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Crystal structures reveal metal binding plasticity at the metallo- β -lactamase active site of PqqB from *Pseudomonas putida*

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Abstract

PqqB is an enzyme involved in the biosynthesis of pyrroloquinoline quinone (PQQ) and a distal member of the metallo- β -lactamase (MBL) superfamily. PqqB lacks two residues in the conserved signature motif HxHxDH that makes up the key metal-chelating elements that can bind up to two metal ions at the active site of MBLs and other members of its superfamily. Here we report crystal structures of PqqB bound to Mn^{2+} , Mg^{2+} , Cu^{2+} and Zn^{2+} . These structures demonstrate that PqqB can still bind metal ions at the canonical MBL active site. The fact that PqqB can adapt its side chains to chelate a wide spectrum of metal ions with different coordination features on a uniform main chain scaffold demonstrates its metal binding plasticity. This plasticity may provide insights into the structural basis of promiscuous activities found in ensembles of metal complexes within this superfamily. Furthermore, PqqB belongs to a small subclass of MBLs that contain an additional CxCxxC motif that binds a structural Zn^{2+} . Our data support a key role for this motif in dimerization.

Keywords

PQQ; PqqB; metallo- β -lactamase; metal binding plasticity; pyrroloquinoline quinone; X-ray crystallography

Introduction

Pyrroloquinoline quinone (PQQ) is an aromatic heterocyclic tricarboxylic acid that acts primarily as a redox cofactor in some bacterial and possibly fungi dehydrogenases, or as an antioxidant for protecting mitochondria from oxidative stress [1–4]. Emerging data have been uncovering its benefits as a nutrient for promoting age-related mitochondrial biogenesis, improving memory and reversing cognitive impairment caused by chronic

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oxidative stress in neuron cells [5, 6]. Biosynthesis of PQQ is enabled by an operon that includes five obligatory genes namely *pqqA*, *pqqB*, *pqqC*, *pqqD* and *pqqE* [7]. *PqqB* encodes a protein that is classified as a new member of the metallo- β -lactamases (MBL) superfamily due to its $\alpha\beta\beta\alpha$ type fold [8]. All well-identified members of the MBL superfamily rely on metal ions for their catalysis. The most common metal is zinc, but some specific members of the superfamily have evolved to use other metals including manganese, nickel, magnesium, and iron. These metal ions bind to the active site to form a di-metal or mono-metal center. Well-identified members of the MBL and its super family share a common HxHxDH signature motif that makes up a key part of their active site and is crucial in chelating catalytic metals ions in the active site [9]. Phylogenetic analysis reveals that PqqB is a distal member of this super family [10]. PqqB shares low sequence similarity to other MBLs, the highest homology being to PhnP (18% sequence identity), a phosphodiesterase [11, 12]. PhnP has the typical signature motif and binds two Mn^{2+} , forming a di-metal center at its active site [11]. However, sequence analysis shows that *Pseudomonas putida* (Pp) PqqB lacks the complete metal-binding sequence, with Asp and Gln replacing the first two His of the motif (Asp88-x-Gln90-x-Asp92-His93 (Supplemental Fig. S1). However, PqqB does retain the potential for a single metal binding site equivalent to one in PhnP, a 2-His/1-carboxylate facial triad that is characteristic of nonheme iron oxygenases, although PhnP has an additional ligand, Asp164, that bridges between the two Mn^{2+} sites that is absent in PqqB [13]. Structural comparison between the previously determined 2.2 Å resolution structure of *Pseudomonas putida* PqqB (PpPqqB) that had no metal in the canonical MBL active site, and the 1.4 Å resolution structure of di- Mn^{2+} -bound PhnP further demonstrates this loss (Fig. 1) [8, 12]. As a consequence, the question is raised whether PqqB retains the ability to bind metal ions at the canonical MBL active site.

PqqB contains an unusual CxCxC motif that forms a zinc binding site in the crystal structure [8]. This motif is absent from most MBLs, but is also present in PhnP [11]. The zinc is not at the active site and has been proposed to play a structural role in PhnP. The majority of the MBLs are monomeric, except for membrane-associated NDM-1 and its subclass B3 L1 [14]. PhnP is a dimer, with crystal contacts suggesting PqqB is also a dimer [8, 11]. Thus, another question is whether PqqB is monomeric or dimeric in solution.

