtype enzyme and C190A variant. The D197A variant stood out among all other enzymes in that it did not take up protons in conjunction with preQ₀ binding ($n_{\text{H}^+} \approx 0$). Interestingly, the D197H variant recovered proton uptake to a level almost analogous to that seen with the wildtype enzyme. The two variants of Cys190 showed reduced proton uptake as compared to the wildtype enzyme. In the H229A variant, the preQ₀ binding involved proton uptake larger than that in the wildtype enzyme.

Enzymatic reduction of preQ₀

Conversion of preQ₀ into preQ₁ was measured directly using HPLC.^{17,27} As shown recently for certain ecQueF variants, 7-formyl-7-deazaguanine is released during a preQ₀ reduction

wherein the imine intermediate can hydrolyse due to its incomplete sequestration from the solvent in the enzyme (Scheme 1B).²⁷ Therefore, reactions were also analysed for 7-formyl-7-deazaguanine.

Under assay conditions routinely used with the wildtype enzyme¹⁷ that involve measurement of NADPH consumption by absorbance at 340 nm, only the H229A variant showed activity. The k_{cat} of the variant was $0.30 (\pm 0.06) \text{ min}^{-1}$, that is, 24-fold lower than the k_{cat} of the wildtype enzyme ($7.2 \pm 0.1 \text{ min}^{-1}$).¹⁷ Initial rates of the H229A variant showed saturation at low concentrations ($\leq 1 \mu\text{M}$) of both preQ_0 and NADPH. Determination of K_{m} was therefore not pursued. Using 4R-D-NADPH as a coenzyme, the kinetic isotope effect of $2.2 (\pm 0.2)$ on the k_{cat} of the H229A variant was determined. This is similar to the kinetic isotope effect of 2.4 on the k_{cat} of the wildtype enzyme.¹⁷ When incubated for longer times (96 h) at high enzyme concentration ($50 \mu\text{M}$), a low level of nitrile reductase activity was confirmed for ecQueF Cys190 and Asp197 variants.

The loss of activity compared to the wildtype enzyme was substantial, about 10^4 -fold for the Cys190 variants (C190A: $0.21 \times 10^{-3} \text{ min}^{-1}$; C190S: $0.25 \times 10^{-3} \text{ min}^{-1}$) and 10^5 -fold for the Asp197 variants (D197A: $0.04 \times 10^{-3} \text{ min}^{-1}$; D197H: $0.10 \times 10^{-3} \text{ min}^{-1}$). Residual activity in these variants is interesting for it implies a reduction of the nitrile group that proceeds in the absence of a covalent ecQueF- preQ_0 intermediate. Careful control was therefore necessary to ascertain this activity beyond doubt.

First of all, a close balance between preQ_0 consumption and preQ_1 formation was demonstrated in all the reactions. 7-Formyl-7-deazaguanine was not released. Non-enzymatic conversion of preQ_0 by NADPH was not detected, indicating that catalysis from the ecQueF variants was required for the reaction.

Secondly, to ensure that the activity could not arise from an endogenous QueF contaminating the recombinant enzyme preparations used, we applied the *E. coli* cell extract to preQ_0 conversion. One cell extract was from the expression of the D197A variant, which was the least active among the enzymes

analysed here. The other was from an *E. coli* strain harbouring the empty plasmid vector and treated identically as the positive control. Whereas the cell-extract containing D197A variant showed preQ_1 formation, the negative control was completely inactive. We also considered that proteins were purified by affinity *via* the His tag, and that the His tag was present in the recombinant but absent in the native enzyme.

Therefore, upon applying a purification procedure selective for the His-tagged target protein, it was inconceivable that a contaminating activity not detectable in the cell extract could become enriched to above detection limit in the course of purification.

Finally, interference from translational error, resulting in amino acid mis-incorporation to yield an active enzyme species, was unlikely due to the particular triplet codon changes used for site-directed mutagenesis. All codon changes involved substitutions of the first or second codon base (C190S, TGC $\rightarrow$ AGC; C190A, TGC $\rightarrow$ GCC; D197H, GAT $\rightarrow$ CAT, D197A, GAT $\rightarrow$ GCG; H229A, CAC $\rightarrow$ GCC). Translational misreading is however known to occur chiefly at the third base.^{38–40} In summary, therefore, these results reinforced the suggestion that Cys190 and Asp197 variants of ecQueF retained a low level of nitrile reductase activity that was intrinsic to their respective active sites.

