Structural Basis of Biological Nitrile Reduction*

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Background: QueF is the only nitrile reductase known in biology. QueF catalyzes the reduction of the nitrile group of 7-cyano-7-deazaguanine (preQ0) to 7-aminomethyl-7-deazaguanine (preQ1), the only nitrile reductase reaction known in biology. We describe here two crystal structures of Bacillus subtilis QueF, one of the wild-type enzyme in complex with the substrate preQ0, trapped as a covalent thioimide, a putative enzyme intermediate in the reaction, and the second of the C55A mutant in complex with the substrate preQ0 bound noncovalently. The QueF enzyme forms an asymmetric tunnel-fold homodecamer of two head-to-head facing pentameric subunits, harboring 10 active sites at the intersubunit interfaces. In both structures, a preQ0 molecule is bound at eight sites, and in the wild-type enzyme, it forms a thioimide covalent linkage to the catalytic residue Cys-55. Both structural and transient kinetic data show that preQ0 binding, not thioimide formation, induces a large conformational change in and closure of the active site. Based on these data, we propose a mechanism for the activation of the Cys-55 nucleophile and subsequent hydride transfer.

Until recently, the known biological transformations of nitriles (1) included nitrilase and nitrile hydratase-catalyzed hydrolysis, giving amides or carboxylic acids, respectively, the oxygenase-catalyzed conversion of nitriles to cyanohydrins, and the cleavage of cyanohydrins to HCN and aldehydes or ketones by hydroxynitrilase lyase. Notably, although the 4-electron reduction of nitriles to the corresponding primary amines is well preceded in organic chemistry, nitrile reduction was unknown in biology with the exception of the nitrogenase-catalyzed reduction of hydrogen cyanide to methylamine (2, 3), a nonspecific reaction that does not appear to have any biological relevance.

In 2005, we reported the discovery of a new dehydrogenase enzyme family involved in nitrile metabolism, QueF (4, 5), which catalyzes the NADPH-dependent reduction of a nitrile group to a primary amine in the biosynthesis of the tRNA-modified nucleoside queuosine (Q),4 a structurally complex nucleoside found at the wobble position of tRNAs possessing the GUN anticodon (those for Tyr, His, Asp, and Asn). The biosynthetic pathway to Q is unique in RNA modification in that a substantial portion of the pathway occurs outside the context of the tRNA and leads to the formation of the modified base 7-aminomethyl-7-deazaguanine (preQ1) (Fig. 1). preQ1 is subsequently inserted into the tRNA by the enzyme tRNA-guanine transglycosylase (6), and the remainder of the transformation to queuosine occurs at the level of the tRNA (7). QueF catalyzes the final step of the tRNA-independent portion of the Q pathway, the 2-fold reduction of the advanced intermediate 7-cyano-7-deazaguanine (preQ0) to preQ1. This reaction constitutes the only example of biologically relevant nitrile reduction identified to date and represents a new biochemical transformation. The importance of nitrile reduction in industrial chemical processes and the unique activity of QueF have positioned it as a potential new candidate for industrial biocatalysis.

Based on their amino acid sequences, QueF enzymes are proposed to belong to the tunneling-fold (T-fold) superfamily (8), a structural superfamily of functionally distant proteins that bind planar pterin/purine substrates. Members of the superfamily all possess the T-fold domain (8), a small (120–160 residues) protein domain composed of an antiparallel β-sheet of four strands, with a pair of antiparallel α-helices between the second and third strands (ββααββ). Functional proteins of this superfamily are formed from a common oligomerization of T-fold domains to form a β2α2α2 barrel (8), and two barrels join in a head-to-head fashion to form an oligomeric structure with tunnel-like center. The T-fold superfamily can be divided into two

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4 The abbreviations used are: Q, queuosine; preQ0, 7-cyano-7-deazaguanine; preQ1, 7-aminomethyl-7-deazaguanine; T-fold, tunneling-fold; GCYH, GTP cyclohydrolase; DMSO, dimethyl sulfoxide; SE, sedimentation equilibrium; SV, sedimentation velocity; r.m.s.d., root mean square deviation.
QueF enzymes have members from both structural subfamilies (5). The unimodular subfamily, represented by the enzyme from Bacillus subtilis, is composed of 160-amino acid proteins that contain a single T-fold domain with an embedded QueF motif (E(S/L)K(S/A)hK(L/Y)(Y/F/W), where h is a hydrophobic amino acid) bracketed on the N- and C-terminal sides by an invariant Cys and Glu, respectively. The bimodular subfamily, represented by the Escherichia coli enzyme, is composed of ~280-amino acid proteins that contain two tandem T-fold domains in which the QueF motif and the invariant Cys and Glu residues are spatially separate, being located in the weakly homologous N- and C-terminal T-fold domains, respectively. Gel filtration studies of an enzyme from each subfamily, the B. subtilis and E. coli QueF, were consistent with homododecameric and dimeric structures for the unimodular and bimodular enzymes, respectively (5). The latter result was surprising as it implies that the enzyme lacks the canonical T-fold quaternary architecture of all other bimodular T-fold enzymes (9, 10), but recent crystallographic analysis of the bimodular enzyme from Vibrio cholerae also revealed a homodimer structure for this subfamily (11), confirming that this enzyme does lack the canonical quaternary structure of the superfamily.

