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Biogenesis of the peptide-derived redox cofactor PQQ

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Abstract

Pyrroloquinoline quinone (PQQ) is a peptide-derived redox cofactor produced by prokaryotes that also plays beneficial roles in organisms from other kingdoms. We review recent developments on the pathway of PQQ biogenesis, focusing on the mechanisms of PqqE, PqqF/G, and PqqB. These advances may shed light on other, uncharacterized biosynthetic pathways.

Introduction

The 1960s and 1970s saw the emergence of bacterial glucose and alcohol dehydrogenases that act independently of nicotinamide or flavin-based cofactors, showing features of a novel quinone-like prosthetic group instead.[1–4] This “mystery cofactor” was later characterized by X-ray crystallography to be PQQ, also referred to as methoxatin (Figure 1A).[5,6]

The PQQ-dependent glucose and alcohol dehydrogenases (Figure 1B) participate in methylotrophic metabolism and ethanol/glucose utilization in prokaryotes.[7–9] More than 80% of PQQ-producing bacteria are proteobacteria, including a variety of opportunistic pathogens displaying antibiotic resistance (Figure 1C).[10–12] Bacteria can capture PQQ exogenously via a PQQ-binding protein, consistent with the proposal of inter-cellular PQQ trafficking.[13] There are recent reports of PQQ-dependent enzymes produced by archaea and fungi[14–16] implicating their symbiotic relationship with PQQ-producing microorganisms, although more biological studies are needed to explore this aspect further. The detection of PQQ in mammal fluid[17] and tissue[18] led to an early proposal that PQQ would be a eukaryotic vitamin.[19] This idea has been replaced by the concept of a “longevity vitamin,” whereby PQQ is not essential for humans but necessary for long-term health.[7,19–21] Echoing animal studies[22,23] that feeding PQQ improves mitochondria-related cellular functions is the finding that PQQ binds to human L-lactate dehydrogenase and regulates its activity, suggesting the active participation of PQQ within mammalian cells.[24] Given that many PQQ-producing bacteria, such as *Acinetobacter* and *Pseudomonas,* are associated with the human microbiome, the rising spotlight on the

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essential role of the human microbiota in health[25] introduces an intriguing new direction for the function of PQQ in eukaryotic organisms.

Unlike the pyridine nucleotide or flavin-based redox cofactors, our understanding of the biological origin of PQQ is a relatively recent event that began with the discovery of six conserved genes, *pqqABCDEF/G*, among PQQ-producing organisms (Figure 1D).[26–35] As the only known widespread redox cofactor that is derived from a ribosomally synthesized peptide, it is gratifying to see decades of research coalescing into a robust understanding of the molecular basis of PQQ production.

PqqA/D/E: a radical beginning for the pathway

Early feeding experiments established the link between PQQ production and cellular tyrosine and glutamate levels.[36,37] Through transposon insertions into *pqqA* and point mutations that shift its reading frame, it was confirmed that *pqqA* expression is essential for PQQ production.[27,31,35] The *pqqA* is translated into a short peptide that contains the fully conserved residues, glutamate and tyrosine; site-direct mutagenesis identified these two residues on PqqA as the best candidate for the precursor to PQQ (Figure 2A).[31] No function could be proposed for PqqD or PqqE, even though their conservation in the pathway was well recognized via genetic knockouts experiments in various PQQ-producing bacteria.[26–35]

Meanwhile, a group of enzymes within the radical *S*-adenosyl-l-methionine (SAM) superfamily[38] was linked to the biogenesis of important metabolites, such as biotin[39], lipoic acid[40], heme[41] and molybdopterin[42]. PqqE contains a conserved CxxxCxxC motif that coordinates a [4Fe-4S] cluster near the N-terminus, a common feature of radical SAM enzymes.[43] The ability of PqqE to conduct SAM cleavage was tested in the presence of the reducing agent sodium dithionite, confirming that PqqE is indeed a radical SAM enzyme that converts SAM to 5’-deoxyadenosine.[44] The iron content, electronic paramagnetic resonance (EPR)[44,45] and Mössbauer spectroscopy[46,47] of PqqE further suggested that the C-terminal domain, later classified as a SPASM domain[48], contains two more iron-sulfur clusters, the auxiliary site I (AuxI) and auxiliary site II (AuxII) (Figure 2B).