The pH dependence of preQ_0 reduction by the D197A variant was analysed by comparing reaction rates at pH 6.0, 7.5 and 9.0. The residual enzyme activity at pH 6.0 and pH 9.0, compared to the maximum activity at pH 7.5, was 19% and 24%, respectively. In a previous study,²⁴ the wildtype enzyme was shown to exhibit a similar pH dependence of activity in the pH range 6.0–9.0. Therefore, these results suggest that Asp197 is not responsible for the pH dependence of activity of ecQueF.

Catalytic proton transfer coupled to covalent catalysis in ecQueF

QueF crystal structures capture the elementary steps of preQ_0 binding.^{18,21} Upon noncovalent complex formation, the

Fig. 6 Conformational flexibility of His and Asp residues in the vcQueF active site and binding of the preQ_1 product to the enzyme. (A) A structural overlay of vcQueF structures, used in Fig. 1 and 6, is shown: apo-enzyme (blue, C194A variant), holo-enzyme (deep blue, preQ_0 -R262L variant; light blue, guanine-C194A variant; teal blue, preQ_0 -H233A; violet, preQ_1 -C194A variant) and ternary complex structures (purple blue, preQ_0 -R262L variant-NADPH). (B) The covalent thioimide complex of the vcQueF H233A variant with preQ_0 (PDB code: 4GHM, teal blue) is shown. The structure reveals that Asp201 interacts with water molecules both inside (red sphere) and outside (green sphere) the active site. This could establish a proton relay despite the absence of His. (C) The complex of vcQueF with preQ_1 (C194A variant, PDB code: 3RZP, violet) is shown. Crystal structures of vcQueF are from ref. 21 and from unpublished data in the database.

substrate is anchored tightly between the N-terminal ends of two helices ($\alpha 2$ and $\alpha 5$, in ecQueF; $\alpha 1'$ and $\alpha 2$, in bsQueF), as shown in Fig. 1D and E. The reactive nitrile group so becomes oriented towards the active-site loop comprising Cys190 and Asp197. The side chains of Asp197 and His229 adopt relatively flexible conformations that enable a dynamic, water-mediated interaction between the two residues (Fig. 6A). Upon covalent complex formation, the substrate nitrogen atom develops a hydrogen bond with Asp197, which in turn remains linked *via* a water molecule to His229 (Fig. 1A, B, and F). Biochemical evidence from the current study assigns proton relay function to this active-site network of hydrogen bonds.

The mechanism in Scheme 3 is proposed. Asp197 is central for proton transfer into and within the ecQueF active site. When preQ₀ binds, Asp197 picks up a proton from water to protonate His229. The relative proton amount taken up by the enzyme (~ 0.8 protons per active site) reflects the protonation state of the histidine at the pH of 7.5 used. Replacement of Asp197 by an alanine, whose side chain is incompetent in the proton transfer function considered, disrupts completely the proton uptake in conjunction with preQ₀ binding. Replacement by a histidine, by contrast, restores the proton uptake to a level (~ 0.6 protons per active site) comparable to that of the wildtype enzyme. The extra positive charge developed on the protonated His229 might assist in catalysis to covalent thioimide formation. It could do so by promoting charge separation in the reactive nitrile group. Nucleophilic attack on carbon and proton transfer to nitrogen would thus be facilitated. Proton uptake by the H229A variant during preQ₀ binding was larger than it was in the wildtype enzyme. This probably reflects the protonation of a water molecule in the H229A active site (Scheme 4). Indeed, the covalent complex structure of the His233A variant of *Vibrio cholerae* QueF reveals a candidate water molecule hydrogen-bonded to the active-site aspartate (Fig. 6B).²¹ Interestingly, as demonstrated by proton uptake measurements for the C190A and C190S variants of ecQueF, the catalytic cysteine is not essential for proton uptake during preQ₀ binding.

Scheme 4 Proton uptake upon covalent thioimide formation in the ecQueF wildtype (A) and H229A variant (B) is shown at pH 7.5. The wildtype enzyme takes up about 0.78 protons due to the pK_a of His229 while the H229A variant takes up 1 proton by water. Amino acid numbering of ecQueF is used. The dashed line indicates hydrogen bonds.

The absolute requirement for Asp197 in covalent thioimide formation is explained by a twofold role of this residue in catalytic proton transfer. First, Asp197 activates through deprotonation of Cys190 for function as the catalytic nucleophile. Second, Asp197 promotes the covalent thioimide through catalytic proton transfer to the nitrile nitrogen atom. The nucleophilic attack on carbon probably occurs in concert with the Asp197-mediated proton transfer events, as shown in Scheme 3. Evidence in support of this notion is that the D197A and D197H variants were both completely inactive to form the covalent thioimide even under conditions ($pH \geq 9.0$) in which a cysteine would be expected to become (partially) deprotonated anyway.