Based on data from biochemical studies of the QueF-catalyzed reaction, we previously proposed a catalytic mechanism in which preQ0 first reacted with a conserved active-site Cys residue to form a covalent thioimide intermediate, which we proposed was the relevant species that underwent the first reductive cycle (14). A covalent thioimide intermediate has also been proposed in the nitrilase-catalyzed hydrolysis of nitriles to carboxylic acids (12); however, no direct structural evidence for such an adduct has been reported. Here we present the crystal structures of wild-type QueF from B. subtilis as well as an inactive mutant (C55A), both crystallized in the presence of preQ0. In the case of wild-type QueF, the enzyme has crystallized in a form in which the preQ0 has reacted to form the thioimide intermediate, whereas in the mutant, which lacks the conserved Cys residue, preQ0 is bound noncovalently. We also present the results of transient kinetic studies of preQ0, binding and thioimide formation that allow us to calculate the rate constants for these steps in the reaction and to correlate the timing of substrate binding and reaction with enzyme structural changes. Taken together, the structural and kinetic data demonstrate the central role that substrate binding has on the structure of QueF and provide the first direct view of a covalent thioimide linkage between an enzyme and its substrate.

**EXPERIMENTAL PROCEDURES**

Crystallography, X-ray Data Collection, and Crystal Structure Determination—Wild-type QueF and the single-site QueF mutant C55A from B. subtilis were overproduced and purified as described previously (13, 14). For both the wild type and the C55A mutant, the enzyme-preQ0 complex was crystallized at 293 K using the hanging drop method as described previously (13). Briefly, the enzyme (4 mg/ml in 100 mM Tris pH 7.5, 100 mM KCl) was mixed with preQ0 at a 1:5–1:10 molar ratio. Hanging drops were set up by mixing equal volumes of sample with reservoir solutions containing 16–20% PEG 500 monomethyl ether, 60 mM imidazole, 40 mM imidazole-Cl (final pH 7.4), and 30 mM MgCl2 (for the wild-type enzyme) or CaCl2 (for the C55A mutant). Rod-shaped crystals were harvested in 18 days and cryo-protected in liquid nitrogen.

The x-ray diffraction data were collected using synchrotron radiation at the Stanford Synchrotron Research Laboratory, beamline 9–1, and processed using HKL2000 (15) (see Table 1). The crystal structure of wild-type QueF complexed with substrate was determined using the molecular replacement Bayesian protocol in the program Phaser (16) and a search model generated from the structure of V. cholerae QueF (Protein Data Bank (PDB) ID 3BP1 (11)) by replacing nonhomologous residues with alanines. A solution with highest likelihood gain was obtained in the P3221 space group. The partial model obtained in Phaser and describing the asymmetric unit was used for automatic tracing and refinement in the program Arp/Warp (17), which produced a significantly more complete model with a crystallographic R-factor of 0.35. The crystal structure of the C55A mutant complexed with preQ0 was determined by the difference Fourier method using the refined wild-type structure as a search model. Both structures were refined using Refmac (18) and Coot (19) and analyzed using a variety of programs in the CCP4 suite (20). The asymmetric unit of either structure contains five QueF monomers (labeled A–E), four preQ0 molecules, a polyethylene glycol molecule, and four metal cations. All protein subunits are disordered in their N-terminal stretch of 21 residues. Subunit A is also disordered in the C-terminal region following Pro-159. A homodecamer is generated by applying the crystallographic 2-fold symmetry operation on the asymmetric unit.

Analytical Ultracentrifugation—Sedimentation equilibrium (SE) and velocity (SV) experiments were performed in a ProteomeLab XL-I (Beckman Coulter) analytical ultracentrifuge.
using the eight-place An-50 Ti rotor. For the SE experiments, protein samples at concentrations of 0.5, 0.25, and 0.125 mg/ml were loaded in six-channel equilibrium cells and spun at 10,000 and 14,000 rpm, 20 °C until equilibrium was achieved at each speed. For the SV experiments, protein samples at 0.5 mg/ml were loaded in two-channel velocity cells and spun at 35,000 rpm, 20 °C for 10 h. All samples contained 100 mM Tris (pH 7.4), 100 mM KCl, preQ₀ dissolved in DMSO, was added to a final enzyme:preQ₀ molar ratio of 1:10 (the final DMSO content in the sample was 5%). The SE data were analyzed using HeteroAnalysis software (23). The XL-A/XL-I data analysis was performed using the SV data and calculate sedimentation coefficients (s). The Sednterp software (24) was used to convert s to s²0,w values and for shape modeling.

