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Nitrile Reductase as Biocatalyst: Opportunity and Challenge

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Nitrile-containing compounds are widely manufactured and extensively used in the chemical and pharmaceutical industries as synthetic intermediates or precursors. Nitrile hydratase and nitrilase have been successfully developed as biocatalysts for the production of amides and carboxylic acids from nitrile precursors. The discovery of a family of nitrile reductases that catalyse the reduction of nitrile to amine raised the hope of developing environmentally sustainable nitrile-reducing biocatalysts to replace metal hydride catalysts. However, ten years after the discovery of the QueF nitrile reductases, little progress has been made towards the development of nitrile reductase biocatalysts with altered or broadened substrate specificity. In this article, we analyse and review the structure and catalytic mechanism of QueF nitrile reductases and other structurally related T-fold family enzymes. We argue that the poor evolvability of the T-fold enzymes and the kinetically sluggish reaction catalysed by QueFs pose formidable challenges for developing this family of enzyme into practically useful biocatalysts. The challenges do not seem to be mitigated by current computational design or directed-evolution methods. Searching for another family of nitrile reductase or engineering a more evolvable protein scaffold to support the nitrile-reducing chemistry may be more viable strategies to develop a nitrile reductase biocatalyst despite another set of foreseeable challenges.

Introduction

Nitrile-containing intermediates or precursors are widely manufactured and extensively used in the fine chemical and pharmaceutical industries. Efficient and environmentally benign methods of transforming nitrile to amide, carboxylate and amine groups are highly valuable given the ubiquitous presence of these functional groups in small-molecule active pharmaceutical ingredients (APIs) (Figure 1). Nitrile-containing metabolites or natural products have been found in bacteria, fungi, plants and arthropods¹. Hence, it is not surprising that nature has evolved enzymes to use nitrile-containing compounds as substrates^{2, 3}. Microbial nitrile hydratase, nitrilase and amidase have been successfully developed as biocatalysts for the production of amides and carboxylates from nitriles. Nitrile hydratases are being employed for the large-scale production of some of the common commodity chemicals such as glycine, nicotinamide and acrylamide³⁻⁶. Nitrile hydratases are exploited for the synthesis of (S)-3-(thiophen-2-ylthio) butanoic acid and (S)-2,2-dimethylcyclopropane carboxylic acid, which are the precursors for the carbonic anhydrase inhibitor Dorzolamide and dehydropeptidase inhibitor Cilastatin respectively^{7, 8}. Nitrilases have been developed to produce the commercially important chemical acrylic acid from acrylonitrile and other carboxylic acids as pharmaceutical intermediates or precursors⁹⁻¹¹.

Nitriles can be converted to amines by catalytic hydrogenation or using complex metal hydrides. By using a complex metal hydride, one can control the reduction of nitrile

to yield either imine or the fully-reduced amine. When a nitrile is reduced with lithium aluminium hydride, a primary amine will be produced. When a nitrile is reduced with the less reactive lithium trialkoxyaluminum hydride or diisobutylaluminum hydride, an imine will be formed. Since the imine can be hydrolysed to give an aldehyde, this method is also used to prepare aldehydes from nitriles. Some of the nitrile-to-amine transformations currently employed in the synthesis of drug candidate or APIs are shown in Figure 1. The chemical methods used to catalyse these conversions employ non-selective transition metal or metal hydride catalysts that inevitably generate toxic by-products and solvent waste. Given the success of the nitrile hydratase and nitrilase biocatalysts, discovery and development of environmentally sustainable biocatalysts to replace the small molecule catalysts for nitrile-to-amine conversion could potentially shorten the production process and reduce waste production.