As shown in Scheme 3, conversion of the covalent thioimide to preQ₁ involves partial protonation-deprotonation, hence positive charge development, at the reactive nitrogen atom of the substrate. Proton relay *via* Asp197-water-His229 would help delocalizing the charge and could thus facilitate the hydride reduction from NADPH. Upon breakdown of covalent thiohemiaminal to noncovalently bound imine, Cys190 would become re-protonated *via* Asp197 in a reversion of the initializing proton transfer event. According to the mechanism proposed (Scheme 3), the preQ₁ product would feature an unprotonated amino group and proton uptake by preQ₁ probably takes place in solution. The notion is supported by

Scheme 3 Proposed enzymatic mechanism of reduction of preQ₀ to preQ₁ showing the proton transfers involved. Amino acid numbering of ecQueF is used. The dashed lines indicate hydrogen bonds. Protons and the NADPH hydride involved in the reaction are marked green.

Scheme 5 Possible mechanisms of enzymatic nitrile reduction by NADPH in variants of ecQueF unable to form a covalent thioimide enzyme intermediate. (A) Direct hydride reduction of nitrile. (B) Hydration nitrile followed by reduction. Both reactions may involve facilitation from an unassigned enzyme acid (BH) or base (B⁻).

the structure of *V. cholerae* QueF in complex with preQ₁ (Fig. 6C). The structure shows the amino group of the product oriented towards the imidazole ring of His229. The orientation is suggestive of a cation- π interaction between the two groups, assuming the amino group to be protonated. The positioning of preQ₁ in the enzyme complex structure thus appears to be unproductive catalytically. Of note, the preQ₁ is not detectably oxidized by NADP⁺ in the presence of ecQueF within a pH range (7.5–9.0) accessible to a stable enzyme.¹⁷ The amino group pK_a of preQ₁ is predicted to be 8.39 (ChemSpider database). It may thus be difficult for the enzyme to bind preQ₁ in the presumably active, unprotonated form. From the evidence presented, an auxiliary role of His229 in each catalytic step of the QueF reaction is suggested (Scheme 3). This is consistent with the k_{cat} of the H229A variant being about 24-fold lower than the k_{cat} of the wildtype enzyme.

QueF and nitrilase appear to utilize a highly similar mechanism to promote the covalent thioimide intermediate (Scheme 1A).^{9,14,41} In the nitrilase, a triad of cysteine/glutamic acid/lysine constitutes the active-site apparatus.^{8,16,41} Like Asp197 in QueF, the glutamic acid is central for catalytic nucleophile activation and for acid-base catalysis. The role adopted by the positively charged lysine in nitrilase might be similar to that proposed for the protonated His229 in QueF (Scheme 3).

Enzymatic nitrile reduction in the absence of covalent catalysis

The conversion of preQ₀ to preQ₁ by the C190A variant implies a nitrile reduction by NADPH that proceeds in the absence of a covalent enzyme intermediate. The Asp197 variants and the C190S variant both could form covalent intermediates with preQ₀ in principle, but no evidence in support of

such intermediates was found in these enzymes. There is ample precedence for hydride reduction of nitriles to amines in synthetic organic chemistry.^{42–44} Borane reagents are often used as hydride sources/donors.⁴³ The chemical conversions involve a twofold reduction at the nitrile carbon *via* the imine intermediate.^{42,44} The catalytic reactions of the ecQueF variants might proceed analogously, using NADPH as the hydride donor (Scheme 5A). From its one-electron redox potential,^{45–47} NADPH is able to reduce a nitrile group directly. An alternative possibility, shown in Scheme 5B, is that the reaction proceeds *via* base-catalysed attack of water on the nitrile group. Such conversion of the nitrile to iminol (amide) is well studied chemically.⁴⁸ The resulting iminol could then undergo two-fold reduction to the amine as shown. Note that our LC-MS analysis of the conversion of preQ₀ excludes the release of an amide (the iminol tautomer) or a carboxylic acid product in the enzymatic reactions. In both scenarios of Scheme 5, the enzyme might provide some catalytic facilitation from a general acid or base. Cys190 and Asp197 are candidates in the corresponding enzyme variants, but no clear assignment is possible on the evidence presented. Our results furthermore show that besides having the ability to position the nitrile substrate for the initial reaction (reduction or hydration-reduction; Scheme 5), the enzyme variants retain imine intermediate sequestration as a characteristic feature of the catalytic function of the ecQueF active site.²⁷ Therefore, each preQ₀ reduced by the variants, however slowly, makes it through to the preQ₁ product.