Although members of the SPASM subfamily catalyze protein/peptide modification in the presence of substrate, SAM, and reductant, these components failed to produce any detectable chemical change in PqqA upon the addition of PqqE.[44] The missing factor has turned out to be PqqD, a small, cofactor-less protein that primarily exists as a free protein (and occasionally as a C-terminal fusion protein of PqqC or an N-terminal fusion of PqqE) (Figure 1D).[12] EPR and hydrogen-deuterium exchange analyses suggested that PqqD interacted directly with PqqE.[45] Further studies, using native mass spectrometry, surface plasmon resonance and isothermal titration calorimetry (ITC), supported a tight-binding (nanomolar) complex between PqqD and PqqA.[49] The confirmation of a “substrate chaperone” role for PqqD finally came from the *in-vitro* demonstration of Glu-Tyr cross-linking within PqqA when PqqD was incubated with PqqE.[50] While the X-ray structure of PqqD initially showed a “domain-swapped” dimer[51], solution NMR[52,53] and small-angle X-ray scattering[49] data have implicated a monomeric structure (Figure 2C). The PqqD monomer is highly similar to the peptide recognition domain in other peptide

modification proteins, such as CteB[54] and SuiB[55], in support of a functional monomeric form of PqqD (Figure 2C). NMR studies also identified residues on PqqD that interact with PqqA and PqqE (Figure 2C).[53] In this manner, the interrelated functions of PqqA, PqqD, and PqqE in the first step of PQQ biogenesis were uncovered (Figure 2D).

When the first (and thus far only) crystal structure of PqqE was resolved,[56] a [2Fe-2S] cluster appeared in the AuxI site of the SPASM domain, distinct from other SPASM proteins that dominantly incorporate [4Fe-4S] at this site. This [2Fe-2S] cluster in PqqE was confirmed spectroscopically by EPR and Mössbauer spectroscopy.[47,56,57] Further EPR investigations on PqqE have also shown a low potential [4Fe-4S] cluster in AuxI that requires Ti(III)citrate for reduction.[57] Such a low redox potential contrasts with the majority of iron-sulfur clusters in other radical SAM enzymes that readily undergo reduction by sodium dithionite (with the exceptions being the low potential auxiliary [4Fe-4S] cluster in BtrN[58,59] and NeoN[60]). Native mass spectrometry and computational modeling studies of PqqE have alternatively supported the formation of either a [4Fe-4S] or [2Fe-2S] cluster within the AuxI site.[47,57,61] Site-specific mutagenesis, in which a cysteine ligand in the AuxI site is replaced by histidine, leads to an [4Fe-4S] only AuxI site, completely abrogating [2Fe-2S] cluster formation.[61] Of mechanistic significance, the Cys-to-His variant catalyzes reductive cleavage of SAM but loses all ability to cross-link the peptide substrate.[61] These findings set up a stage for uncovering the catalytically active form of PqqE at the AuxI site.[61]

PqqF/G: A labile element within the *pqq* operon

Following the modification of PqqA by PqqE in the presence of PqqD, it was clear that a peptidase would be required to generate PQQ. A protease-like protein encoded by *pqqF* in the *pqq* operon has been reported to be essential for PQQ biosynthesis in *K. pneumoniae*, [64] yet this gene was not detected in the *A. calcoaceticus pqq* operon nor did it seem essential for PQQ production in this organism.[26,27] In *Serratia sp. Fs14*, PqqF represents the type of protease that typically presents itself within the *pqq* operon. The first crystal structure of this protein uncovered an 84 kDa M16 metalloprotease containing a His-His-Glu-His motif for zinc-binding in the active site (Figure 3A).[65] In *M. extorquens*, the *pqq* operon does not contain a canonical *pqqF*.[8,32] Instead, two co-existing genes, *pqqF* and *pqqG*, that are located outside of the *pqq* operon, encode the protease associated with PQQ production.[8,32] A recent study reveals that *M. extorquens* PqqF and PqqG form a tight complex, catalyzing the hydrolysis of multiple peptide bonds in PqqA, with a preference for serine residues(Figure 3B).[65] It has been proposed that the heterodimeric PqqF/G first trims the cross-linked product from the PqqA/D/E reaction, with final hydrolysis to a cross-linked di-amino acid requiring the participation of one or more generic cellular proteases. [65]