Discovery, structure and catalytic mechanism of nitrile reductase

Although nitrile hydratases and nitrilases have been discovered and harnessed as industrial biocatalysts for a long time, no such enzyme was previously known to convert nitrile to amine. The addition of a hydride to the cyano group is analogous to the addition of a hydride to the carbonyl group, a common

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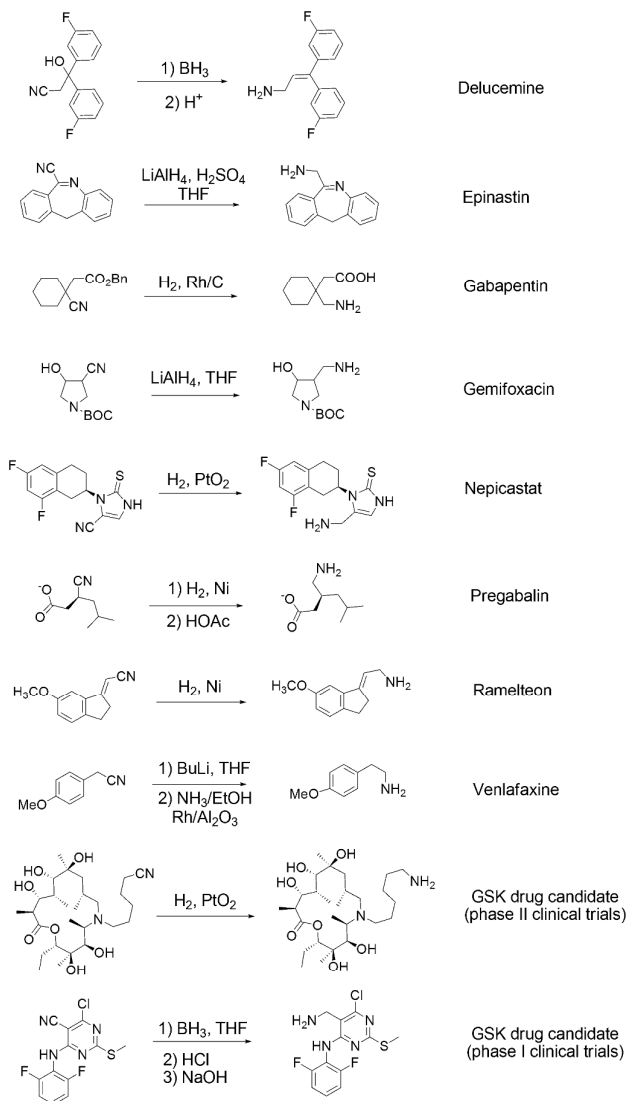


Figure 1. Nitrile-to-amine transformations in the synthesis of APIs or drug candidates.

biological reaction catalysed by NADPH or NADH-dependent reductases or dehydrogenases. However, the reduction of nitrile is kinetically more demanding because the π bond of a nitrile group is intermediate in polarity between the π bond of a carbonyl group and an unactivated alkene. The four-electron reduction of nitrile to amine also would require two reducing hydrides with an imine intermediate that is prone to H_2O hydrolysis. Besides the lack of evolutionary pressure, it was speculated that the kinetic inertness of the nitrile group probably prohibits the evolution of NAD(P)H-dependent nitrile

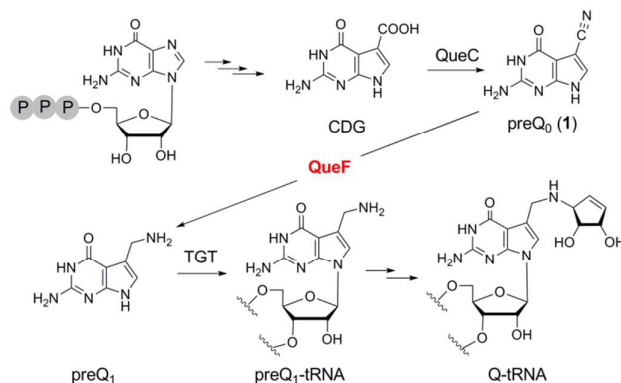


Figure 2. Nitrile reductase QueF and the biosynthetic pathway for queuosine and Q-tRNA.

reductase.