The role of PqqB in PQQ biosynthesis is currently unknown, and knock-out studies indicate low levels of PQQ are synthesized in its absence [15]. Studies have shown that PqqB can hydrolyze CENTA ((6R,7R)-3-[(3-carboxy-4-nitrophenyl)sulfanylmethyl]-8-oxo-7-[(2-thiophen-2-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid) and S-D-lactoylglutathione that are preferred substrates for β -lactamase and Glyoxalase II, respectively, indicating PqqB retains its ancestral capacity of hydrolyzing amide and thioester bonds [10]. But the measured low k_{cat}/K_M suggest that they are only promiscuous substrates for PqqB. Members of the MBL superfamily catalyze a diverse range of chemical reactions, but the retention of a putative 2-His/1-carboxylate facial triad metal binding site has led to the suggestion that PqqB may be an oxygenase [16].

Materials and methods

Cloning, expression and purification of *Pseudomonas putida* PqqB

PqqB gene from *Pseudomonas putida* was synthesized and cloned into NdeI/XhoI sites of pET22b(+) vector (Novagen) in-frame with a DNA sequence encoding a C-terminal hexahistidine tag (LEHHHHHH). The plasmid was validated by DNA sequencing and transformed into competent *E. coli* BL21(DE3) cells. Cells were grown to an OD₆₀₀ of 0.6 at 37 °C and then induced overnight at 20 °C by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Harvested cell pellets were re-suspended and lysed on ice in buffer A (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5mM β-mercaptoethanol and 30 mM imidazole) freshly supplemented with protease inhibitors (cOmplete EDTA-free, Roche) by sonication. Cell lysates were subsequently cleared by ultracentrifugation (12,000g) at 4 °C and loaded onto 4 ml of Ni-NTA affinity resin in a column. This column was washed with 400 ml of buffer B (20 mM Tris-HCl, pH 8.0, 300 mM NaCl and 50 mM imidazole) and then eluted with buffer C (20 mM Tris-HCl, pH 8.0, 300 mM NaCl and 300 mM imidazole). The pooled PqqB protein peak was immediately further purified by gel filtration chromatography conducted in a Superdex 200 10/300 GL column (GE Healthcare) equilibrated and eluted with buffer D (20 mM Tris-HCl, pH 7.5, and 100 mM NaCl).

Molecular weight measurement using size exclusion chromatography (SEC) and Zetasizer light scattering system

The molecular weight of PqqB in solution was determined using both SEC and dynamic light scattering with no additional exogeneous metal added to the preparation. SEC was conducted in a Superdex 200 10/300 GL column (GE Healthcare) connected to an AKTA FPLC (GE Healthcare) with a flow rate of 0.5 ml/min at 4 °C. The elution buffer was 50 mM Tris-HCl pH 8.0 and 100 mM NaCl. 100 µg of protein was injected for each run. The elution volume was monitored by UV absorbance at 280 nm. The column was calibrated using five standards (Sigma) including aprotinin (6.5 kDa, eluted at 18.6 ml), cytochrome *c* (12.4 kDa, eluted at 16.8 ml), carbonic anhydrase (29 kDa, eluted at 15.3 ml), albumin (66 kDa, eluted at 13.5 ml), and alcohol dehydrogenase (150 kDa, eluted at 11.28 ml). The dead volume was determined by blue dextran (Sigma). Purity of the protein was assessed by SDS-PAGE (Supplemental Fig. S2).

Dynamic light scattering was performed using a Zetasizer light scattering system. Purified PqqB sample was diluted to a concentration of 0.1 mg/ml using Millipore water containing 100 mM NaCl. The diluted sample was centrifuged at 10, 000g and 4 °C for 15 min, then transferred into a 200 µl cuvette for determining its molecular size using the Zetasizer light scattering system. Data were analyzed using Zetasizer software.