PqqB: The missing link and a new twist

Although *pqqB* is conserved in the *pqq* operon, conflicting results regarding the necessity of PqqB in PQQ production had been reported.[34,66,67] Sequence alignments, together with X-ray crystal structures, assigned PqqB to the metallo-β-lactamase (MBL) superfamily (Figure 3C).[68,69] Enzymes in this superfamily generally catalyze the hydrolysis of small

molecules using metal centers that range from zinc to manganese, nickel, magnesium, and iron.[69,70] The latter led to the speculation that PqqB might serve as an oxygenase to modify the tyrosine sidechain within PqqA.[71] Metal reconstitutions of PqqB revealed fairly promiscuous and stable incorporation of copper, zinc, manganese, and magnesium, but not iron.[69] Chemical reactivity was pursued with metal reconstituted PqqB via the use of Cys-DOPA as an analog of the postulated Glu-Tyr di-amino acid substrate (Figure 3C). PqqB containing copper, zinc, manganese, or magnesium was, however, unable to catalyze any chemical conversion of Cys-DOPA. A surprising twist arose when PqqB was incubated with iron in the presence of Cys-Tyr or Cys-DOPA, leading not only to a significant increase in iron affinity but to two separate O_2-dependent reactions: the decarboxylation of α-ketoglutatrate in the presence of Cys-Tyr and the aromatic ring hydroxylation of Cys-DOPA (Figure 3C).[72] Detailed kinetic analyses have confirmed that PqqB is a previously unknown iron-dependent hydroxylase, indicating a cross-over between the MBL superfamily and the non-heme iron hydroxylases. A mechanism has been proposed in which the Tyr of a Glu-Tyr di-amino acid is first hydroxylated to generate a 3,4-dihydoxy-intermediate that is subsequently oxidized again to a trihydroxy-derivative of Tyr (Figure 3C).[72] While each step consumes one equivalent of O_2, neither produces hydrogen peroxide leading instead to water (Figure 3C). The organic product from the PqqB-catalyzed reaction with Cys-DOPA has further been shown to undergo a spontaneous cyclization in solution, leading to an analog of 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline-7,9-dicarboxylic acid (AHQQ) which is the substrate of PqqC in the final step of PQQ biogenesis.[73] These discoveries regarding the properties of PqqB have not only provided a critical step toward closing our understanding of the PQQ biosynthetic pathway, but also suggest an emerging presence of nonheme iron hydroxylating enzymes within the metallo-β-lactamase superfamily.[72]

PqqC: The last step but the first to be discovered

The function of PqqC was the first to be characterized among all the enzymes in the pathway. In *pqqC* knockout experiments, the accumulation of a “PQQ-like” intermediate, that disappeared upon PqqC complementation, led to the identification of the substrate for PqqC.[34,75,76] The chemical identity of this “PQQ-like” intermediate is AHQQ.[73] Through a combination of X-ray studies that yielded structures for PqqC with and without bound cofactor[77] and the detailed dissection of the four key oxidative steps[78] of PqqC, a catalytic mechanism has been described (Figure 3D). PqqC catalyzes an overall eight-electron and eight-proton oxidation on AHQQ in the absence of any organic/metal cofactor, consuming three equivalents of O_2 in a single turnover manner. The stepwise utilization of O_2 is accompanied by a progressive conformational change of PqqC, resulting in the reduction of bound product H_2O_2, rather than exogenous O_2, during the 3rd step of the reaction.[78]

Overview of PQQ biogenesis

We can now propose the chemical steps that lead from PqqA to PQQ (Figure 4A). PqqA interacts with PqqD and guides the to-be-modified Glu and Tyr side chains of PqqA to the active side of PqqE. In the presence of reducing reagent and SAM, the [4Fe-4S] cluster in PqqE promotes the cleavage of SAM to 5’-deoxyadenosyl radical to initiate a series of steps

culminating in a Glu-Tyr crosslink within PqqA. This product, PqqA', is subsequently hydrolyzed by the protease PqqF (or a PqqF/ PqqG complex) that acts to trim away both N- and C-termini amino acids; the action of other (as yet unidentified) cellular proteases appears necessary in the generation of a Glu-Tyr di-amino acid substrate for PqqB. PqqB first hydroxylates the cross-linked Tyr to form Glu-DOPA, which is then further functionalized to a Glu-OH-DOPA quinone product. The latter is sufficiently reactive to undergo spontaneous cyclization, yielding AHQQ as the substrate for the final oxidative steps of the pathway catalyzed by PqqC.

Outlook

Beyond what is shown here, many intriguing questions remain in PQQ biogenesis. Perhaps the most compelling is the question of how the individual steps of the pathway coordinate with each other such that anaerobic reaction and oxygen-dependent reactions can operate sequentially, and whether this is related to the highly conserved order of open reading frame within the *pqq* operon. While the formation of a functional ternary complex of PqqA/D/E is established, it is intriguing to speculate that additional transient protein-protein interactions may function to segregate the aerobic and anaerobic portions of the pathway. The deeper understanding of the PQQ biosynthesis pathway will encourage further studies into antibiotic design targeting PQQ biogenesis selective against opportunistic pathogens. The utilization of PQQ by non-PQQ producing organisms, especially in eukaryotic cells, is also an intriguing aspect for future research in microbiology and systems biology.