It came as a delightful surprise in 2005 when the *Bacillus subtilis* enzyme YkvM or QueF, which was initially predicted to be a GTP cyclohydrolase, was found to catalyse an NADPH-dependent reduction of nitrile to primary amine¹². QueF is from the biosynthetic pathway of queuosine, a 7-deazaguanine modified nucleoside found at the wobble position of tRNAs in Bacteria and Eukarya^{13, 14}. The biosynthetic intermediate 7-cyano-7-deazaguanine (preQ₀) that contains the nitrile group originates from GTP, with the cyano nitrogen derived from ammonia (Figure 2). The intermediate preQ₀ is subsequently reduced by QueF to give 7-aminomethyl-7-deazaguanine (preQ₁), which is later incorporated into the tRNA to form preQ₁-tRNA and Q-tRNA through several additional enzymatic steps.

So far, the recombinant proteins of the QueF homologs from *B. subtilis*, *Vibrio cholera*, *Escherichia coli*, *Geobacillus kaustophilus* have been cloned and biochemically characterized. Crystal structures of the QueF homologs from *V. cholerae* and *B. subtilis* have been determined (PDB IDs: 3RJ4; 3BP1; 3UXJ; 3UXV; 4GHM; 4IQI; 4F8B)¹⁵⁻¹⁷. The crystal structures revealed that the QueF enzymes from *V. cholerae* and *B. subtilis* form homodimer and homodecamer respectively (Figures 3A & 3B). The subunit of *B. subtilis* QueF adopts a T-fold or tunnelling fold that is composed of four β -strands and two α -helices. The β -strands form a highly twisted antiparallel β -sheet to form a two-layer sandwich with the two α -helices packed against the concave side of the β -sheet. The T-fold domains oligomerize in a head-to-tail arrangement to form a pentamer, which further dimerizes to form the decamer. In contrast, the subunit of the dimeric *V. cholerae* QueF is a pseudodimer that contains two head-to-tail T-folds within the same polypeptide. In both proteins, the substrate preQ₀ is bound in the active site located at the interface of two T-folds (Figures 3C & 3D). As a result, QueFs possess multiple symmetry-related active sites in their tunnel-shaped and oligomeric $\beta_{2n}\alpha_n$ barrel complexes. The crystal structures of the two QueF enzymes also reveal that the

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substrate specificity toward preQ₀ is defined by hydrophobic interaction and specific hydrogen bonding interaction between the substrate and active site residues (Figures 3D & 3E).