Conclusions

We show in this study that proton relay through an active-site network of hydrogen bonds is central for ecQueF to promote nitrile reduction to amine *via* a covalent thioimide intermediate. Asp197 is the key residue to manage the interplay between

nucleophilic catalysis by Cys190 and the catalytic proton transfers. His229 has an auxiliary role. Deepened insight into the catalytic mechanism of ecQueF was thus obtained. The results have relevance in advancing mechanistic understanding of biological transformations of the nitrile group.

Conflicts of interest

There are no conflicts to declare.

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References

- 1 A. J. M. Howden, C. Jill Harrison and G. M. Preston, *Plant J.*, 2009, 57, 243–253.
- 2 A. J. M. Howden and G. M. Preston, *Microb. Biotechnol.*, 2009, 2, 441–451.
- 3 V. M. Luque-Almagro, P. Cabello, L. P. Sáez, A. Olaya-Abril, C. Moreno-Vivián and M. D. Roldán, *Appl. Microbiol. Biotechnol.*, 2018, 102, 1067–1074.
- 4 P. W. Ramteke, N. G. Maurice, B. Joseph and B. J. Wadher, *Biotechnol. Appl. Biochem.*, 2013, 60, 459–481.
- 5 J.-S. Gong, J.-S. Shi, Z.-M. Lu, H. Li, Z.-M. Zhou and Z.-H. Xu, *Crit. Rev. Biotechnol.*, 2017, 37, 69–81.
- 6 C. Brenner, *Curr. Opin. Struct. Biol.*, 2002, 12, 775–782.
- 7 J.-S. Gong, Z.-M. Lu, H. Li, J.-S. Shi, Z.-M. Zhou and Z.-H. Xu, *Microb. Cell Fact.*, 2012, 11, 142.
- 8 L. Zhang, B. Yin, C. Wang, S. Jiang, H. Wang, Y. A. Yuan and D. Wei, *J. Struct. Biol.*, 2014, 188, 93–101.
- 9 S. Jiang, L. Zhang, Z. Yao, B. Gao, H. Wang, X. Mao and D. Wei, *Catal. Sci. Technol.*, 2017, 7, 1122–1128.
- 10 B. Wilding, M. Winkler, B. Petschacher, R. Kratzer, A. Glieder and N. Klempier, *Adv. Synth. Catal.*, 2012, 354, 2191–2198.
- 11 B. Wilding, M. Winkler, B. Petschacher, R. Kratzer, S. Egger, G. Steinkellner, A. Lyskowski, B. Nidetzky, K. Gruber and N. Klempier, *Chem. – Eur. J.*, 2013, 19, 7007–7012.
- 12 S. G. Van Lanen, J. S. Reader, M. A. Swairjo, V. de Crécy-Lagard, B. Lee and D. Iwata-Reuyl, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, 102, 4264–4269.
- 13 L. Yang, S. L. Koh, P. W. Sutton and Z.-X. Liang, *Catal. Sci. Technol.*, 2014, 4, 2871.
- 14 B. C. M. Fernandes, C. Mateo, C. Kiziak, A. Chmura, J. Wacker, F. van Rantwijk, A. Stolz and R. A. Sheldon, *Adv. Synth. Catal.*, 2006, 348, 2597–2603.
- 15 H. C. Pace, S. C. Hodawadekar, A. Draganescu, J. Huang, P. Bieganski, Y. Pekarsky, C. M. Croce and C. Brenner, *Curr. Biol.*, 2000, 10, 907–917.
- 16 J. E. Raczynska, C. E. Vorgias, G. Antranikian and W. Rypniewski, *J. Struct. Biol.*, 2011, 173, 294–302.
- 17 J. Jung, T. Czabany, B. Wilding, N. Klempier and B. Nidetzky, *J. Biol. Chem.*, 2016, 291, 25411–25426.
- 18 V. M. Chikwana, B. Stec, B. W. K. Lee, V. de Crécy-Lagard, D. Iwata-Reuyl and M. A. Swairjo, *J. Biol. Chem.*, 2012, 287, 30560–30570.
- 19 B. W. K. Lee, S. G. Van Lanen and D. Iwata-Reuyl, *Biochemistry*, 2007, 46, 12844–12854.
- 20 L. Gjonaj, M. Pinkse, E. Fernández-Fueyo, F. Hollmann and U. Hanefeld, *Catal. Sci. Technol.*, 2016, 6, 7391–7397.
- 21 Y. Kim, M. Zhou, S. Moy, J. Morales, M. A. Cunningham and A. Joachimiak, *J. Mol. Biol.*, 2010, 404, 127–137.
- 22 D. Iwata-Reuyl, *Bioorg. Chem.*, 2003, 31, 24–43.
- 23 P. Domínguez de María, *ChemCatChem*, 2011, 3, 1683–1685.
- 24 K. Moeller, G.-S. Nguyen, F. Hollmann and U. Hanefeld, *Enzyme Microb. Technol.*, 2013, 52, 129–133.
- 25 M. Li, Z. Zhou, Z.-J. Zhang, H.-L. Yu and J.-H. Xu, *J. Mol. Catal. B: Enzym.*, 2016, 131, 47–54.
- 26 Z. Zhou, M. Li, J.-H. Xu and Z.-J. Zhang, *ChemBioChem*, 2018, 19, 521–526.
- 27 J. Jung and B. Nidetzky, *J. Biol. Chem.*, 2018, 293, 3720–3733.
- 28 A. J. M. Ribeiro, L. Yang, M. J. Ramos, P. A. Fernandes, Z.-X. Liang and H. Hirao, *ACS Catal.*, 2015, 5, 3740–3751.
- 29 B. El Yacoubi, M. Bailly and V. de Crécy-Lagard, *Annu. Rev. Genet.*, 2012, 46, 69–95.
- 30 F. Harada and S. Nishimura, *Biochemistry*, 1972, 11, 301–308.
- 31 A. Mohammad, A. Bon Ramos, B. W. K. Lee, S. W. Cohen, M. Kiani, D. Iwata-Reuyl, B. Stec and M. Swairjo, *Biomolecules*, 2017, 7, 30.
- 32 W. Wang and B. A. Malcolm, *BioTechniques*, 1999, 26, 680–682.
- 33 A. G. Kozlov and T. M. Lohman, *Biochemistry*, 1999, 38, 7388–7397.
- 34 S. Leavitt and E. Freire, *Curr. Opin. Struct. Biol.*, 2001, 11, 560–566.
- 35 R. N. Goldberg, N. Kishore and R. M. Lennen, *J. Phys. Chem. Ref. Data*, 1999, 31, 231–370.
- 36 S. L. Pival, M. Klimacek and B. Nidetzky, *Biochem. J.*, 2009, 421, 43–49.
- 37 M. Klimacek, M. Brunsteiner and B. Nidetzky, *J. Biol. Chem.*, 2012, 287, 6655–6667.
- 38 J. M. Ogle and V. Ramakrishnan, *Annu. Rev. Biochem.*, 2005, 74, 129–177.
- 39 J. M. Ogle, D. E. Brodersen, W. M. Clemons Jr., M. J. Tarry, A. P. Carter and V. Ramakrishnan, *Science*, 2001, 292, 897–902.
- 40 M. Johansson, J. Zhang and M. Ehrenberg, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, 109, 131–136.

