Structural and Biochemical Evidence for an Enzymatic Quinone Redox Cycle in *Escherichia coli*

IDENTIFICATION OF A NOVEL QUINOL MONOOXYGENASE*[§]

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Naturally synthesized quinones perform a variety of important cellular functions. *Escherichia coli* produce both ubiquinone and menaquinone, which are involved in electron transport. However, semiquinone intermediates produced during the one-electron reduction of these compounds, as well as through auto-oxidation of the hydroxyquinone product, generate reactive oxygen species that stress the cell. Here, we present the crystal structure of YgiN, a protein of hitherto unknown function. The three-dimensional fold of YgiN is similar to that of ActIVA-Orf6 monooxygenase, which acts on hydroxyquinone substrates. YgiN shares a promoter with “modulator of drug activity B,” a protein with activity similar to that of mammalian DT-diaphorase capable of reducing menadione. YgiN was able to reoxidize menadion, the product of the “modulator of drug activity B” (MdaB) enzymatic reaction. We therefore refer to YgiN as quinol monooxygenase. Modulator of drug activity B is reported to be involved in the protection of cells from reactive oxygen species formed during single electron oxidation and reduction reactions. The enzymatic activities, together with the structural characterization of YgiN, lend evidence to the possible existence of a novel quinol redox cycle in *E. coli*.

Quinones are biologically active molecules that function as lipid electron carriers for the transportation of hydrogen and electrons between the protein complexes of the electron transport chain. Both ubiquinone and menaquinone are synthesized by *Escherichia coli* for this purpose. However, although these compounds are essential for normal electron transport, it has been demonstrated that such quinonoids are also capable of diverting electron flow from the respiratory chain, and in doing so, cause increased intracellular production of superoxide radicals and hydrogen peroxide (1). Benzoquinones and naphthoquinones, such as menaquinone, are highly electrophilic and exhibit substantial thiol reactivity that results in their rapid reduction to semiquinone intermediates. This high redox potential is responsible for quinone cytotoxicity (2), which causes the oxidation of cellular macromolecules (3). It is therefore extremely important that the cell maintains sufficient quinone levels for respiration while minimizing their toxicity.

In addition to potential damage from endogenously synthesized quinones, organisms are also at risk of from environmental quinones, such as from the natural antibiotic compounds synthesized by Gram-positive bacteria. The ubiquitous distribution of quinonoids thus requires organisms to possess a specific defense mechanism.

The metabolism and toxicity of menadione* (2-methyl-1,4-naphthoquinone) have been extensively studied in mammalian cells (4, 5). Menadione is an analogue of menaquinone that differs only in the lack of an extended alkyl chain. DT-diaphorase, an NAD(P)H: oxidoreductase, is believed to reduce a variety of quinone substrates by means of a two-electron reduction mechanism. It has been demonstrated that the activity of this enzyme protects rat hepatocytes from menadione toxicity by competing with the potentially toxic one-electron reduction pathway employed by the electron transport chain and avoiding the generation of a semiquinone intermediate (6–8). The protein “modulator of drug activity B” (MdaB) was first identified on the basis of the protection it provided from the toxic effects of DMP840, adriamycin, and etoposide when overexpressed in *E. coli* (9). It was later confirmed through protein sequencing that this protein corresponded to a DT-diaphorase-like enzyme previously purified from *E. coli* that demonstrated reductase activity toward menadione (10). Also, MdaB was reported to be up-regulated more than 20-fold in the cytoplasmic fraction of *E. coli* in response to 0.2–0.3 mM menadione (11). Although early work suggested FMN and NADH dependence, later studies found MdaB to be FAD- and NADPH-dependent (10). Confusion as to the co-factor identities still remains. It has been suggested that induction of this enzyme may be an adaptive cellular response to minimize the toxicity of menaquinone and other quinone compounds. Recent studies of the MdaB homologue from *Helicobacter pylori* have demonstrated that the growth of *mdab*-deficient mutants is significantly inhibited by 10% oxygen environments and that these bacteria are diminished in their ability to colonize the stomachs of mice when compared with wild-type *H. pylori* (12). Although this work suggests that MdaB is particularly important for the protection of cells against oxidative stress, the quinol products of the reduction reaction are also capable of generating reactive oxygen species through auto-oxidative processes. Therefore, a