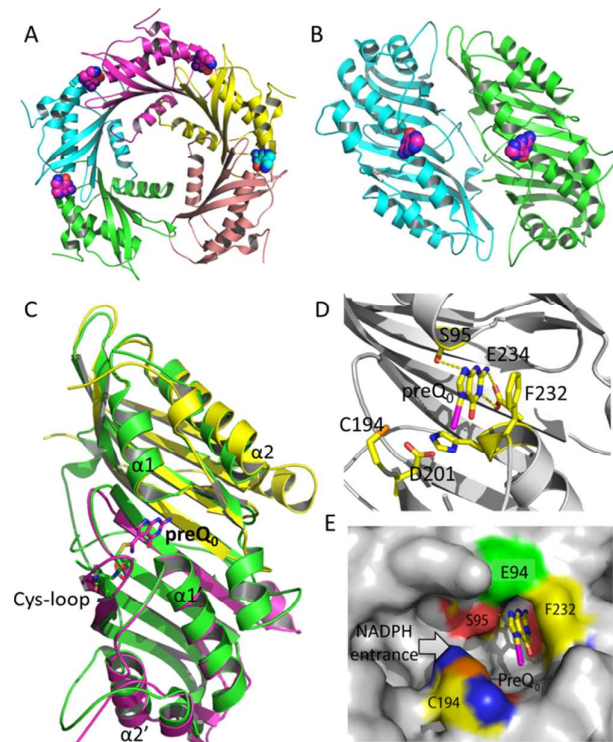


Figure 3. Crystal structures of QueFs. A. Structure of *B. subtilis* QueF (PDB: 4F8B) with only half of the decameric structure showing here. B. Structure of dimeric *V. cholerae* QueF. (PDB: 3BP1)¹⁵⁻¹⁷. The substrate preQ₀ is shown in spheres in A & B. C. Superimposed subunit structures of the *B. subtilis* QueF (yellow and magenta) and *V. cholerae* QueF (green). D-E. Substrate-binding pocket of *V. cholerae* QueF and binding mode of preQ₀.

Based on the crystal structures and enzyme kinetic studies, a catalytic mechanism that involves a nucleophilic reaction and two hydride transfer steps was proposed^{16,18,19}. Two conserved polar residues (Cys194, Asp201) in the active site of *V. cholerae* QueF were suggested to play major catalytic roles. As illustrated in Figure 4, the reduction of nitrile starts with the nucleophilic attack of the thiol group of Cys194 (*V. cholerae* QueF) to the nitrile, leading to the formation of a covalent thioimidate intermediate. Formation of the thioimidate covalent intermediate was borne out by X-ray crystallographic studies. Reduction of the thioimidate intermediate by an equivalent of NADPH yields a thiohemiaminal intermediate. Collapse of the thiohemiaminal intermediate is followed by a second NADPH reduction step to yield the amine preQ₁. It was suggested that the collapse of the thiohemiaminal intermediate only occurs after the binding of the second NADPH. Because the imine

intermediate is prone to hydrolysis in the presence of a water molecule, the presence of NADPH would be crucial for protecting the imine from H₂O and safeguarding the turnover of imine to amine. In addition to the nucleophilic Cys194

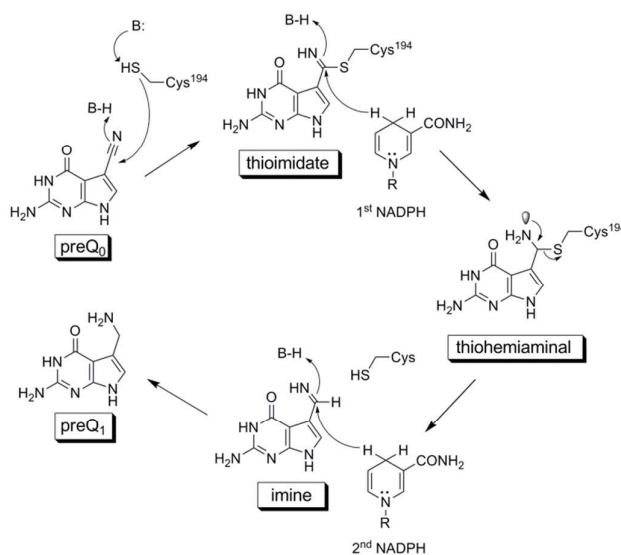


Figure 4. Proposed catalytic mechanism for QueF nitrile reductase.

residue, Asp201 is the most likely general base/acid catalyst needed for the reduction of nitrile. Although the overall mechanism of nitrile reduction is new, the chemistry is not unprecedented. It is well known that the nitrile group is prone to nucleophilic attack by thiol. This has been exploited for designing irreversible inhibitors for cysteine proteases²⁰. The overall mechanism is reminiscent of the catalytic mechanism of guanosine monophosphate (GMP) reductase, which uses a nucleophilic cysteine residue to attack the substrate GMP to generate a thioimidate intermediate that is subsequently reduced by an equivalent of NADPH to yield the product inosine monophosphate²¹. Similar two-step transformations are also observed in aldehyde dehydrogenases, which contain a nucleophilic cysteine and feature a highly flexible binding site for the NADH cofactor^{22,23}.