- 41 L. Martínková and V. Křen, *Curr. Opin. Chem. Biol.*, 2010, **14**, 130–137.
- 42 D. B. Bagal and B. M. Bhanage, *Adv. Synth. Catal.*, 2015, **357**, 883–900.
- 43 D. Haddenham, L. Pasumansky, J. DeSoto, S. Eagon and B. Singaram, *J. Org. Chem.*, 2009, **74**, 1964–1970.
- 44 C. Bornschein, S. Werkmeister, B. Wendt, H. Jiao, E. Alberico, W. Baumann, H. Junge, K. Junge and M. Beller, *Nat. Commun.*, 2014, **5**, 4111.
- 45 K. Burton and T. H. Wilson, *Biochem. J.*, 1953, **54**, 86–94.
- 46 A. W. Munro, M. A. Noble, L. Robledo, S. N. Daff and S. K. Chapman, *Biochemistry*, 2001, **40**, 1956–1963.
- 47 T. E. DeCoursey, D. Morgan and V. V. Cherny, *Nature*, 2003, **422**, 531–534.
- 48 T. E. Schmid, A. Gómez-Herrera, O. Songis, D. Sneddon, A. Révolte, F. Nahra and C. S. J. Cazin, *Catal. Sci. Technol.*, 2015, **5**, 2865–2868.