Crystallization and diffraction data collection

Crystals were grown in a 19°C incubator using a traditional hanging drop method. Two microliters of protein sample were mixed with an equal volume of reservoir solution and equilibrated against 500 µl of reservoir solution. The concentrated PqqB was diluted to a concentration of ~2.5 mg/ml of PqqB buffered in 50 mM NaCl and 10mM Tris-HCl pH 7.5 using Millipore water. The reservoir solution for crystallizing unliganded PqqB contained

11% PEG4000, 0.2 M NaCl and 0.1 M Bis-Tris pH 6.7. This reservoir solution further contained 1 mM MnCl_2 and 1 mM malic acid, 1 mM MgCl_2 and 1 mM malic acid, 50 μM CuCl_2 , or 80 μM ZnCl_2 to co-crystallize PqqB with each of the respective metal ions. Visible crystals appeared within one week and were harvested in about one month. Crystals were soaked in cryoprotectant solution (reservoir solution with glycerol concentration elevated to 25%) for ~30 s and then immediately flash-cooled in liquid nitrogen. Diffraction data were collected at beamline 24-ID-C for Mn^{2+} - or Cu^{2+} -bound PqqB structures, 19-BM for Mg^{2+} -bound PqqB structure and 19-ID for Zn^{2+} -bound PqqB structure, respectively, at the Advanced Photon Source of Argonne National Laboratory.

Structural determination and analyses

Oscillation diffraction data were processed, reduced and scaled using software XDS [17] or HKL3000 [18]. The 1.68 Å resolution unliganded PqqB structure had its initial phases derived from the previously solved 2.2 Å PpPqqB resolution structure (PDB ID 3JXP) using a molecular replacement method implemented in the software Phaser [8, 19]. Although the PpPqqB sequences were identical between the two structures, the construct used to generate protein for the prior structure had an additional Met on the N-terminus and a second hexa-His tag (LEHHHHHH) on the C-terminus. Active site metal bound PqqB structures had their initial phases similarly derived from this high resolution unliganded structure. Iterative model building and structural refinement were carried out with Coot [20] and Phenix [21]. Anomalous difference Fourier maps were calculated using programs from the CCP4 suite [22]. Composite omit maps and simulated annealing omit maps were calculated with CNS [23]. The anisotropic B factor or TLS was refined at the final stage of refinement. The final models and structural factors were deposited in the Protein Data Bank (<http://www.pdb.org>) under accession codes 4Z6X, 4Z67, 4Z5Z, 4Z60 and 4Z5Y. All final models were assessed for their stereochemical quality using the program MolProbity [24]. Structural alignment was achieved by secondary structure matching [25] implemented in the Coot software. Figures were made with software PyMOL [26]. Sequence similarity/identity was calculated using the SIAS server (<http://imed.med.ucm.es/Tools/sias.html>).

Results

The structure of PpPqqB with no metal bound in the canonical MBL active site was previously solved to 2.20 Å resolution [8]. Herein we significantly enhanced the resolution to 1.68 Å, which allowed us to model missing residues and define the structure more accurately, but with very similar rmsd on main chain atoms of 0.338 Å. This higher resolution structure crystallizes in a condition containing 11% PEG 4000, 0.2 M NaCl and 0.1M Bis-Tris pH 6.7, and is essentially isomorphous to the two previously determined crystal structures [8]. Compared to the previously reported crystallization conditions, this condition is free of divalent metal ions and has a slightly acidic pH, which is more suitable for co-crystallization with various divalent metals. We tried a variety of metal ions and successfully obtained PqqB structures with either Mn^{2+} , Cu^{2+} , Zn^{2+} or Mg^{2+} bound at the canonical MBL active site. Both Zn^{2+} - and Mg^{2+} -bound PqqB structures were solved to ~2.5 Å resolution, while Mn^{2+} - and Cu^{2+} -bound PqqB structures were solved to 1.50 Å and 1.56 Å resolutions, respectively. All metal ions have reliable electron densities

(Supplemental Fig. S3) and were verified by anomalous difference Fourier map, where applicable. All final models have good geometry as validated by MolProbity [24]. Detailed data processing and structural refinement results are shown in Table 1.

Structures of PqqB display metal binding plasticity at the canonical MBL active site