Challenges in the development of nitrile reductase as biocatalyst

Considering the importance of the nitrile-to-amine conversion to the pharmaceutical and fine chemical industries, it was hoped that the QueF nitrile reductases can be exploited as biocatalysts to replace the metal hydride catalysts²⁴. However, recent studies suggested that development of the nitrile reductases into useful biocatalysts for non-native substrates could be very challenging for some of the reasons detailed below.

All QueF enzymes characterized so far possess stringent substrate specificity. Besides the native substrate preQ₀, the QueF homolog from *E. coli* was found to act on 5-cyanopyrrolo[2,3-d]pyrimidin-4-one (**1**, Figure 5), a substrate

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that differs from preQ₀ by the absence of a single amine group^{19, 25}. The QueF homolog from the thermophilic bacterium *G. kaustophilus* exhibited catalytic activity towards **1** and 2-amino-5-cyanopyrrolo[2,3-d]pyrimidine (**2**), another substrate that shares high structural similarity with preQ₀²⁶. A survey of the substrate scope of the *B. subtilis* QueF by us with a panel of commercially available nitrile compounds demonstrated that this homolog is also highly specific, with none of the compounds showing detectable turnover to amine. The high substrate specificity is in agreement with the small (volume ~ 387 Å³) and well-defined substrate-binding pocket observed in the crystal structures. According to the crystal structures of *V. cholerae* QueFs, preQ₀ is bound in a solvent-exposed binding pocket with the side chain of Phe232 packed against the planar preQ₀¹⁶. The substrate specificity is further defined by specific polar interactions between preQ₀ and Glu234, Ser95 and Glu94. With the narrow substrate specificity, the hope of using QueF proteins as biocatalysts is hinged on whether we can broaden or alter the substrate specificity by rational design or directed-evolution methods.

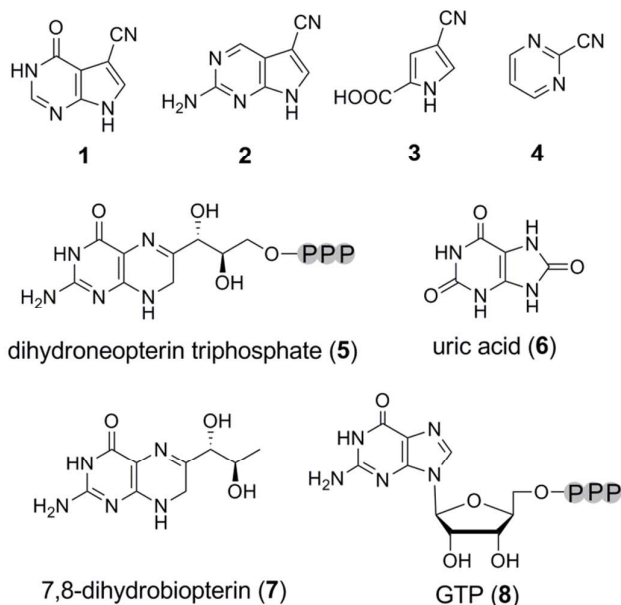


Figure 5. Non-native nitrile substrates (**1 - 4**) identified for QueF mutants and the purine or pterin substrates (**5 - 8**) for other T-fold enzymes.

Klempier and co-workers generated several mutants of the *E. coli* and *G. kaustophilus* QueF nitrile reductases by replacing the residues predicted to form hydrogen bonds with preQ₀^{19, 26}. Among a dozen nitrile compounds that are structurally related to preQ₀, only one (**1**) showed detectable activity for a few of the *E. coli* mutants. A few mutants of *G. kaustophilus* QueF exhibited activity towards **1**, and very low activity towards **2**. This is discouraging because as mentioned above, **1** and **2** could also be accepted by the wild type enzyme as substrates. In our laboratory, mutants of *V. cholerae* QueF generated by site-directed and random mutagenesis were tested

against a panel of structurally more diverse nitrile compounds, including several pharmaceutical intermediates shown in Figure 1. In addition, mutants that contain up to nine replacements were computationally designed for some of the pharmaceutical nitrile intermediates shown in Figure 1 by using Rosetta enzyme design protocol. Only low enzymatic activity towards two small non-natural substrates (**3** & **4**) could be observed for the *V. cholerae* QueF mutants that contain the Ser95Ala or Ser95Gly replacement (to be published). The observations suggest that the QueF enzymes have highly substrate specificity that cannot be altered or broadened easily.