Size Exclusion Chromatography—Size exclusion chromatography was carried out using a Bio-Sep-Sc-4000 column (Phenomenex) with a mobile phase of 25 mM phosphate (pH 7.2), 25 mM KCl, and a flow rate of 1 ml/min. The retention times of QueF were determined in the absence and presence of preQ₀. QueF (100 μM) was preincubated with preQ₀ (200 μM) for 10 min prior to analysis. Standard curves were generated from the elution behavior of the standards carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa), β-amyloc (200 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa).

Absorbance Measurements of Thioimide Breakdown—For the preparation of the QueF-preQ₀ complex, 1 ml of 200 μM QueF and 600 μM preQ₀ was incubated for at least 10 min in 50 mM phosphate (pH 6.5), 50 mM KCl, 250 μM MnCl₂, and 1 mM DTT before the excess preQ₀, was removed by dialysis (Slide-A-Lyzer, Pierce) against 4 liters of the same buffer for 2 h. Thioimide decomposition was then monitored with QueF-preQ₀ against 4 liters of the same buffer for 2 h. Thioimide concentrations were measured using stopped-flow spectrophotometry under pseudo first-order conditions, where the QueF concentration was maintained high and preQ₀ concentration was varied. One syringe was filled with solution A (124 μM QueF, 50 mM potassium phosphate (pH 6.5), 50 mM KCl, 250 μM MnCl₂, and 1 mM DTT), whereas the second syringe was filled with solution B (i.e. solution A without QueF but with preQ₀). Five or more shots were performed in the absence of preQ₀ to flush the stopped-flow lines and to obtain a base line. After the flushing was complete, several shots were performed with varying concentrations of preQ₀. The final concentrations of preQ₀ in the reactions were 11.8, 17.0, 21.5, 26.3, and 37.7 μM, whereas the final QueF concentration was 62 μM.

The reactions were carried out at 30 °C and monitored at 370 nm for 5 s. Equal volumes (60 μl) of the enzyme solution were mixed with the preQ₀ solution. The rates of the spectral changes at 370 nm were obtained from the best fits with the smallest residuals.

Data Analysis—For data analysis with DynaFit (21), a model for each reaction was defined to allow the program to calculate the rates for the various steps in the formation of the thioimide intermediate. The fitting function (model equation) for each individual data set is

\[ s(t) = s_0 + \sum_{i=1}^{n} r_i c_i(t) \]  

(Eq. 1)

where \( s(t) \) is the experimental signal observed at time \( t \); \( s_0 \) is the offset on the signal axis (a property of the instrument); \( n \) is the number of unique molecular species participating in the reaction mechanism; \( c_i(t) \) is the concentration of the \( i \)th species at time \( t \); and \( r_i \) is the molar response coefficient of the \( i \)th species.

The molecular species participating in the mechanism are defined for each of the various reactions. The concentrations of these molecular species at time \( t \) are computed from their initial concentrations (at time 0, \( t = 0 \)) by solving an initial value problem defined by a system of simultaneous first-order ordinary differential equations.

RESULTS

Tunnel-fold Homodecamer and Conserved Interface Catalysis—We determined two crystal structures of the unimodular QueF from B. subtilis. The crystal structure of wild-type

Structure of B. subtilis QueF
unimodular QueF was determined at 2.5 Å using molecular replacement methods and a search model generated from the crystal structure of *V. cholerae* QueF (PDB ID 3BP1) (Fig. 2, A and B, and Table 1) (11). The structure of the C55A enzyme mutant was then determined by the difference Fourier method using calculated model phases from the wild-type structure (Table 1). The two overall structures are similar and superpose with r.m.s.d. 0.37 Å over 164 Cα atoms. Five monomers, A–E, were identified in the asymmetric unit, and a homodecamer, not a homododecamer as originally proposed (5), is formed by applying the crystallographic two-fold symmetry operation on the asymmetric unit. The structure reveals a nonsymmetric homodecamer of two head-to-head facing pentamers, each composed of a cyclic arrangement of monomeric β2α2 barrels with the classic T-fold architecture characteristic of this superfamily (8) (Fig. 2A). The monomer is a globular subunit composed of a highly twisted antiparallel β-sheet (β2, β3, β5, β6) layered on its concave side with two antiparallel α-helices (α1,
Structure of B. subtilis QueF