It is noteworthy that PqqB binds Mn^{2+} , Mg^{2+} , Cu^{2+} and Zn^{2+} using different residues and with different coordination geometries (Fig. 2). Both Mn^{2+} and Mg^{2+} require the presence of malic acid to obtain high occupancy in the canonical MBL active site, while Zn^{2+} and Cu^{2+} bind PqqB without the addition of exogenous ligand (Supplemental Table S1). The metal-binding site lies at the PqqB surface within a wide depression and is very accessible to ligands, which likely also contributes to metal lability. All attempts to co-crystallize PqqB with iron bound in the MBL active site led to no discernible electron density for metal (aerobically as Fe^{3+} , anaerobically as Fe^{2+} , both with and without malic acid present). Mn^{2+} is ligated by Asp92, His269, a water molecule, and a malic acid molecule, forming a octahedral coordination geometry with distances of 2.1 – 2.3 Å (Fig. 2A). In contrast, Mg^{2+} binds Asp92, a malic acid, and His269, forming a T-shaped geometry wherein this central Mg^{2+} linearly coordinates two carboxylate groups from Asp92 and a malic acid at distances of 1.9 Å ~ 2.0 Å, while binding the His269 along the vertical axis with a distance of 2.8 Å (Fig. 2B). Zn^{2+} adopts a distorted tetrahedral geometry, coordinating Asp92, His93, and His269 at distances of ~2.4 Å in a nearly trigonal planar geometry, with an apical water molecule at ~2.5 Å (Fig. 2D). In contrast, the copper in the PqqB assumes a less distorted tetrahedral geometry wherein the central copper is ligated by His93, His269, and two water molecules at distances of 2.1 Å ~ 2.5 Å (Fig. 2C). It should be noted that although crystallized with Cu^{2+} , the copper could have been photoreduced to Cu^+ during data collection. As such, although the crystal is cryocooled to 100K, the metal-to-ligand distances may be more reflective of a Cu^+ oxidation state.

Conformational changes induced by metal ions at the canonical MBL active site

The newly solved 1.68 Å PqqB structure without additional metal has an identically structured MBL active site to that of the previously reported 2.20 Å structure (PDB ID 3JXP) and serves as a reference for the metal bound structures [8]. The negatively charged carboxylate group of Asp92 in this reference structure is 2.6 Å away from the positively charged guanidinium group of Arg101 of the dimeric partner, forming a weak intermolecular salt bridge well defined in the 2Fo-Fc electron density map. Both Mn^{2+} and Mg^{2+} enlist Asp92 as a ligand, whereby the carboxylate group of Asp92 undergoes a distinct conformational change rotating ~120° about the C α -C β bond to ligate the metal (Fig. 2A,B). It is notable that this conformational change leads to movement of His93 and Asn55, wherein the His93 imidazole group moves ~2.0 Å away from where it lies in the reference structure (Fig. 2A). The presence of malic acid causes a conformational change in Gln90, whereby the amine group of Gln90 rotates 90° degree about the C α -C β bond to interact with the malic acid hydroxyl group at a distance of 2.8 Å. Both Zn^{2+} and Cu^{2+} occupy a similar position ~1.4 Å away from where Mn^{2+} and Mg^{2+} bind (Fig. 2). Like manganese and magnesium, Zn^{2+} also recruits Asp92 as a ligand, but the carboxylate group of Asp92 only rotates ~90° about its C α -C β bond due to the slight change in metal position compared to Mn^{2+} and Mg^{2+} (Fig. 2D). This rotation requires no accompanying conformational changes

in His93 and Asn55 as observed in the Mn^{2+} - and Mg^{2+} -PqqB structures (Fig. 2). It is unclear if the different metal positions of Mn^{2+} and Mg^{2+} co-crystals compared to Zn^{2+} and Cu^{2+} are a consequence of the requirement to have malic acid present to obtain significant metal occupancy (Supplemental Table S1). The Cu^{2+} is the only metal that does not ligate Asp92, so no significant conformational changes are observed in Asp92, His93, or Asn55 (Fig. 2C). Here the carboxylate group of Asp92 remains in a salt bridge with the positively charged guanidinium group of Arg101 of the other monomer in the dimer, as observed in the unbound structure. Unlike Mn^{2+} and Mg^{2+} , both Zn^{2+} and Cu^{2+} directly coordinate His93, which requires no reorientation of this side chain. In the structure without metal in the MBL active site, the $\text{Zn}^{2+}/\text{Cu}^{2+}$ is replaced by a water molecule that is 2.7 Å away from His93, but the distance shortens to 2.4 Å and 2.5 Å in the Cu^{2+} - and Zn^{2+} -bound structures, respectively. The same effect is observed with His269: the coordination distance shortens when either Cu^{2+} or Zn^{2+} replaces the water molecule of the unbound structure.