The protein structures of QueFs and other T-fold enzymes offer some clues on why it is so difficult to alter the substrate specificity of the QueF enzymes. The core domain of QueF nitrile reductases adopts a T-fold or tunnelling fold with the active site located between two T-fold domains. Several other enzymes that include GTP cyclohydrolase I, 6-pyruvoyl tetrahydropterin synthase, 7, 8-dihydroneopterin aldolase, 7, 8-dihydroneopterin triphosphate epimerase, uricase and the ApbE Protein (TM1553) of *T. maritima* also contain a T-fold catalytic domain²⁷⁻³⁴. Despite the different types of reactions catalysed by T-fold enzymes³⁵, they seem to only act on substrates that contain a planar purine or pterin (preQ₀ and compounds **5**, **6**, **7** and **8**, Figure 5). The similarity of the substrates is in accordance with the small and planar substrate-binding pocket of all T-fold enzymes. The active sites of T-fold enzymes also contain a conserved Glu or Gln residue (Glu234 of *B. subtilis* QueF) that forms hydrogen bonds with the substrates²⁷. Hence, despite the diverse reaction types catalysed by the T-fold enzymes, the T-fold seems to be evolutionarily conserved for binding a small class of structurally similar substrates.

Inspection of the structures of QueF and other T-fold enzymes revealed that the residues lining the substrate-binding pocket are mostly from the structural β -strand and α -helices. Only a short loop that contains the nucleophilic cysteine (termed as cys-loop) is relatively mobile or flexible (Figure 3C). This is in sharp contrast to other highly evolvable scaffolds such as the (β/α)₈ barrel and metallo- β -lactase folds, which contain long and mobile loops and exhibit great conformational dynamics in the substrate-binding pocket. A protein scaffold that has a high percentage of structural residues involved in substrate-binding is considered to have low dynamics and a low scaffold-active-site polarity³⁶⁻³⁸. Such low-polarity protein scaffolds are usually associated with poor evolvability because of the strong coupling between protein stability and function. Computational docking studies by us suggest that the substrate-binding pocket of *V. cholerae* QueF is too small for most of the pharmaceutical intermediates shown in Figure 1. Because the size of the preQ₀-binding pocket is restricted by the main chain groups of $\alpha 1$ and $\alpha 1'$ helices (Figure 3C), any significant expansion of the pocket requires an alteration of the main chain conformation, such as the shorting of $\alpha 1$ or $\alpha 1'$ helices. However, shortening the Ser95-containing $\alpha 1$ helix to enlarge the substrate-binding pocket of *V. cholerae* QueF by residue deletion resulted in a loss of thermostability, as indicated by significant decreases in protein melting temperature (Yang et al, to be published). Hence, destabilizing effects of residue replacement or deletion will comprise a major constraint for QueF engineering.

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Alteration or expansion of the small substrate-binding pocket of QueF without perturbing protein folding or stability will be a considerable challenge.