TABLE 1
X-ray data collection parameters and structure refinement statistics

|                          | Covalent thioimide complex | C55A mutant complex |
|--------------------------|----------------------------|---------------------|
| **Data collection**      |                            |                     |
| Space group              | P3,21                      | P3,21               |
| Unit cell (Å)            | 93.51, 193.70              | 93.05, 193.65       |
| Wavelength (Å)           | 1.00 0003                  | 1.00 0003           |
| Resolution (Å)           | 2.50                       | 2.50                |
| Highest resolution bin (Å) | 2.50–2.57                 | 2.50–2.54           |
| Measured unique reflections | 34,277 (3,103)*             | 31,577 (1,498)      |
| Completeness (%)         | 98.7 (89.9)                | 91.6 (88.8)         |
| Redundancy               | 9.9 (6.3)                  | 4.0 (3.9)           |
| Rmerge*                  | 0.12 (0.38)                | 0.14 (0.65)         |
| Rwork                     | 7.8 (2.1)                  | 6.8 (2.3)           |

**Reefinement**

| Resolution range (Å)     | 41.56–2.50                 | 22.15–2.50          |
| No. of reflections (F > 0 σf) | 30,755/1,720               | 29,866/1,592        |
| No. of atoms             | 6,268                      | 6,345               |
| preQ0/water/Mg2+/Ca2+    | 52/165/4/0                 | 52/205/0/8          |
| PEG/PO4                  | 24/5                       | 24/0                |
| Rmerge/Rwork             | 0.205/0.272                | 0.228/0.305         |
| r.m.s.d. bond lengths (Å) | 0.024                      | 0.016               |
| r.m.s.d. bond angles (°)  | 2.057                      | 1.997               |
| Wilson B-factor (Å²)     | 44.15                      | 46.6                |
| Average B-factor (Å²)    | 42.1                       | 39.3                |
| Protein                  | 39.6                       | 41.7                |
| Metals                   | 64.7                       | 41.3                |
| preQ0                    | 42.9                       | 32.4                |
| Water                    | 42.9                       | 32.4                |
| Ramachandran plot (%)    | 96.0                       | 94.0                |
| Favored                  | 4.0                        | 5.0                 |
| Allowed                  |                            |                     |

* Highest-resolution shell information in parentheses.
** Ramachandran plot (%)
*** Wilson B-factor (Å²)
**** Average B-factor (Å²)
***** The free R-factor was monitored with 5% of the data excluded from refinement.

α2) to form the T-fold core followed by a helix (α3) and a 10-residue C-terminal extension (Fig. 2C).

A large surface area of 6334 Å² (amounting to 21% of the solvent-accessible surface area for each pentamer) is buried in the interpentamer interface, consistent with a stable decamer being the biological enzyme form. The two pentamers are further pinned together at the carboxyl termini from opposing subunits, which form salt bridges at the interpentamer interface via the conserved residues Asp-131 and Arg-164, as well as pairs of high affinity metal sites involving Asp-162 and C-terminal carboxylate of Arg-164 (Fig. 2, A and D). In the wild-type structure, the metal sites are occupied by Mg2+, whereas in the C55A mutant structure, the sites are occupied by Ca2+. The bound metal ions in each structure were modeled based on the metal that was included in the crystallization buffer, MgCl2 and CaCl2 for the wild-type and mutant crystals, respectively. Of the 10 subunits in the decamer, two opposing subunits, one from each pentamer, exhibit disordered C termini; hence the corresponding salt bridges and metal-binding sites are not formed.

The preQ0-binding Pocket—The enzyme active sites are located at the interfaces between subunits (Fig. 2A). In both the wild-type and the C55A mutant structures, electron density corresponding to preQ0 is seen in the difference Fourier maps in 8 of the 10 active sites in the enzyme homodecamer (Fig. 3, A and B). The preQ0-binding pocket is defined by a cleft between the two subunits from the same pentamer. The left and back walls of the cleft are formed by the loops connecting strands β2 and β3 (Leu-54–Pro-61), helices α1 and α2 (Gly-93–Glu-97), and strands β5 and β6 (Phe-122–Ile-130) and the N-terminal half of strand β3 (Asp-62–Ala-64) from one subunit (Fig. 3C). The right wall of the cleft is formed by the C-terminal half of strand β4 (Val-77–Ser-79) and by strand β1 (Leu-30–Pro-34) from the adjacent subunit. Several interface residues make side-chain and backbone interactions with preQ0. From one subunit, the invariant Glu-97 side chain forms hydrogen bonds with the N1 and N2 atoms of preQ0, and the backbone amine of His-96 forms a hydrogen bond with the O6 atom of preQ0. From the other subunit, a hydrogen bond is formed between...
the side chain of Glu-78 (the first residue in the conserved QueF motif) and the N9 atom of preQ0. The backbone amine of Ser-79 and the carbonyl oxygen of Val-77 make hydrogen bonds with the N3 and N2 atoms of preQ0, respectively. The substrate-binding pocket is also lined with hydrophobic residues (Phe-33, Phe-95, and Ile-130) that make Van der Waals contacts with preQ0.