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The atomic coordinates and structure factors (codes 1R6Y and 1TUV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ The on-line version of this article (available at http://www.jbc.org) contains a supplementary figure showing multiple sequence alignment of selected proteins from the novel monooxygenase family.

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1 The abbreviations and trivial names used are: menadione, 2-methyl-1,4-naphthoquinone; MdaB, modulator of drug activity B; QuMo, quinol monooxygenase; DT-diaphorase, NAD(P)H: oxidoreductase.
detoxification mechanism involving MdbA must also include a mechanism for detoxifying the quinol reduction product.

We have expressed a hypothetical open reading frame encoding the putative protein YgiN from E. coli K-12 and determined its three-dimensional crystal structure. Prior to structural determination, the function of this protein was unknown as no sequence conservation with proteins of known function could be detected. Using the RegulonDB data base (www.cifn.unam.mx/Computational_Genomics/regulondb), a search of the E. coli genome positioned the b3029/YgiN gene immediately downstream of the modulator of drug activity B (MdbB) gene and predicted that both proteins would be co-regulated by a single promoter (13). In this report, we show that the three-dimensional fold of YgiN aligns well with that of ActVA-Orf6, a novel quinone monooxygenase from Streptomyces coelicolor thought to function via a two-electron oxidation of quinol substrates in antibiotic biosynthesis pathways (14, 15). We have also determined the structure of YgiN complexed with menadione. Further, we provide in vitro evidence that MdbB and YgiN form an enzymatic quinone reduction-oxidation cycle. We shall therefore refer to YgiN as quinol monooxygenase (QuMo). It is possible that such a redox cycle allows the cell to maintain a stable pool of quinones for electron transport while attenuating the potential toxicity of both quinone and quinol species.

EXPERIMENTAL PROCEDURES

Crystallization and Data Collection—Recombinant QuMo (YgiN) was expressed in BL21 (DE3) E. coli cells under the T7 promoter. The expression construct contained a C-terminal hexa-histidine tag that permitted facile purification using batch elution over nickel-nitritetri-acetic acid resin (Qiagen). The final yield was 380 mg of pure protein/liter. E. coli protein culture with no additional purification steps. All reagents for protein expression and purification were purchased from BIOSHOP Canada. All crystallization reagents were purchased from Hampton Research. The optimal crystallization conditions for the native protein were 1.5 M ammonium sulfate, 0.1 M sodium citrate, pH 5.6, 0.2 M potassium/sodium tartrate, at 298 K using hanging drop vapor diffusion and a protein concentration of 15 mg/ml. QuMo protein was also expressed as a selenomethionine derivative in DL41 (DE3) E. coli in LE Master medium (16) and purified as per native QuMo protein. The crystallization conditions for the selenomethionine protein were unaltered; however the protein concentration was decreased to 2.5 mg/ml, and the volume ratio of protein to reservoir solution was modified from 3:00 to 3:00. M menadione, and 5 mM NaH2PO4, pH 8.0, 0.2 mM NADH, 50 M menadione, and 5 M menadione had been completely reduced by MdaB. The activity of each enzyme was assayed at 22 °C in a reaction mixture containing 50 mM NaH2PO4, pH 8.0, 0.2 mM NADH, 50 μM menadione, and 5 μM enzyme in a total volume of 1.0 ml. The activity was measured by monitoring the decrease in absorbance at 308 nm, resulting from the conversion of menadione to menadionol. QuMo activity was measured by the addition of this enzyme in a 1:1 molar ratio with MdbB to the reaction mixture once menadione had been completely reduced by MdbB. The activity of QuMo was demonstrated by an increase in absorbance at 308 nm corresponding to the oxidation of menadionol to generate menadione.