Over-expressed PpPqqB purifies without metal bound in the canonical MBL active site, but 100% occupancy in the structural Zn^{2+} site, consistent with the MBL active site binding metals more weakly. Although binding of additional metal ions to PqqB causes significant side chain conformational changes to some residues, no distinct main chain displacement is observed in any of the residues involved in metal binding (Fig. 3A). All structures have very consistent overall conformations except in a mobile loop (residues 159–181) that forms one side of the entrance to the active site (Fig. 3B). Thus, these side chain conformational changes take place within a relatively rigid protein main chain scaffold (Fig. 3).

PqqB is a dimer in solution

The recombinant PpPqqB is composed of 311 residues with a His-tag at the C-terminus, corresponding to a theoretical molecular mass of 34.5 kDa. The molecular mass in solution was measured using dynamic light scattering and size exclusion chromatography. It was found that PqqB consistently appeared as a monodisperse peak with an estimated molecular weight of ~72 kDa, which clearly indicates that PqqB is dimeric in solution (Fig. 4A and Supplemental Fig. S4). No exogenous metal was added, so only the structural zinc binding site would be occupied in the protein sample. Although there is a monomer in the asymmetric unit, crystal packing analyses by PISA [25] revealed 2184 Å² of interface area between pairs of monomers, equating to 15% of the total surface area of each monomer. Therefore, the dimer observed in the crystal is also found to exist in solution.

All PqqB crystal structures contain a bound zinc that is approximately 15 Å away from the canonical MBL active site. This zinc ion has well-defined electron density and nearly 100% occupancy (Supplemental Table S1). No metal ions were supplemented during any of the cell culture, lysis or protein purification steps demonstrating the tight binding of this metal. This zinc is coordinated by the highly conserved Cys19-x-Cys21-xx-Cys24 motif and Asn272 in PpPqqB. Further examination of the dimer interface uncovers a structural role for this zinc ion, which forms a scaffold that organizes residues important for intersubunit interactions. Indeed, residues around this zinc account for the majority of these interactions (Supplemental Fig. S5). Trp17 forms the most extensive interactions at the dimer interface, interacting with residues Met70, Leu64, Ile81, Leu100, and Gly 103 of a hydrophobic core

of the second monomer (Fig. 4B). Asn20 interacts with the side chain of Gln71 of the other monomer at a distance of 2.9 Å, the main chain oxygen of Ala75 at a distance of 2.8 Å, and the main chain oxygen of Leu76 at a distance of 2.7 Å, respectively. Other intersubunit interactions at the dimer interface are detailed in Supplemental Table S2.

As previously mentioned, there is an additional salt bridge between Arg101 and Asp92 of adjacent monomers when Asp92 is not a metal ligand within the canonical MBL active site. This forms on one side of the wide surface depression at the bottom of which the metal bound in the MBL active site sits. The solvent accessibility of the salt bridge is consistent with the observed weak, monodentate binding mode. This appears to contribute little to dimer formation, as all the co-crystallizations contain identical dimers irrespective of whether Asp92 is a metal ligand, and the equivalent dimer of PhnP does not contain this salt bridge [11].

Conclusion and discussion

In terms of homology, PqqB is most closely related to the phosphodiesterase, PhnP, a member of the MBL superfamily [11, 12]. PhnP contains a di-Mn²⁺ core, and the MBL signature HxHxDH metal binding motif is divided between the two Mn²⁺ sites; in *Escherichia coli* (Ec) PhnP, Asp80 and His81 ligate Mn(I) and His76 and His78 ligate Mn(II) (Fig. 1) [9]. As well as His76 and His78, the Mn(II) site of EcPhnP is ligated by the strictly conserved His143 and Asp164, the latter being a bridging ligand between Mn(I) and Mn(II). In contrast, PpPqqB has Asp88, Gln90, Arg162 and Gly199 at the corresponding positions, signifying loss of metal binding at this site. This includes loss of the PhnP metal bridging Asp, which is a Gly in PqqB. In EcPhnP, the Mn(I) site is formed by Asp80, His81, Asp164 and His222, with Asp164 being the bridging ligand between the two Mn²⁺ sites (Fig. 1). Other than the loss of the bridging ligand, the three other Mn(I) ligands in PhnP are strictly conserved in PqqB (Asp92, His93, and His269 in PpPqqB, Fig. 1). Taken together, these data are consistent with a mono-metal binding capability for PqqB, as observed in our crystal structures. The juxtaposition of Asp92, His93, and His269 in PpPqqB is reminiscent of a 2-His/1-carboxylate facial triad, the characteristic binding motif of nonheme iron oxygenases [13]. Therefore, it was highly surprising when attempts to bind iron in the canonical MBL active site failed. We were able to co-crystallize PpPqqB with Mn²⁺, but it required the addition of malic acid as an exogenous ligand to stabilize the metal at high occupancy (Fig. 2A, Supplemental Table S1). Even more surprising was that His93 was not a ligand to the Mn²⁺, such that Asp92 and His269 were the only protein ligands. The malic acid adopted a “claw-like” conformation to occupy three ligand sites on the Mn²⁺, with a water as the sixth ligand. It should be noted that malic acid also supplemented the metal-chelating elements in the first solved PhnP crystal structure [11].