**Structural Observation of the Covalent Thiimide Intermediate**—In the wild-type structure, but not the C55A mutant, the electron density from preQ0 seamlessly continues into the invariant active-site Cys-55 side chain, reflecting formation of a thioimide covalent linkage (Fig. 3, A and B). This covalent adduct was first proposed based on spectroscopic measurements in solution (14) and later predicted from numerical simulations on the V. cholerae enzyme (11). Asp-62 from strand β3 forms a hydrogen bond (3.1 Å) via its Oδ1 atom with the nitrogen of the thioimide (Fig. 3C). In the C55A mutant structure, the electron density of bound preQ0 is clearly discontinuous from the enzyme, and the cyan carbon of preQ0 is 4 Å from the Ala-55 side chain (Fig. 3B).

**Substrate Binding Induces Closure of the Active Site and Tightening of the Pentamer**—Two of the 10 active sites in the QueF homodecamer are unoccupied, providing insight into the structure of the unliganded enzyme. Three-dimensional superposition of an empty active site (e.g., site AE) with any of the preQ0-occupied active sites (e.g., site BA) shows a dramatic conformational change associated with preQ0 binding (Fig. 4, A and B). In the empty site, the subunit contributing the QueF motif is displaced by a rigid body movement relative to its position in the occupied site.

This rigid body displacement renders it off the noncrystallographic five-fold symmetry by a rotation k = 25.51° around an axis defined by the polar angles ω = 15.38° and φ = −21.65°. This shift widens the empty site by 13 Å and reduces the area of the intersubunit interface from 1427 to 546 Å². In the preQ0-occupied sites, the average distance between the Cα atoms of the preQ0 liganding residue Cys-55 in one subunit and the first residue in the QueF-motif, Glu-78, in the other subunit is ~12 Å. In the empty site, this distance is ~23 Å. Further, in the case of the empty site, the 6-residue stretch at the C terminus of the Cys-55-contributing subunit (subunit A) is disordered and thus does not form a divergent metal bridge or the salt bridges between the two pentamers. These observations suggest that preQ0 binding induces closure of the active sites and subsequent tightening of the pentamer and stabilization of the functional decamer.

**Gel Filtration and Analytical Ultracentrifugation**—To investigate the effect of preQ0 binding on the structure and stability of the enzyme homodecamer in solution, we conducted gel filtration and analytical centrifugation experiments. When subjecting QueF to size exclusion chromatography in the absence of preQ0, the enzyme exhibits a broad, asymmetric peak with a retention time of 8.57 min (Fig. 4C), corresponding to a molecular mass of 200.7 kDa. The retention time of QueF in the presence of preQ0 shifts to 8.33 min and exhibits a sharper peak, indicating a tighter decamer. The apparent average molecular mass of QueF from an SE run is 182.1 kDa, slightly lower than the theoretical molecular mass of the decamer (192.4 kDa), indicating the presence of smaller multimeric species. The best fit of the SE data were achieved by assuming two multimeric species in equilibrium: pentamer and decamer with an apparent Kd = 190 ± 50 nm. No good fit was found using a monomer-decamer equilibrium model. These results are consistent with pentamer formation as a step in the oligomerization of QueF. In the presence of preQ0, the apparent average molecular mass of QueF is 189.5 kDa, higher than the apo enzyme, indicating a shift in equilibrium toward the decamer. Fitting the data assuming two multimeric species in equilibrium, pentamer and decamer, yielded an apparent Kd = 36 ± 1 nm, lower than that of the apo enzyme, indicating a substrate-induced tightening of the decamer. For both runs, the Kd value was consistent for two centrifugation speeds, indicating that no higher order aggregates than the decamer are present in the sample.

When subjecting QueF to UV ultracentrifugation, only a single sedimenting species, i.e., decamer, was resolved in SV scans in the absence or presence of preQ0. The SV data were fitted to obtain apparent sedimentation coefficients (s), which were converted to s20,w to account for the differences in sample buffer composition. Resulting s20,w values are 9.24 and 9.37 for QueF and QueF-preQ0 complex, respectively. The experimental error for s did not exceed 0.5%. The increase in s20,w value indicates that in the presence of preQ0 the decamer adopts a more compact (less extended) shape.