Looking to the future, as new organisms and new biosynthetic pathways are discovered, much exciting chemistry is anticipated. The combination of genetic knockout/editing technologies, genome sequencing, bioinformatics tools (e.g., RODEO[79], antiSMASH[80]), and machine learning[81] is enabling rapid progress. Mycofactocin (MFT) biosynthesis [82–84] is an example of the ability of experimental and bioinformatics methods to drive new peptide-derived cofactor discovery.[82](Figure 4B) Even though the molecular function of MFT remains to be confirmed,[83] its structure and properties strongly suggest its role as a second, peptide-derived, prokaryotic redox cofactor.[84]

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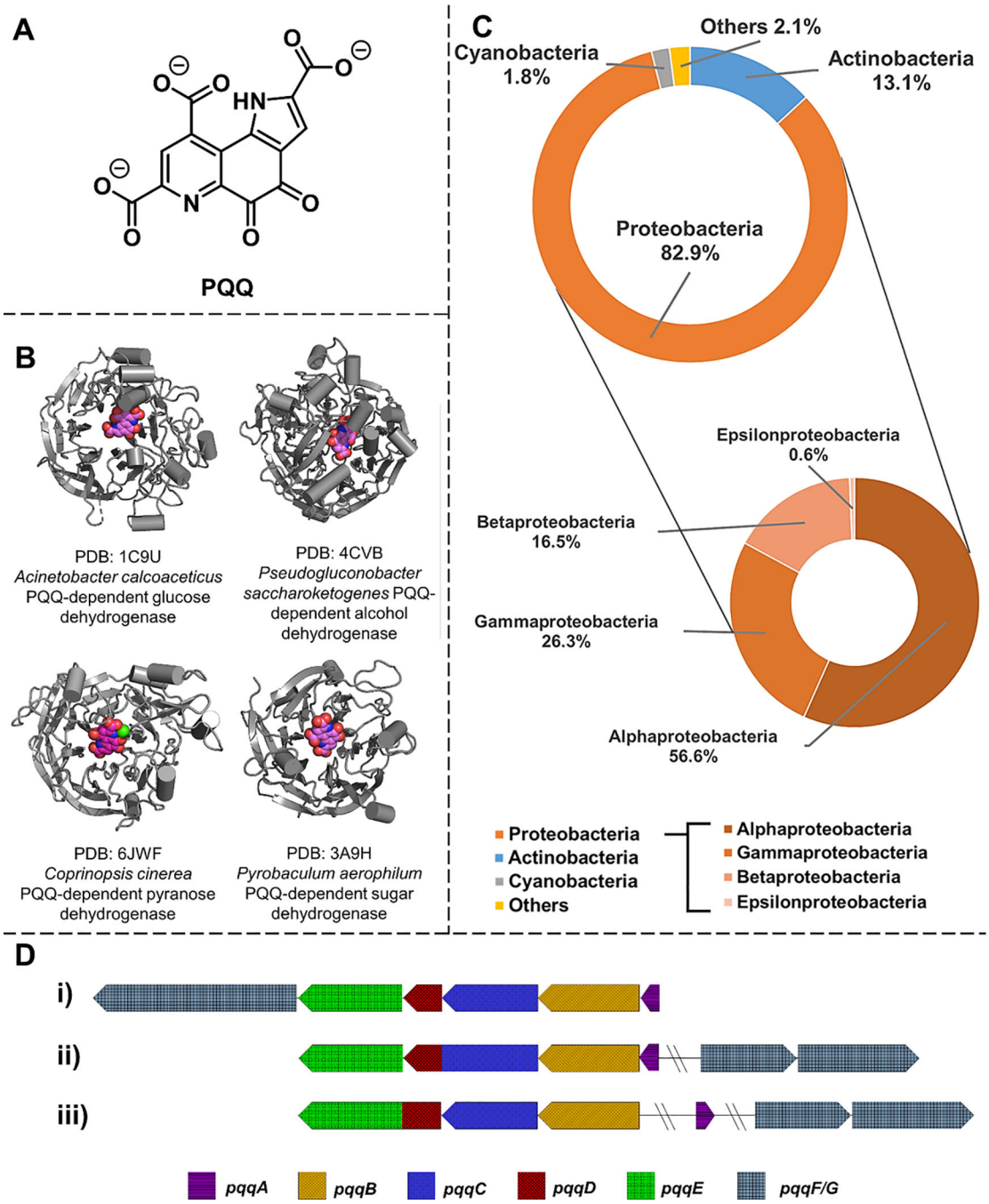

Figure 1. PQQ, PQQ-dependent enzymes and PQQ biogenesis in bacteria.

