Allosteric regulation of menaquinone (vitamin K₂) biosynthesis in the human pathogen *Mycobacterium tuberculosis*

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**Editors’ Pick**

The atomic coordinates and structure factors (codes 6O04, 6O0G, 6O0J, and 6O0N) have been deposited in the Protein Data Bank (http://wwpdb.org/). This article contains Table S1 and Figs. S1 and S2. This article was selected as one of our Editors’ Picks.

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Edited by Chris Whitfield

Menaquinone (vitamin K₂) plays a vital role in energy generation and environmental adaptation in many bacteria, including the human pathogen *Mycobacterium tuberculosis* (*Mtb*). Although menaquinone levels are known to be tightly linked to the cellular redox/energy status of the cell, the regulatory mechanisms underpinning this phenomenon are unclear. The first committed step in menaquinone biosynthesis is catalyzed by MenD, a thiamine diphosphate–dependent enzyme comprising three domains. Domains I and III form the MenD active site, but no function has yet been ascribed to domain II. Here, we show that the last cytosolic metabolite in the menaquinone biosynthesis pathway, 1,4-dihydroxy-2-naphthoic acid (DHNA), binds to domain II of *Mtb*-MenD and inhibits its activity. Using X-ray crystallography of four apo– cofactor-bound *Mtb*-MenD structures, along with several spectroscopy assays, we identified three arginine residues (Arg-97, Arg-277, and Arg-303) that are important for both enzyme activity and the feedback inhibition by DHNA. Among these residues, Arg-277 appeared to be particularly important for signal propagation from the allosteric site to the active site. This is the first evidence of feedback regulation of the menaquinone biosynthesis pathway in bacteria, identifying a protein-level regulatory mechanism that controls menaquinone levels within the cell and may therefore represent a good target for disrupting menaquinone biosynthesis in *M. tuberculosis*.

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*Mycobacterium tuberculosis* (*Mtb*),² the causative agent of tuberculosis in humans, is able to adopt a persistent phenotype, resulting in long treatment times and a hard-to-eradicate latent infection (1). To combat this latent state, there has been a growing interest in menaquinone (vitamin K₂, MK), a small redox molecule that is essential for energy generation in both actively growing and persistent *Mtb* (2). MK also plays a role in triggering persistence in *Mtb* through its capacity to signal redox status (3). Previous studies have shown that inhibition of MK biosynthesis enzymes can significantly reduce growth of persistent state–like and drug-resistant *Mtb* (4–6). Therefore, a fundamental understanding of the MK biosynthesis pathway and its regulatory mechanism would provide a deeper insight into the underlying complexity of *Mtb* biology, opening up novel approaches for anti-TB therapeutics.

MK levels are known to be tightly linked to the redox potential in bacteria (2, 3, 7–10); however, the molecular mechanisms that regulate this phenomenon are unclear. The first committed step in MK biosynthesis in *Mtb* is catalyzed by the thiamine diphosphate (ThDP)-dependent enzyme MenD (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexadiene-1-carboxylate synthase, SEPHCHC synthase). Like other members of the ThDP-dependent pyruvate oxidase (POX) family, which are dimers or tetramers (comprising two interfacing dimers), MenD is tetrameric, with each monomer comprising three domains (Fig. 1, C and D) (11, 12). Domains I and III have known roles in catalytic function; domain I from one monomer in the dimer pairs with domain III of the other monomer (and vice versa) to form two paired active sites per dimer, with residues from both domains contributing to each active site (13–15). Domain II, however, is much less conserved and does not appear to participate in cofactor or substrate binding (11, 15). Various roles have been suggested for domain II in other POX-family enzymes; for
example, substrate and substrate-like allosteric activators bind in domain II of pyruvate decarboxylase (PDC) and phenylpyruvate decarboxylase (11, 12, 14–18), and nucleotides bind in other enzymes (e.g. FAD in POX and ADP in oxalyl-CoA decarboxylase) (11, 19–21). However, the function of domain II in MenD remains unexplored.

The classical MK biosynthesis pathway (Fig. 1A) starts with the synthesis of the naphthoquinone headgroup precursor (1,4-dihydroxy-2-naphthoic acid (DHNA)) in the cytosol (22, 23), followed by prenylation and methylation by membrane-bound enzymes to produce the lipid-soluble MK (24, 25). In addition to its electron transport role, the prenyl tail of MK can be further modified, and these modified quinones have been shown to regulate virulence of Mtb infection (7, 26, 27). Mtb-MenD is situated at a key step in MK biosynthesis, the first committed step (Fig. 1, A and B) (28, 29), and we hypothesized that it might be involved in controlling flux through this pathway.

We previously determined a series of crystal structures of Mtb-MenD showing each step in the MenD catalytic cycle, as its substrates α-ketoglutarate and isochorismate are successively added to the ThDP cofactor before the final product is released (13). We have now identified a downstream metabolite of the MK biosynthesis pathway (DHNA) that binds to domain II of Mtb-MenD and inhibits its catalytic activity. Herein, we characterize DHNA binding to Mtb-MenD at the molecular level, providing evidence for protein-level allosteric regulation and feedback inhibition of the classical MK biosynthesis pathway in Mtb.

Results

Search for MenD regulators

We aimed to determine whether Mtb-MenD is subject to feedback regulation. As a first step toward identifying possible regulatory molecules, we crystallized Mtb-MenD in its cofactor-bound form, as described previously (13) and then soaked these crystals in mother liquors containing a range of substrates, metabolites, and metabolite-like compounds from the MK biosynthesis pathway. By these means, we discovered that DHNA, a downstream metabolite of MK biosynthesis, binds to a site in domain II of Mtb-MenD (Fig. 1, C and D).