**Decomposition of the Thiimide Intermediate**—Previous experiments suggested that the thioimide intermediate was stable over several hours (14), and therefore the breakdown of this covalent adduct could be ignored in evaluating kinetic data under pre-steady state conditions. Nevertheless, to quantitate the phenomenon, we isolated the enzyme after incubating with preQ0 and then, after removing the unbound substrate by dialysis, monitored the breakdown of the thioimide by UV spectroscopy (Fig. 5). Global analysis of the data using a variety of models, from a simple first-order process to a multistep mechanism, provided calculated rate constants that were virtually identical, ranging from 4.81 × 10^-6 s^-1 (first-order decay) to 5.41 × 10^-6 s^-1 (multistep mechanism), and the residuals were not significantly different. This is likely due to the fact that the rate constant for thioimide breakdown is so disproportionately small when compared with all the other rate constants that it dominates any proposed model. The best fit of the models was a second-order process with k = 5.17 (± 0.05) × 10^-6 s^-1.

**Kinetics of preQ0 Binding and Thiimide Formation**—QueF has a single tryptophan residue at position 119, and when irradiated at 295 nm, the protein fluoresces with an emission maximum at 334 nm (Fig. 6A). Binding and/or reaction of preQ0 is associated with a blue shift in the emission maximum (334–328 nm) coupled with quenching of the emission by 70%.

To determine whether fluorescence quenching was due to binding of preQ0 to QueF, thioimide formation, or both, the fluorescence properties of preQ0 binding were first determined with the C55A mutant of QueF, which is incapable of reacting to form the thioimide (14). Binding was monitored by measuring the change in the intrinsic tryptophan fluorescence under pre-steady state conditions at 20 °C. Under these conditions, binding was observable from quenching of the fluorescence, and the data were monophasic (Fig. 6B), consistent with
a one-step binding mechanism. The association rate constant from global analysis of the data was calculated to be $1.06 \pm 0.31 \mu M^{-1} s^{-1}$, with a dissociation rate constant of $1.35 \pm 0.26 s^{-1}$.

To investigate preQ$_0$ binding and thioimide formation, fluorescence measurements were conducted on wild-type QueF. In this case, the data were biphasic (Fig. 6C), consistent with a two-step mechanism comprising reversible binding of preQ$_0$ followed by reaction to give the thioimide. From global analysis of the data, the association constant was calculated to be $0.17 \pm 0.05 \mu M^{-1} s^{-1}$, 10-fold lower than with the C55A mutant. The dissociation constant was calculated to be $1.29 \pm 0.19 s^{-1}$, identical within experimental error to the value calculated with the C55A mutant, and the rate of thioimide formation was calculated to be $1.30 \pm 0.05 s^{-1}$.

The kinetics of thioimide formation were also investigated by directly observing the appearance of the thioimide absorbance band at 370 nm (Fig. 6D). Again, the data were biphasic and fit to a two-step mechanism comprising preQ$_0$ binding followed by thioimide formation, identical to that utilized in the fluorescence experiments. Using this model, global analysis of the data provided a rate for thioimide formation of $2.78 \pm 0.01 s^{-1}$ at 30 °C, in close agreement to the value calculated above from the fluorescence experiments carried out at 20 °C. These data suggest that the structural changes that occur in the Trp environ-

**FIGURE 4. Substrate-induced closure of the active site.** A, superposition of the empty active site at the interface between subunits A and E (site AE, purple) with the preQ$_0$-occupied active site between subunits B and A (site BA, green). The subunits contributing the thioimide ligand Cys-55 (dark colors) were superposed with r.m.s.d. 1.3 Å over all C$_\alpha$ atoms. B, surface representation of the empty (left) and occupied (right) sites showing cleft closure in the presence of preQ$_0$. C, HPLC analytical gel filtration analysis of QueF (blue) and the QueF-preQ$_0$ complex (red). QueF-preQ$_0$ complex elutes at 8.33 min.
ment are associated with preQ₀ binding but not thioimide formation (Fig. 7).

**DISCUSSION**

Prior to their discovery, QueF enzymes had been misannotated in genomic databases as GTP cyclohydrolase I (GCYH-I)-like enzymes (5). Both the QueF and the GCYH-I enzymes belong to the T-fold superfAMILY, and both are represented by unimodular (22) and bimodular (10) subfamilies where a module is built around a T-fold core (Fig. 8). The tertiary and quaternary structural similarity between the unimodular members of these two enzyme families is striking despite disparate sequences and catalytic functions (Fig. 8). In both cases, the enzymes form decamers generated from the association of two pentamers. In contrast, the reported structure of the bimodular QueF (11) is quite distinct from that of bimodular GCYH-I (10), and indeed all bimodular T-fold members, as the bimodular QueF lacks the canonical T-fold quaternary structure of head-to-head association of two βαβαα barrels formed from oligomerization of subunits. Consequently, catalysis in bimodular QueF occurs at the intrasubunit interface between the N- and C-terminal T-fold modules, not at the intersubunit interface as in bimodular GCYH-I and other bimodular T-fold enzymes (9).