RESULTS

Three-dimensional Fold of QuMo Suggests That It Is a Monooxygenase—The three-dimensional structure of QuMo from E. coli K-12 was determined using the single anomalous dispersion method and refined to 2.2 Å (Fig. 1a). The model was refined to 20.84 and 24.95% R and R-free values, respectively, with 94% of residues in the most favorable regions of the Ramachandran plot and the remaining 6% in allowed regions (Table I). The overall fold is of the α+β fold class and comprises a ferredoxin-like split βββ-fold (27) (Fig. 1a). A structural homology search yielded a good alignment with the structure of ActVA-Orf6, a novel monooxygenase from S. coelicolor, with a 1.9 Å root mean square deviation (15) (Fig. 1b). Both structures contain two βββ motifs that form an antiparallel sheet in which strand B1 is positioned between strands B3 and B4. Poor overall identity is observed in multiple sequence alignments of QuMo with established members of the monooxygenase family. However, there is good alignment, both structurally and sequence-wise, for several conserved residues, including the QWES motif found in many
members of this family, which is conserved in QuMo (see Supplemental Material).

A homodimer of QuMo is formed with a symmetry-related molecule from a neighboring asymmetric unit through an extensive hydrophobic interface that has a buried surface of 1728.5 Å² (Fig. 2a). From dynamic light scattering experiments, QuMo also forms a homodimer in solution (data not shown). The interface between the two monomers of QuMo forms a hydrophobic pocket, primarily containing leucine and isoleucine residues. ActVA-Orf6 also forms a homodimer, in which the interface involves intermolecular interactions of tyrosine-tyrosine stacking and a tyrosine-histidine hydrogen bond at the entrance to the active site cleft, whereas ActVA-Orf6 is hydrophobic with small regions of positive charge (blue) at the entrance to the active site. c, despite a lack of sequence identity, amino acid substitutions at the type II (green), and ActVA-Orf6 is shown in pink (36).

Although the surface of each monomer consists of polar amino acids in the solvent-exposed areas, the charge localization of QuMo is opposite to that of ActVA-Orf6 (Fig. 2b). The entrance to the active site of ActVA-Orf6 displays a swapped β-strand interaction in which the extended loop between strands B2 and B3 of one monomer contributes to the β-sheet of the other monomer by lying antiparallel to strand B4 (Fig. 2a). In ActVA-Orf6, this swapped β-strand is formed by an extended portion of the C terminus (residues 103–113) as the extended loop region is absent. The swapped strand contributes to the formation of the bottom of the active site and appears to be important for dimer stability, particularly in the absence of contributions from Tyr-63 and His-52 of ActVA-Orf6.

Although the surface of each monomer consists of polar amino acids in the solvent-exposed areas, the charge localization of QuMo is opposite to that of ActVA-Orf6 (Fig. 2b). The entrance to the active site of ActVA-Orf6 displays a swapping of positively charged residues (Arg-73, Arg-96, and Lys-100). The active site entrance of QuMo, however, is substantially negatively charged and lacks any sequence identity to ActVA-Orf6. Similarly, the opposite face of the protein is negatively charged in ActVA-Orf6, whereas it is positively charged in QuMo.

A β-turn positioned between helix A2 and strand B3 is also conserved between the two proteins and conforms to the class of type II β-turns. In contrast to previously identified monooxygenases, QuMo does not contain the strictly conserved Pro-44. Rather, Pro-44 is substituted by Glu-35. Glycine 45 of ActVA-Orf6, however, is structurally conserved by Gly-36 of QuMo. Substitutions within this region do not appear to alter the topology of the β-turn (Fig. 2c). Additionally, a type III β-turn
located between strand B3 and helix A3 of ActVA-Orf6 is conserved. Although Ser-68 from ActVA-Orf6 is conserved in QuMo, Glu-69, Gln-70, and Ala-71 in ActVA-Orf6 are substituted by isoleucine, alanine, and histidine, respectively, in QuMo. These substitutions do not disrupt the topology of the turn (Fig. 2c). Multiple alignments of various homologues demonstrate that the Ser-68 is highly conserved, and our results are consistent with the hypothesis that this residue is important in maintaining the backbone of the N-terminal residues of helix A3.