Therefore, we co-crystallized with other metals to examine the breadth of metal types that could be bound by PqqB. First, we co-crystallized with magnesium, an alkaline metal. Again malic acid was required, and the binding had similarities to that of Mn²⁺ with malic acid, although His269 was 2.8 Å from the metal and malic acid bound in a monodentate fashion (Fig. 2B). Although copper is an unknown physiological active site metal in MBLs, its redox capabilities and the requirement for an unidentified oxygenase/oxidase in PQQ biosynthesis

led us to co-crystallize with the metal. It bound without the need for malic acid, but ligated to only two of the potential metal coordinating amino acids, this time His93 and His269, with two additional water ligands in a tetrahedral coordination geometry (Fig. 2C). The bond lengths between the amino acids and the copper were long (2.4 and 2.5 Å, respectively) consistent with relatively weak binding compared to other copper-containing enzyme active sites, although this could also be reflective of photoreduction in the X-ray beam to Cu⁺. One of the water ligands (Wat1) occupies the ligand site in the putative MBL-equivalent substrate pocket open to bulk solvent, where malic acid binds in the Mn²⁺- and Mg²⁺-PqqB structures (Fig. 2). However, the other coordinating water (Wat2) lies in a small pocket tucked between the metal and protein where an additional protein ligand might be expected. Zinc is the most common MBL metal, and indeed a single Zn²⁺ is bound at high occupancy in the canonical MBL active site of Zn²⁺-PpPqqB [9]. It is the only metal coordinated by all three of the conserved MBL active site PqqB residues, along with a water in a distorted tetrahedral geometry (Fig. 2D). Surprisingly, the coordination position of this water is not the equivalent of Wat1 of the Cu²⁺-PpPqqB structure that lies in the substrate pocket of MBLs, but sandwiched between the zinc and protein in the small pocket occupied by Wat2 (Fig. 2C,D). This PqqB pocket has little room for a ligand of more than a few atoms, and is certainly not large enough to accommodate any of the substrates and products of PQQ biosynthesis, if the PqqB active site is located equivalent to other MBLs.

This unexpected finding is also inconsistent with the observed exogenous ligand binding site in known mono-Zn²⁺-dependent MBL enzymes from subclass B2, the subclass in which binding of an additional zinc to the active site is inhibitory [9, 27, 28]. The *Aeromonas hydrophila* carbapenemase, AhCphA, is one of the best studied B2 MBLs, and in its 1.7 Å resolution crystal structure the zinc is coordinated in a tetrahedral geometry with three protein and one exogenous ligand [29]. An overlay with PpPqqB demonstrates that the AhCphA ligands Asp120 and His263 ligands are equivalent to the PpPqqB ligands Asp92 and His269, both in terms of sequential position and spatial position (Fig. 5). However, the CphA and the MBL B2 subclass are sequentially far removed from PqqB, and the third protein ligand to the zinc is a cysteine (Cys221 in AhCphA) [10, 30]. This has very different properties to the His93 ligand of PqqB, which forms part of the signature HxHxDH motif, and in CphA this position is an Arg. The Cys of CphA spatially comes from a different part of the structure than His93 of PqqB, and leads to a different zinc position with the apical exogenous ligands in the two structures being on opposite faces of the zinc; in the case of CphA the face accessing the substrate pocket open to bulk solvent, and in PqqB the face where the small pocket sits between the zinc and protein (Fig. 5). If the active site of PqqB is the same as other MBLs, then unless substrate displaces one of the protein ligands, or the zinc undergoes a change in geometry, it is difficult to believe that PqqB could be natively Zn²⁺-dependent.