Based on prior biochemical studies, we had predicted the formation of a thioimide intermediate in the reaction (14), and the structures of the wild-type and C55A mutant QueF from *B. subtilis* validate this prediction. In the C55A mutant, the electron density is clearly discontinuous between preQ₀ and the enzyme, with the linear structure of the –CN group distinct. In contrast, in the structure of the wild-type enzyme, the electron density is continuous from Cys-55 through the substituent at the 7-position of preQ₀, consistent with the presence of the thioimide. Although a covalent thioimide adduct has also been proposed in the nitrilase-catalyzed hydrolysis of nitriles to carboxylic acids (12), the thioimide reported here is the first structural observation of such an intermediate.

Interestingly, although preQ₀, was included in the crystallization sample in excess of the enzyme (5–10-fold), we trapped asymmetric, partially occupied enzyme complexes in the crystal for both the C55A mutant and the wild-type enzymes. It is not clear whether this asymmetric structure represents a true biological state of QueF or is an outcome of the crystallization conditions and crystal lattice contacts. Because the enzyme does not produce crystals that diffract to high resolution in the absence of substrate, the presence of unliganded subunits in both structures is fortuitous in that they provide insight into the potential structure of the free enzyme. The looser, more open structure of the free enzyme implied by these unoccupied subunits is consistent with the gel filtration and analytical ultracentrifugation data.

A consequence of the conformational change that occurs upon preQ₀ binding is seen around Trp-119, the only tryptophan in *B. subtilis* QueF (Fig. 7), and clearly explains the origin of the fluorescence quenching observed upon binding of preQ₀. Trp-119 sits in a solvent-free pocket made by the side chains of Lys-121, Tyr-133, and His-154. In the substrate-free subunit A, where the C-terminal stretch is disordered, Trp-119 makes two additional hydrophilic interactions with the backbone of the most C-terminal, ordered residue Pro-158 and with the side chain of Asp-131 from the same subunit. In the substrate-bound subunits (e.g. subunit C), these two interactions are eliminated by the ordering of the C-terminal stretch and subsequent conformational change in Asp-131 side chain to form a salt bridge with Arg-164 from the opposite subunit. The interactions in the unoccupied and occupied subunits are identical in both structures and are consistent with the observed Trp fluorescence quenching arising from structural changes associated with preQ₀ binding, but not thioimide formation.

As seen from the crystal structures, the divalent metal sites in QueF can accommodate both Ca²⁺ and Mg²⁺; the similarity of both structures suggests that either metal should result in a functional enzyme, and preliminary biochemical investigations have shown that both metals are capable of supporting efficient catalysis, as is Mn²⁺.

5 Inspection of ~100 unimodular QueF sequences (Fig. 8) for conservation of the metal-binding site (represented by Asp-162 as the only side-chain ligand to the metal ion; Arg-164 contributes the C-terminal carboxylate) and conservation of the salt-bridge pair show that 80% of the sequences contain the residues for the metal site, the salt bridge, or both. The salt bridge is more prevalent than the metal site, with 70% of the sequences containing an Asp-Arg pair at homologous positions to Asp-131–Arg-164, whereas 35% of sequences contain a metal-chelating side chain at or near position 162. These sequence conservation patterns suggest that stabilization of the decamer via salt-bridge formation and/or metal binding at the interpentamer interface is important for the biological function of unimodular QueF.

5 V. M. Chikwana, B. Stec, B. W. K. Lee, V. de Crécy-Lagard, D. Iwata-Reuyl, and M. A. Swairjo, unpublished data.
Although preQ0 does not bind in the interpentamer interface, the ultracentrifugation data indicate that preQ0 does affect the apparent pentamer-decamer equilibrium in solution, decreasing the apparent $K_D$ by over 5-fold. The crystal structures of both the wild-type and the C55A mutant enzyme provide a rationale for this observation as the salt bridges and metal sites are disrupted in the unliganded subunits.