The Active Site—Monooxygenases of this type are small enzymes that oxidize relatively large, multiringed aromatic substrates. It is therefore not surprising that all secondary structural elements are involved in the formation of the active site. A slight disruption at the center of the helices results in the formation of helical arches over a rigid β-sheet floor. Although the rear of the ActVA-Orf6 cavity is closed by the swapped β-strand, the dimer interface of QuMo is not involved in the formation of the active site cavity. Rather, the side chains of helix A1 and strand B2 close the posterior of the QuMo active site. The studies of ActVA-Orf6 have implied that the enzyme does not require a metal ion or prosthetic group for catalysis (14, 15, 28). This is also the case for QuMo and likely for other monooxygenases of this family. There is no density that could be attributed to a metal ion, nor a clustering of appropriate residues that could serve as a metal coordination site.

The native structure of ActVA-Orf6 suggested that 4 residues may be important for binding and/or catalysis of the hydroxyquinone substrate: Tyr-51, Asn-62, and Trp-66, belonging to the β-sheets, and Tyr-72, hanging from helix A3. Tryptophan 66 is the only residue of these to be conserved in QuMo; the others substituted with Pro-42, Met-62, and Leu-72, respectively. These differences may reflect alterations for the accommodation of different substrates.

The ability of QuMo to bind quinonoid compounds was confirmed through co-crystallization of QuMo with menadione, a natural reaction product. The structure was refined to 1.65 Å with menaquinone clearly defined in the active site (Fig. 3). Two water molecules were also positioned in the active site, helping to coordinate menadione in the pocket via hydrogen bonding (Fig. 3b). The oxygen in the C1 position of menadione is hydrogen-bonded to a water molecule, which in turn hydrogen-bonds with the side chain γ- oxygen of Ser-85. The second coordinating interaction occurs between the first and second water molecules, the latter hydrogen-bonding to the backbone oxygen of Leu-76. The positioning of menadione permits the extension of the alkyl group out of the pocket, similar to the positioning of substrate analogues of Actva-Orf6, whereby the active site can accommodate much larger polyketide structure than the natural product (15).

QuMo Works in Concert with MdaB to Form a Quinone Cycle—MdaB co-purified with an associated flavin. This was made obvious by the intense yellow color of the purified protein, as well as a characteristic absorbance spectrum with a maximum at 320 nm. Activity was observed only with NADH supplementation; no activity was observed when NADPH was employed. This confirms the initial biochemical work, which suggested that this DT-diaphorase-like enzyme in E. coli was NADH-dependent (11). On the basis of prior experiments, this also suggests that MdaB is FMN-dependent. MdaB was able to completely reduce 50 μmol of menadione in 100 s, as measured by the consumption of menadione (308 nm) (Fig. 4). This is in agreement with previously reported data for the consumption of menadione by a DT-diaphorase-like enzyme purified from E. coli when NADH was provided for reaction (11).

The oxidation of menadiol to menadione was measured in the presence and absence of QuMo. QuMo at a final concentration of 5 μM was added to the MdaB reaction once menadione had been completely reduced to menadiol. A significant increase in the absorbance is notable after 60 s in the presence of QuMo (Fig. 4). No reduction of menadione was observed in control reactions containing MdaB alone or NADH alone. There was no detectable auto-oxidation of menadiol in the absence of QuMo over this time course, indicating that QuMo is indeed responsible for the oxidation of menadiol to menadione. Neither reaction was inhibited by the addition of heptyl-4-hydroxyquinoline-N-oxide.