A. Structure of PQQ. **B**. Crystal structure of PQQ-dependent enzymes. PQQ-dependent dehydrogenases have been found in bacteria (e.g., *Acinetobacter calcoaceticus* and *Pseudogluconobacter saccharoketogenes*) as well as archaeon (e.g., *Pyrobaculum aerophilum*) and fungi (e.g., *Coprinopsis cinerea*). The bound-PQQ molecule is highlighted in magenta. **C**. Predicted PQQ producing bacteria are mostly γ-bacteria in the proteobacteria phylum. Doughnuts represent the distribution of species that are predicted to contain PQQ biosynthesis pathways. The initial pool of bacteria was selected from those that contain

PqqA (PF08042)in the Pfam 32.0 database (https://pfam.xfam.org) since PqqA is absolutely required in PQQ biogenesis. **D**. Genes involved in PQQ biogenesis can be organized differently in various organisms: i) all six genes present in the *pqq* operon in *Acinetobacter calcoaceticus*; ii) *pqqF/G* located outside of the *pqq* operon containing *pqqA-E* in *Methylobacterium extorquens*; *pqqD* is fused with *pqqC*; iii) *pqqA* and *pqqF/G* are both distal from *pqqB-E* in *Methylosinus trichosporium* and *pqqD* is fused with *pqqE.*