PhnP has evolutionarily gained a novel CxCxxC motif while diverging from MBLs, and PqqB retains this motif [11]. Previous studies have shown that this motif is critical to PhnP stability [11]. PqqB is a dimer in solution and in the crystal, as is PhnP, substantiating a role for this motif in dimerization [8, 11]. Other MBL families do not have this motif, and none of these form a dimer in solution except for NDM-1, sequentially far removed from PqqB and PhnP [31–34]. This motif creates a structural zinc site wherein these three Cys and an

additional residue coordinate the zinc ion in a tetrahedral geometry. This additional residue is Asn for PqqB or His for PhnP, so PqqB differs from PhnP by only one residue at this site. The fact that residues around this structural zinc site account for most of the effective intersubunit interactions strongly suggests that it acts as a scaffold to assemble residues for forming the dimer interface. To our best knowledge, PqqB and PhnP are the only members in which oligomerization is mediated by a zinc-finger-like motif, although mutagenesis disrupting this zinc coordination will be required to further confirm this structural postulate.

PqqB can bind a wide variety of metals within the canonical MBL active site with very different properties (Mn^{2+} , Cu^{2+} , Mg^{2+} , and Zn^{2+}), and demonstrates that PqqB has a plasticity towards metal ion binding. Such flexibility has been previously noted in many members of the MBL superfamily; for example, PhnP can bind both nickel and zinc [11]. These metal isomers of MBLs can also display promiscuous activities [35]. The observed plasticity in metal binding, coordination geometries, and resulting catalytic activities has made the MBL superfamily a model for testing the role promiscuity plays in functional divergence within superfamilies [10, 30, 35]. Therefore, the observed multi-metal binding capabilities of PqqB align with the promiscuous nature of other MBLs. Binding of the metals appear weak compared to other MBLs, and so we cannot rule out that physiological PqqB catalysis is metal-independent, and that metal binding is vestigial and an evolutionary carryover.

In conclusion, PqqB can bind a spectrum of metals in the canonical active site of MBLs, but has little binding capacity towards either ferrous or ferric iron. This is despite the presence of a likely 2-His/1-carboxylate facial triad configuration within this region that led to the postulate PqqB could be an iron-dependent oxygenase. Given our observations, this now seems unlikely. Until the substrate of PqqB is defined, metal-dependency tested, and catalytic activity demonstrated, the role of PqqB in PQQ biosynthesis will remain elusive.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PQQ	pyrroloquinoline quinone
MBL	metallo- β -lactamase
Pp	<i>Pseudomonas putida</i>
IPTG	isopropyl β -D-1-thiogalactopyranoside

SEC	size exclusion chromatography
PDB	Protein Data Bank
Ec	<i>Escherichia coli</i>

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Figure 1.

Structure superposition of unliganded PqqB (gray) and its closest homologue PhnP (pink). PhnP (PDB ID 3P2U) contains His76-x-His78-x-Asp80-His81, a signature motif conserved in MBLs that is not conserved in PqqB (PDB ID 3JXP) [8, 12]. This motif together with other residues makes up a di- Mn^{2+} site indicated by Mn(I) and Mn(II), respectively. Bound orthovanadate (VO_4^{3-}) in the PhnP structure is represented by stick. Gly199 is highlighted with a gray sphere. Water molecules and Mn^{2+} are represented by red and violet spheres, respectively.

Figure 2.

The metal binding plasticity revealed by the crystal structures of PqqB. A. Mn^{2+} -bound PqqB; B. Mg^{2+} -bound PqqB; C. Cu^{2+} -bound PqqB; D. Zn^{2+} -bound PqqB. Malic acid coordinates the metals in panels A and B. Water molecules are represented by red spheres labeled with “w”. Metal ions are represented by balls with different colors rather than orange. The coordination bonds are indicated by dotted lines, and distances in Angstroms indicated.

Figure 3. Structure comparison of metal bound structures with the PqqB structure without metal in the canonical MBL active site. A. Close-in view of superposed active sites; B. Overall view of superposed structures. Colors of PqqB structures: unbound (gray), Mn²⁺-bound (cyan), Mg²⁺-bound (magenta), Cu²⁺-bound (blue), and Zn²⁺-bound (green).