**DISCUSSION**

QuMo (YgiN), the b3029 gene product, was selected for structure-based functional discovery as its low sequence homology with proteins of known function made functional annotation difficult. The QuMo crystal structure was refined to 2.2 Å. The protein fold aligned well with that of the monooxygenase ActVA-Orf6 from S. coelicolor with an overall root mean square deviation of 1.9 Å, despite only 19% sequence identity. Most interesting was the structural alignment of the Trp-66 residue, one of which is strictly conserved across all members of this functional group and thought to play an important role in catalysis (14, 15, 28). The maintenance of β-turn topology in both QuMo and ActVA-Orf6 highlights the minimal effect of low sequence identity on the conservation of global protein fold.

To date, this family of novel monooxygenases has been restricted to pathways responsible for the synthesis of large polyketide aromatic compounds, specifically the antibiotic biosynthetic pathways in Gram-positive bacteria. ActVA-Orf6 is such an enzyme, acting as a late-stage tailoring enzyme that confers functionality to dihydrokalaafungin, a compound with...
antifungal activity. The existence of members of the novel monooxygenase family in Gram-negative bacteria, which are not known to synthesize polyketide antibiotics, suggests a hitherto unidentified cellular function for some members of this family and also indicates that the family is much larger than initially suspected. The combination of low sequence identity and lack of conservation of active site residues together with a restriction of structural homologues to Gram-positive bacteria has made confident functional annotation prior to structural determination difficult, if not impossible.

Similar to its Gram-positive counterpart, QuMo does not appear to require metal ions or other co-factors for catalysis. On this basis, a protein radical mechanism in which the enzyme extracts a proton and electron from the substrate to yield a phenolic radical form of the polyketide substrate, which then reacts with molecular oxygen, has been proposed (29, 30). This leaves a radical enzyme species. The conserved Trp-66 found in QuMo is generally considered the most likely candidate as an important residue in the radical reaction. The positioning of menadione in the active site is consistent with the structures of ActVA-Orf6 bound to various substrate and product analogues. The alkyl group in position 2 of menadione points out of the cavity and does not appear to be important for substrate recognition. The large mouth of the cavity possibly allows the accommodation of the partially saturated polisoprenoid alcohol chain, which can contain up to 13 isoprenoid units in in vitro menaquinone derivatives. This can be likened to the ability of ActVA-Orf6 to accommodate much larger non-natural substrates, such as tetracenomycin F (15).

Following the structure determination of ActVA-Orf6, a second enzymatic mechanism based on the auto-oxidation of anthrones was proposed, suggesting that the invariant Trp-66 is essential for the specific recognition of the C-11 hydroxyl from 6-deoxydihydrokalafungin through a hydrogen bond that orients the substrate in the active site with the indole NH acting as a hydrogen donor (15). This hydrogen bond enhances the formation of a network of hydrogen bonds to the substrate and promotes the deprotonation of the hydroxyl group, leading to the formation of a C6 carbanion. In contrast to ActVA-Orf6, QuMo lacks the Tyr-51, Asn-62, and Tyr-72 residues that are believed to be involved in the catalytic mechanism of ActVA-Orf6. The substitution of Asn-62 with Met-62 still permits the stabilization of the peroxy intermediate, whereas the functions of the tyrosine residues must be replaced by other amino acids in the active site, possibly Tyr-40 and Tyr-84. Met-51 and His-75 may also be in appropriate positions to be involved in catalysis. These substitutions may indicate a difference in substrate specificity between the two proteins.

The differences in charge localization around the active site pocket are consistent with the postulated differences in substrate specificity between these monooxygenases. It has been suggested that the positively charged residues localized to the active site entrance of ActVA-Orf6 attract negatively charged hydroxyquinol substrates (15). In contrast, QuMo possesses a strong negative charge in the active site. This suggests that the substrate recognition mechanism of QuMo is more specific than general electrostatic attraction as the enzyme must overcome the anticipated charge repulsion that will occur during interaction of negative substrates in the negatively charged binding pocket.