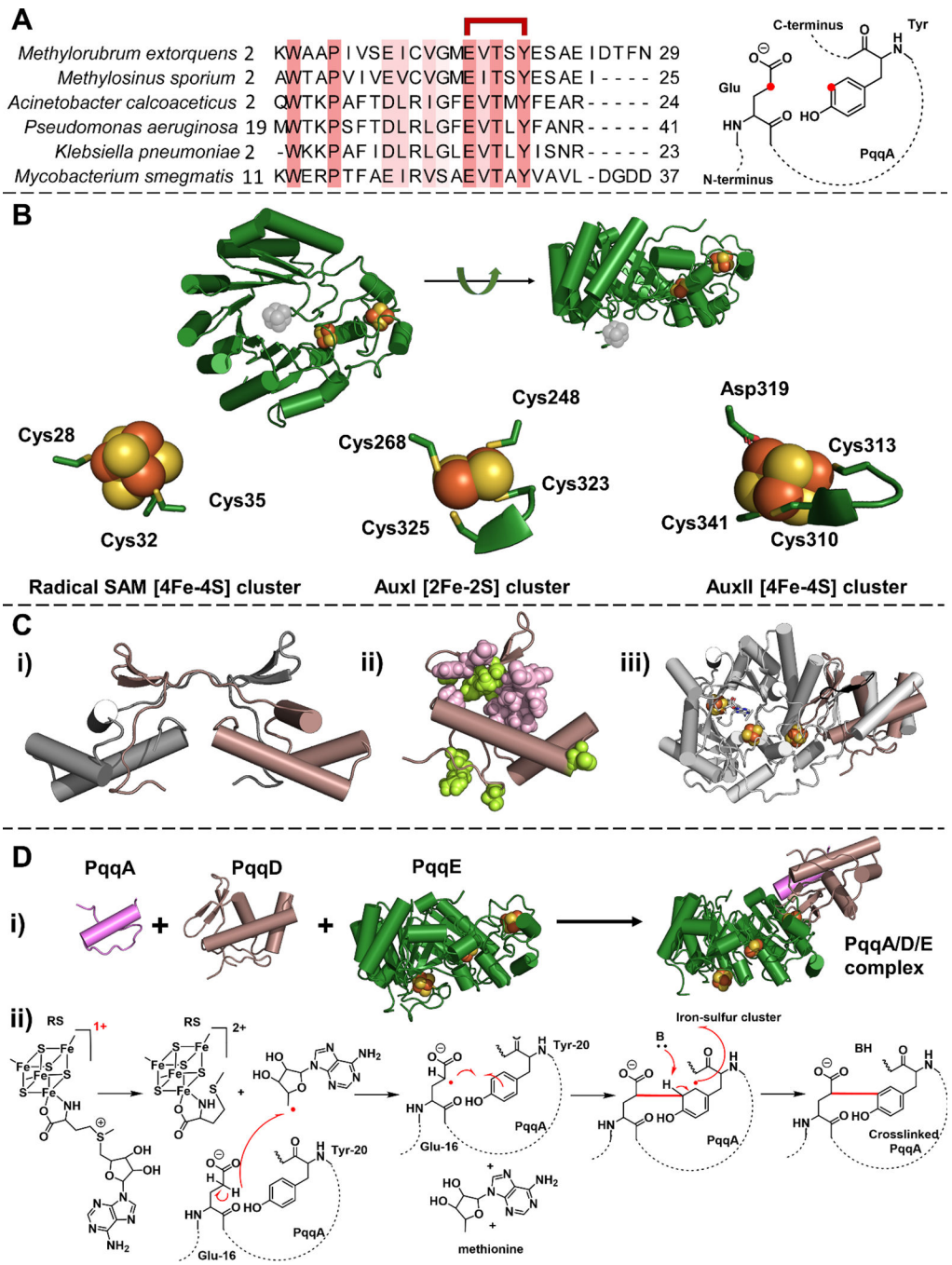

Figure 2. PqqA, PqqD and PqqE participate in the first step of the PQQ biogenesis.

A. PqqA is a short peptide. Left, the sequence alignment shows the conserved residues in PqqA (highlighted in salmon). The glutamate and tyrosine residues used to form PQQ are indicated as linked in red. Right, the representative structure of peptide PqqA. The to-be-crosslinked carbons on the glutamate and tyrosine residue are highlighted in red. **B**. X-ray crystal structure of *Methylorubrum extorquens* PqqE (PDB: 6C8V). The RS cluster in the N-terminal TIM barrel region was not well-resolved, possibly due to oxidative damage during the long delay times imposed by the protein crystallization process. The missing radical

SAM [4Fe-4S] cluster is inserted (in gray). The ligands for the iron-sulfur cluster binding sites in PqqE are presented at the bottom. The ligand environment of the radical SAM [4Fe-4S] cluster is computationally modeled using CteB structure as a template by SWISS-MODEL.[62] **C**. Structures of PqqD. i) X-ray crystal structure of *Xanthomonas campestris* PqqD (PDB: 3G2B). A monomer of the PqqD dimer is shown in brown. ii) NMR structure of *Methylorubrum extorquens* PqqD (PDB: 3SXY) is shown in brown. The residues that interact with PqqA are highlighted in pink and the residues that interact with PqqE are highlighted in light green. iii) The PqqD NMR structure (brown) is superimposed with the peptide-binding domain in another radical SAM enzyme, CteB (gray) (PDB: 5WGG). **D**. The first step of PQQ biogenesis is catalyzed by a PqqA/D/E complex. i) A proposed structure for PqqA/PqqD/PqqE complex. The 3D structure of PqqA was predicted by PEP-FOLD3,[63] and the relative position of three components are modeled using CteB structure as a template by SWISS-MODEL.[62] ii) A proposed chemical mechanism of the reaction catalyzed in the PqqA/D/E complex. In the presence of PqqD and external reducing power, SAM is cleaved by PqqE, leading to the presumptive generation of 5'-deoxyadenosyl radical that abstracts a hydrogen atom from the γ-carbon of the conserved glutamate side chain of PqqA, to initiate C-C crosslinking to a conserved tyrosine.