The reduction of a nitrile to an amine requires the delivery of four electrons and four protons. In the QueF reaction, the electrons and two protons are supplied via hydride delivery from NADPH, leaving two protons to be accounted for. Inspection of the active-site region around the nitrile and thioimide moieties in the C55A and wild-type structures, respectively, indicates that only 2 residues, Cys-55 and Asp-62, both conserved, appear to be positioned appropriately to serve as donors. Thus, in the minimal catalytic mechanism (Fig. 9), proton transfer from Cys-55 to preQ0, followed by nucleophilic attack of the thiolate on the nitrile, produces the covalent thioimide inter-

**FIGURE 6.** Transient kinetic analysis of the QueF reaction. **A,** fluorescence emission spectra of wild-type QueF in the absence (top trace) and presence (bottom trace) of preQ0. Spectra were collected with excitation at 295 nm. RFU, relative fluorescence units. **B,** fluorescence measurements of preQ0 binding to the C55A mutant. QueF(C52A) was present at a final concentration of 200 µM, and the final concentrations of preQ0 in reactions were 6.8, 11.8, 18.2, 23.3, 29.2, 38.8, and 50.0 µM. The reactions were carried out at 20 °C. The experimental data are shown in circles, and the model fit is the solid line. **C,** fluorescence measurements of preQ0 binding and reaction with wild-type QueF. Wild-type QueF was present at a final concentration of 200 µM, and the final concentrations of preQ0 in reactions were: 5.3, 15.4, 22.6, and 30.4 µM. The reactions were carried out at 20 °C. The experimental data are shown in circles, and the model fit is the solid line. **D,** absorbance measurements of preQ0 binding and reaction with wild-type QueF. Wild-type QueF was present at a final concentration of 62 µM, and the final concentrations of preQ0 in reactions were 11.8, 17.0, 21.5, 26.3, and 37.7 µM. The reactions were carried out at 30 °C. The experimental data are shown in circles, and the model fit is the solid line. For clarity, only every third data point that was collected is shown in the graph.

**FIGURE 7.** Conformational changes around Trp-119 induced by preQ0 binding. **A,** the environment of Trp-119 in subunit A where the nearest active site (site EA) is unoccupied with substrate and open, the C-terminal residues are disordered, and metal sites are unformed. **B,** same view of Trp-119 in subunit C where the nearest active site (site BC) is occupied with substrate and closed, the C-terminal residues are ordered, and divalent metal sites are formed and occupied. Residues interacting with Trp-119 at distances <4 Å are shown. Hydrophilic interactions with Pro-158 and Asp-131 seen in subunit A are eliminated in subunit C by the ordering of the C-terminal stretch and subsequent conformational change in Asp-131 side chain to form the salt bridge with Arg-164. These changes in the tryptophan environment cause a blue shift in its fluorescence. As in Fig. 2D, the view is from inside the tunnel. The color scheme is the same as in Fig. 2A.
mediate. This intermediate is subsequently reduced to a hemithioaminal by NADPH, with Asp-62 delivering the necessary proton. Binding of the second equivalent of NADPH is followed by breakdown of the covalent hemithioaminal to the protonated imine, and hydride transfer from NADPH then gives the amine of preQ₁.

Based on molecular dynamics simulations of the bimodular QueF from *V. cholerae* (11), an additional conserved residue, His-233 (*V. cholerae* numbering), was proposed to be responsible for delivering the final proton. In the *B. subtilis* QueF, this residue (His-96) is not in an appropriate geometry or in close enough proximity to the thioimide to function effectively as a proton donor.
**Structure of B. subtilis QueF**

direct proton donor. However, His-96 side chain is within contact distance to Asp-62 (3.3 Å) and may be capable of proton transfer to Asp-62, in which case it could serve indirectly in this process by serving as the source of the proton delivered by Asp-62 in the reduction of the thioimide. In the structure of *V. cholerae* QueF, His-233 is similarly positioned, but the significance of this is not clear as the enzyme has guanine, not preQ0, bound in the active site, and the catalytic Cys residue (Cys-194), as well as the loop it is part of, is disordered in the structure.

Taken together, the kinetic and structural data presented here clearly demonstrate the formation of a covalent thioimide intermediate in the reaction cycle and are consistent with a global structural reorganization of the enzyme upon initial binding of preQ0, but not with formation of the thioimide. The thioimide intermediate is quite stable, with a decomposition rate of ~5 × 10⁻⁶ s⁻¹. The fast rate of thioimide formation (2.78 s⁻¹) relative to *k*₅ (0.011 s⁻¹) (14) indicates that steps later in the reaction, involving NADPH binding and subsequent hydride transfer, are rate-limiting in the reaction. Efforts are currently underway to elucidate the details of these later steps.

The reduction of nitriles to amines is typically carried out synthetically by hydrogenation over various transition metal catalysts or by metal hydride reductions, and there exists a need for more environmentally benign, alternative approaches for the synthetic conversion of nitriles to amines. The utilization of engineered biocatalysts to nitrite reduction is an especially attractive option in this context, and as the only known enzyme catalyzing nitrite reduction, QueF has emerged as a potential attractive option in this context, and as the only known enzyme engineered biocatalysts to nitrile reduction is especially needed. Studies of *B. subtilis* QueF combined with molecular simulations provide insight into enzyme mechanism.

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