Figure 4. PqqB is dimeric. A. Mass measurement by size exclusion chromatography. B. Intermolecular hydrophobic interaction stabilized by the structural zinc ion. The two monomers of the PqqB dimer are colored gray and yellow. The structural zinc ion, represented by a violet sphere, coordinates with Cys19, Cys21, Cys24 and Asn272, as showed by yellow dash lines. The most notable interaction is between Trp17 and the hydrophobic core of the other monomer. The Gly residue within this hydrophobic core is indicated by a yellow sphere, while other hydrophobic residues are showed by sticks.

Figure 5. Superposition of Zn²⁺-PpPqqB (green) and AhCphA (orange, PDB ID 1X8G) [29]. Labels are colored by structure. Zincs are drawn as gray spheres, and coordination bonds by yellow dash lines.

Table 1

Statistics of data collection and refinement

Data collection	Unliganded PqqB	Mn ²⁺ -PqqB	Mg ²⁺ -PqqB	Zn ²⁺ -PqqB	Cu ²⁺ -PqqB
Space group	<i>P</i> ₄ ₃ ₂ ₁ ₂	<i>P</i> ₄ ₃ ₂ ₁ ₂	<i>P</i> ₄ ₃ ₂ ₁ ₂	<i>P</i> ₄ ₃ ₂ ₁ ₂	<i>P</i> ₄ ₃ ₂ ₁ ₂
a, b, c (Å)	86.7, 86.7, 106.9	86.3, 86.3, 107.1	86.2, 86.2, 107.0	86.2, 86.2, 107.2	86.2, 86.2, 106.6
Resolution range (Å) ^a	28.9 – 1.68 (1.71 – 1.68)	29.4 – 1.50 (1.53 – 1.50)	30 – 2.54 (2.58 – 2.54)	30–2.50 (2.54 – 2.50)	29.3 – 1.56 (1.6 – 1.56)
Unique reflections	46,746	65,318	13,539	14,603	438,378
R _{merge} ^b	0.045 (0.602)	0.074 (0.846)	0.144 (0.472)	0.105 (0.518)	0.081 (0.812)
CC(1/2) ^c	100 (0.67)	0.99 (0.52)	0.99 (0.69)	0.99 (0.81)	0.99 (0.79)
<I>/<σ(I)> ^d	18.2 (2.3)	19.2 (2.2)	9.4 (2.4)	20.3 (3.5)	16.7 (2.8)
Redundancy	4.5 (4.0)	8.9 (5.0)	6.3 (4.9)	8.7 (7.5)	7.6 (7.4)
Completeness (%)	99.4 (90.0)	100 (99.9)	97.3 (97.1)	99.9 (100)	99.9 (98.7)
Refinement Statistics					
Resolution range (Å)	27.9 – 1.68	29.4 – 1.50	23.9 – 2.54	29.3 – 2.50	29.3 – 1.56
R _{work} /R _{free} (%) ^e	13.7/17.5	14.4/17.4	18.8/22.4	16.1/21.3	13.6/16.6
Root-mean-square deviation from idea values					
Bond length (Å)	0.007	0.016	0.003	0.005	0.012
Bond angle (°)	0.997	1.602	0.699	0.806	1.456
Ramachandran plot (%) ^f					
favored	97.4	97.8	97.1	97.7	97.7
allowed	2.6	1.9	2.6	2.0	2.3
outliers	0	0.3	0.3	0.3	0
PDB ID	4Z6X	4Z67	4Z5Z	4Z60	4Z5Y

^a Selected statistics from data collection and refinement are shown. Values in parentheses are data from the highest resolution shell.

^b R_{merge} is $\sum hkl \sum j |I_j - \langle I \rangle| / \sum hkl \sum j I_j$; $\langle I \rangle$ is the mean intensity of *j* observations of reflection *hkl* and its symmetry equivalents.

^c CC(1/2) is half-dataset correlation coefficients calculated by splitting the data randomly in half.

R_{work} is $\frac{\sum |F_o| - |F_c|}{\sum |F_o|}$ where F_o is an observed amplitude and F_c is a calculated amplitude; R_{free} is the same statistic calculated over a subset of the data that was not used for refinement.

σ_p is the mean intensity divided by the mean error.

R_{Ram} Ramachandran plot is calculated by MolProbity.