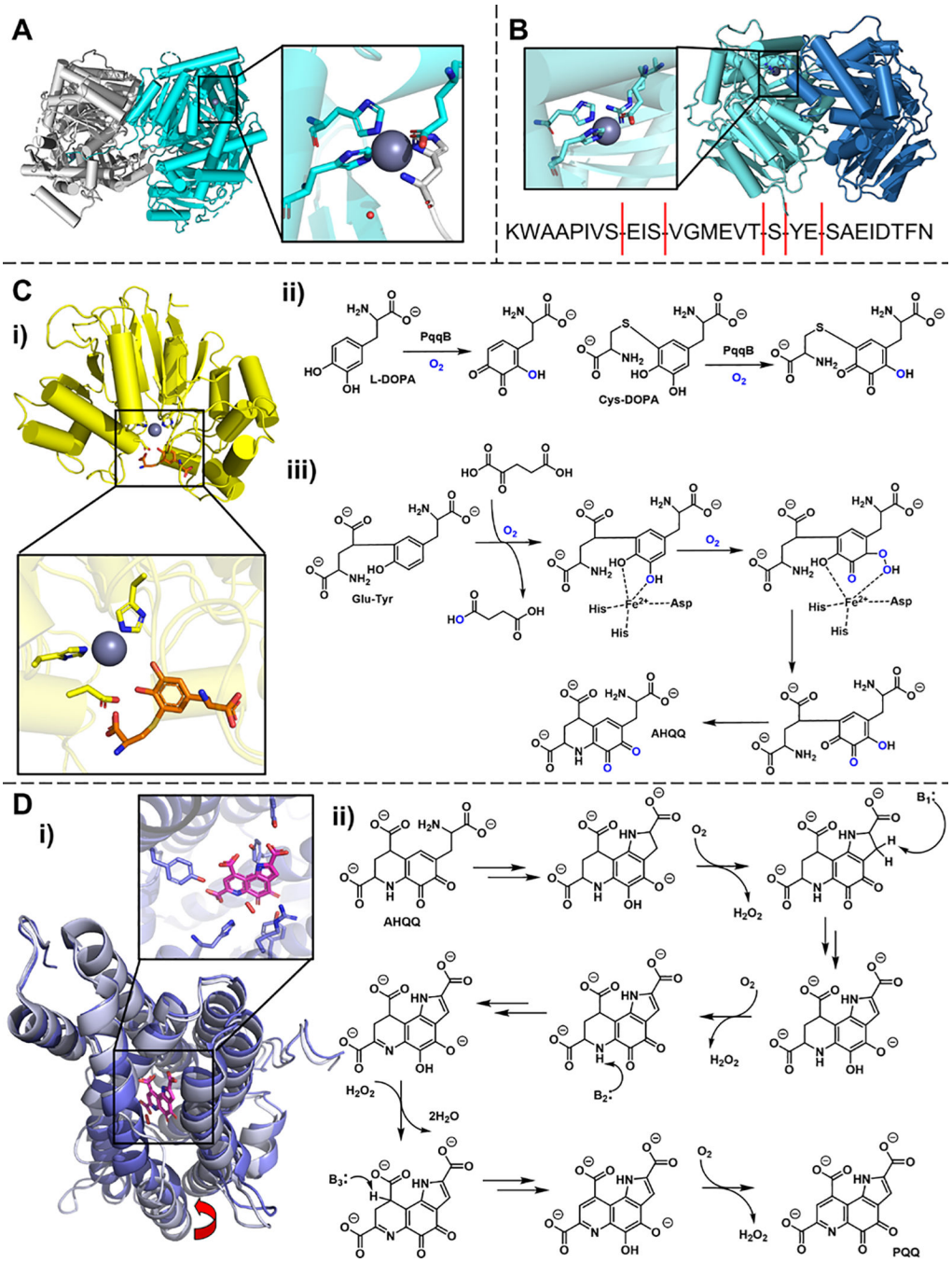

Figure 3. Properties of PqqF/G, PqqB and PqqC (proposed to catalyze the second, third and final steps in PQQ biogenesis).

A. Crystal structure of *Serratia sp.* PqqF (PDB: 5CIO). The monomer in the dimerized crystal structure is in cyan. The zoom-in picture shows the zinc-binding site in one of the monomers. Zn is shown in the purple sphere, a water molecule is in the red dot, the Zn-ligands from one monomer is shown in cyan stick, and the his-tag from another monomer bound to the active site Zn is shown in the gray stick. **B**. Structural model of *Methylorubrum extorquens* PqqF/G constructed via SWISS-MODEL using PDB: 5CIO as the template.

PqqF is in cyan and PqqG is in blue. The zoom-in picture shows the modeled zinc-binding site in PqqF. The dominant protease cleavage sites on *Methylorubrum extorquens* PqqA by PqqF/G are highlighted by red lines at the bottom. **C**. Crystal structure of *Pseudomonas putida* PqqB (PDB: 6E13) and the chemical reaction catalyzed by PqqB. i) Cys-DOPA (orange), a substrate analog, can bind to the metal center of PqqB. The metal in the crystal structure is Zn (catalytically inactive) shown in the slate sphere. Metal-binding residues are shown in purple sticks. ii) PqqB is able to hydroxylate Cys-DOPA and L-DOPA in an O_2-dependent manner. iii) A proposed mechanism for PqqB, based on the behavior of Cys-Tyr and Cys-DOPA. The mechanism is illustrated using the proposed native substrate, Glu-Tyr. The last step involves a spontaneous series of reactions to generate AHQQ, the substrate for PqqC. **D**. Crystal structures of PqqC and its catalytic mechanism. i) Crystal structure of *Klebsiella pneumoniae* PqqC in a complex with PQQ in a closed conformation (purple, PDB:1OTW) is superimposed with the open conformation (light purple, PDB: 1OTV). The bound PQQ in the closed conformation is in magenta, and the bound H_2O_2 is in red sticks. The red arrow at the bottom points to the large movement of the helix to accommodate the binding of PQQ. The zoom-in picture shows the ligands that interact with PQQ in the closed conformation. ii) Proposed reaction mechanism of PqqC.[74]