Theoretically, it is still feasible to engineer the QueF enzymes to accept non-native substrates that are smaller or not significantly larger than preQ₀ by side chain replacement. Directed-evolution methods by random mutagenesis or homologous recombination may be considered. Although the enzymatic activity of QueFs can be assayed by monitoring NADPH depletion using absorption of fluorescence spectroscopic methods, a colony or cell lysate-based high-throughput screening method must be developed for directed-evolution. We have tested several fluorescent and colorimetric methods for the detection of dye conjugates formed by the amine product of QueF in cell lysate or *E. coli* colonies. Largely due to high background, none of the methods was considered to be suitable for the screening of nitrile reductase activities. Because of the sluggish reaction catalysed by the wild type enzymes ($k_{\text{cat}} = 0.12 \text{ s}^{-1}$ for *E. coli* QueF (30 °C), $k_{\text{cat}} = 0.065 \text{ s}^{-1}$ for *G. kaustophilus* QueF (55 °C) and $k_{\text{cat}} = 0.01 \text{ s}^{-1}$ for *B. subtilis* QueF (30 °C))^{15, 18, 19, 26}, screening for QueF variants with low nitrile reductase activity will require a highly sensitive and selective method.

The hope of altering or expanding the substrate specificity of the QueF nitrile reductase by using computational methods faces additional challenges. The first step of the enzyme mechanism involves the nucleophilic cysteine residue located on a mobile loop. Given the small size of the substrate-binding pocket, any change in the active site will potentially alter the mobility of the loop and change the surroundings of the cysteine. Such changes may affect the conformation and nucleophilicity of the cysteine adversely. Current computational methods are incapable of predicting such a subtle effect. Second, the precise binding mode of NADPH is uncertain. Only a portion of the NADP⁺ is visible from the electron density map obtained from NADP⁺ co-crystallized enzyme. The missing nicotinamide seems to suggest a high degree of flexibility for the NADPH-binding residues. Prediction of the binding mode of NADPH will be required for computational design, but the conformational flexibility will be a challenge for current computational methods. And lastly, because a significant enlargement of the substrate-binding pocket cannot be achieved by replacement of only side chains, engineering of QueFs to act on substrates larger than preQ₀ would require the alteration of protein backbone structure. Current computational methods are still not able to predict accurately the alteration of main chain conformation (flexible backbone design) caused by residue replacement or deletion.

Conclusions

Development of the QueF nitrile reductases as practical biocatalysts for non-native nitrile substrates will be challenging because of the high substrate specificity and poor adaptability of the evolutionarily conserved substrate-binding pocket. Maybe it is time to consider other options to develop nitrile-reducing biocatalysts. Is there another family of natural nitrile reductases of different protein fold? Assuming the catalytic mechanism is preserved, we need to search for a family of functionally unknown proteins with a NADPH-binding domain

and a conserved cysteine residue. If such a bioinformatics search turns out to be unsuccessful, could we retrofit a more evolvable scaffold to support the nitrile-reducing chemistry? The approach of re-designing the active site of existing protein scaffolds has been adopted for computational enzyme design recently³⁹⁻⁴⁶. Considering the large number of crystal structures available for NAD(P)H-dependent imine reductases or medium-chain and short-chain dehydrogenases, it may be feasible to find a suitable scaffold that allows us to install a cysteine residue and general base/acid catalyst to introduce the nitrile-reducing activity. Aldehyde dehydrogenases are probably even better starting scaffolds because they already possess a nucleophilic cysteine residue and a NADPH binding site. Guanosine monophosphate reductase could also be a good scaffold considering the highly evolvable (β/α)₈-fold also harbours a nucleophilic cysteine residue and a NADPH binding site.

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Notes and references

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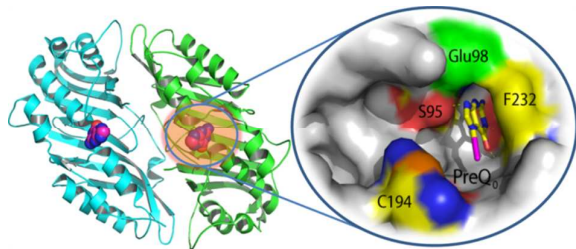
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The review highlights recent progress and challenges in developing a family of nitrile reductases as biocatalysts for nitrile-to-amine transformation.