As QuMo is not part of an antibiotic biosynthetic operon, the genomic organization of E. coli was examined for insights as to a possible natural substrate of QuMo. Both QuMo and MdaB are highly conserved proteins. Orthologues of both proteins can be found in all kingdoms of bacteria and also the Archaea, and not only are the proteins themselves conserved but their genomic organization as well. The co-localization of QuMo and MdaB on the same promoter indicates that their functions are related, an argument strengthened by the fact that both act on quinone-derived substrates. From earlier work related to MdaB, menadione was identified as a possible substrate due to its overall structural similarity to substrates of the previously identified monooxygenases and the known ability of MdaB to reduce menadione, an analogue of menaquinone. We have been able to confirm the menadione reductase activity previously reported for MdaB and have shown it to be NADH-dependent. In addition, we have demonstrated the ability of QuMo to regenerate menadione from menadiol. As shown in Fig. 4, QuMo is able to rescue ~50% of the initial amount of menadione, indicating at least an in vitro ability of QuMo to oxidize the reaction product of MdaB and form an enzymatic redox cycle. This work is the first to postulate the existence of the quinone...
discovery of a possible novel quinone redox cycle in E. coli, as well as to suggest that the function of MdaB may be coupled to the activity of another protein. Previous studies have demonstrated that MdaB expression is upregulated more than 20-fold in response to menadione (11), indicating QuMo expression to be likewise up-regulated. The endogenous expression of QuMo in E. coli K-12 has been reported previously (31). The response of the shared promoter of QuMo and MdaB to menadione coupled to the reported endogenous expression of these proteins under normal cellular conditions suggests that these proteins have a regular functional role in the cell, potentially in the protection of cells from oxidative damage.

The role of an enzymatic redox cycle may be two-fold. Firstly, it is important for E. coli to maintain an intracellular pool of menadione, which is preferentially employed under anaerobic growth conditions, as well as ubiquinone. This would allow a seamless transition from aerobic to anaerobic respiration under low oxygen environmental conditions. Due to the instability and insolubility of quinone compounds, it would be exceptionally difficult for cells to safely maintain such internal stores without the formation of reactive oxygen species. A redox cycle, such as the one formed by QuMo and MdaB, avoids the formation of these toxic entities by employing catalytic mechanisms that bypass the formation of a semiquinone intermediate (Fig. 4). This is highlighted by the insensitivity of either reaction to heptyl-4-hydroxyquinoline-N-oxide (indicated by HOQNO in the figure), a typical inhibitor of single electron reduction reactions, which indicates that both proteins act through two-electron transfer mechanisms. Secondly, quinones are widely distributed in nature and are internalized by many cells, including those of mammals. Once inside the cell, these quinones are also able to generate reactive oxygen species. Thus, the QuMo-MdaB cycle would also act as a quinone buffer, owing to the broad substrate specificity for these types of proteins (12, 14).

In conclusion, we have solved the structure of the first member of the novel monooxygenase family not involved in the biosynthesis of polyketide aromatic compounds. This indicates that these proteins may be more widespread than initially anticipated and suggests that many have not yet been identified because of the low overall sequence identity between these and more traditional family members. The structure of QuMo maintains the ferredoxin-like fold, similar to ActVA-Orf6, despite low sequence identity. However, differences in the positioning of active site residues, as well as differences in charge localization in the region, suggest a difference in substrate specificity between the two proteins. Most importantly, we have provided evidence that the QuMo protein may function along with MdaB to form a quinone redox cycle in E. coli that minimizes the formation of free radical species that otherwise pose a substantial threat to the cell. This work has allowed us to establish not only a functional annotation for the individual protein initially targeted but also to clarify the role of a closely related enzyme with opposing activity, thereby leading to the discovery of a possible novel quinone redox cycle in E. coli.
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