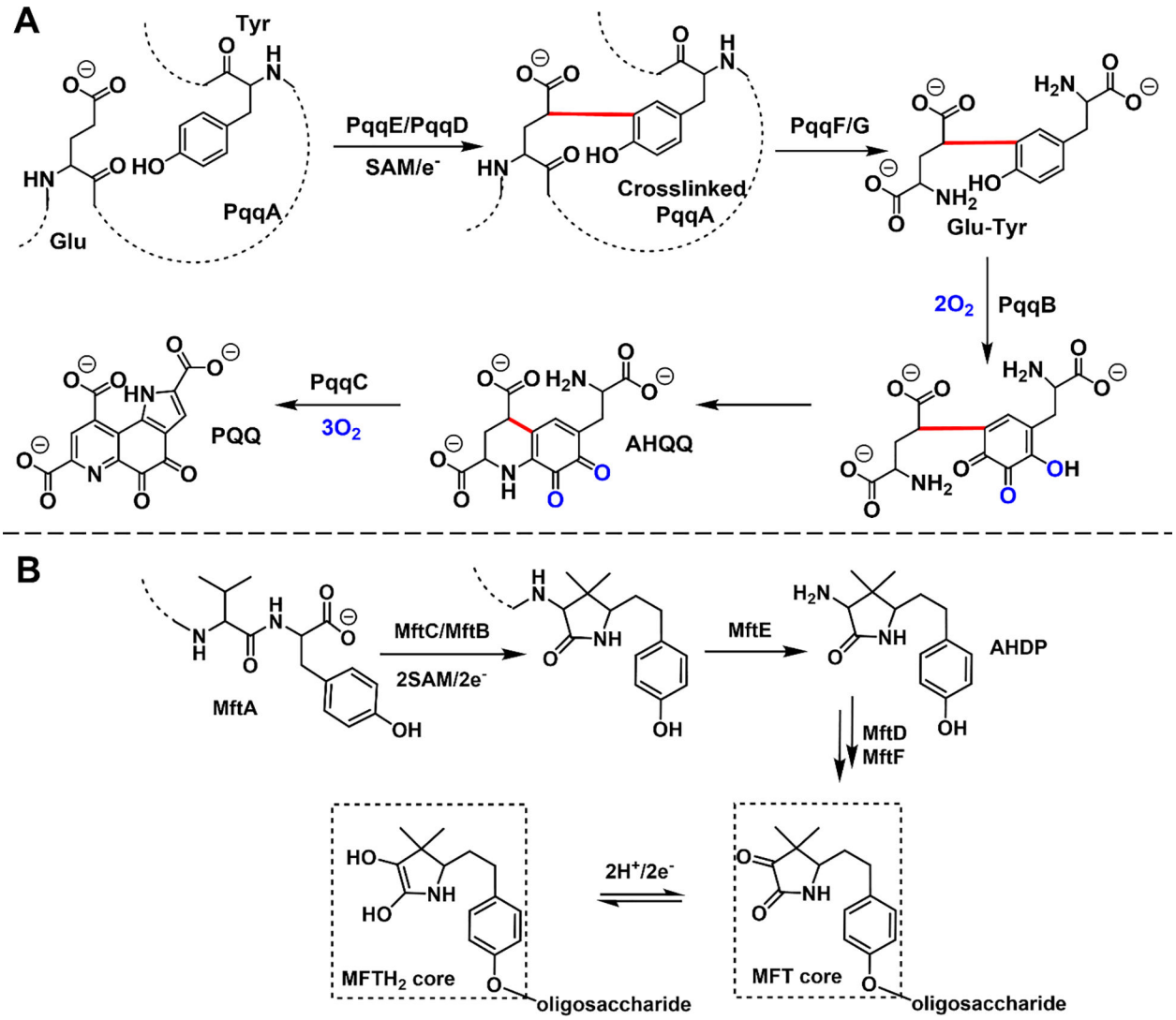

Figure 4. Key features of the proposed PQQ biosynthetic pathway and the newly proposed mycofactocin pathway.

A. The production of one molecule of PQQ requires the participation of at least six proteins, five equivalents of O_2, one equivalent of SAM and the participation of an electron donor system to initiate the reductive SAM cleavage. **B.** The newly proposed redox cofactor mycofactocin (MFT) biosynthetic pathway shares high similarity to PQQ biogenesis. [82,84] In the proposed pathway, MftA, a short peptide, is modified by radical SAM enzyme, MftC, in the presence of a chaperone, MftB. The modified MftA is hydrolyzed by protease, MftE, followed by oxidative deamination and glycosylation. The mature MFT contains a presumed redox-active MFT core that can be reduced to $MFTH_2$.[84]