Definition of the DHNA-binding site

To further characterize the interactions between Mtb-MenD and DHNA, we determined four crystal structures representing different functional states of the enzyme: its apo (cofactor-free) form complexed with DHNA, a cofactor (ThDP)-bound DHNA complex, and two covalent cofactor intermediate-bound (intermediates I and II, Fig. 1B) DHNA complexes (Table 1 and Fig. S1). In all cases, DHNA bound between domains I and II of each MenD subunit, in a cleft formed by residues 94–97, 232–235, 276–278, and 299–306 and capped by residues 112–120 from a neighboring subunit (Fig. 2A). This cleft is located ~20 Å away from the closer of the paired active sites in the dimer and ~30 Å from the more distant one (Figs. 1C and 2B).

The binding cleft for DHNA (Fig. 2A) is essentially the same in all four structures. The DHNA molecule occupies an “arginine cage” formed by three arginine residues, Arg-97, Arg-277, and Arg-303, arranged such that the side chains of Arg-277 and Arg-303 pack on either side of the planar dihydroxynaphthoic acid ring and the side chain of Arg-97 hydrogen-bonds to the DHNA carboxylate. Additional hydrogen bonding interactions are made by the DHNA hydroxyl groups with the carboxyl oxygens of Tyr-95 and Arg-303 and the Arg-303 guanidinium group. In each structure, for two of the four sites per tetramer, the binding cleft is closed off by residues 112–120 from a flexible active-site loop belonging to the other subunit of the MenD dimer. Particularly notable in this interaction is Gly-115, which makes van der Waals contacts with the DHNA ring, as well as Thr-114, which makes contacts across to Asp-306 in the allosteric binding cleft.

Whether there is functional connectivity between the four allosteric DHNA-binding sites in the Mtb-MenD tetramer is unclear. Unlike the half-of-sites occupancy observed for the Mtb-MenD active site (13), DHNA binding to Mtb-MenD was consistently observed in all four subunits of each structure (i.e. both with and without the ThDP cofactor and in the reaction intermediate–bound forms). However, in two of the four active sites per tetramer, the DHNA-binding site was not complete, with disorder exhibited in the 112–120 region that capped the binding site. There is also a hydrogen-bonding network traceable from domain II residues 299–306 at the DHNA-binding site via residues Arg-97, Ala-170, Arg-159, and Arg-168 to the same region in a neighboring Mtb-MenD subunit ~25 Å away. This suggests that binding events on one subunit could be transmitted to the others through such a network.

Changes upon DHNA binding highlight connectivity between the allosteric and active sites

Comparison of DHNA-bound and DHNA-free structures revealed only subtle changes in the binding cleft residues, although striking changes occurred for the domain I flexible active site loop (residues 105–125). This latter loop not only contributes to the DHNA binding site but contributes residues to the closest active site ~20 Å from the DHNA-binding site (Fig. 2B).

In the DHNA-free structures, the 105–125 loop is partly disordered in the apo-state but becomes fully ordered when its associated active site is occupied, which happens in two of the four monomers in the tetramer (Fig. 3A). In association with those changes, residues 79–82 at the N terminus of a domain I α-helix also rearrange. This region encloses the catalytic Glu-55 in active sites that are unoccupied (13) and rearranges in occupied sites, allowing Glu-55 to contribute to the binding pocket for the 4′-aminopyrimidine (AP) ring of the ThDP cofactor. These changes are required to generate the catalytically competent state of the enzyme and presumably also to enable product release.

In many respects, the structural changes that occur as DHNA binds to the apo-enzyme mirror key changes in domain I that occur when ThDP binds. In the apo-enzyme without DHNA, all four monomers show the same “active site–unoccupied conformation” for the above two domain I regions. When DHNA is bound to the apo-enzyme, however, residues 112–120 become fully ordered as they complete its binding site. This is associated also with reorganization of residues 78–82, as a new hydrogen bond is made between Ser-79 and Gln-118 (Fig. 3B). The hydrogen-bonding environment of the catalytically essential Glu-55 is also changed as a result, to a state partway between that seen in...
occupied and unoccupied active sites. This occurs for two of the four active sites, creating asymmetry in apo-structures that has previously only been observed in cofactor-occupied structures.

**DHNA is a potent inhibitor of Mtb-MenD SEPHCHC synthase activity**

To determine whether DHNA does indeed have a regulatory role in MK biosynthesis, we studied its effect on *Mtb-MenD* activity. An NMR-based activity assay, similar to that previously reported (13), was first used and showed that the activity of *Mtb-MenD* at a concentration of 5 μM was reduced in the presence of 20 μM DHNA to only 24% of its noninhibited activity (Fig. 4A). Further increases in DHNA concentration resulted in only small increases in inhibition (data not shown), consistent with saturation of the enzyme at low micromolar concentrations.

A UV spectrophotometry–based assay, in which the consumption of isochorismate is monitored at 278 nm, was then used to examine the inhibition over a lower DHNA concentration range (0.1 nM to 10 μM). We found that DHNA inhibited *Mtb-MenD* with an IC50 of 53 nM under the conditions of this assay (Fig. 4B and Table 1). In combination with our structural complexes, these assays establish DHNA as a potent allosteric inhibitor of *Mtb-MenD*.

**Arginine cage residues support inhibition by DHNA and dramatically affect enzyme activity**

The three arginine residues (Arg-97, Arg-277, and Arg-303) that form a cage around DHNA in its binding site are candidates for signaling between the allosteric and active sites. All three residues interact directly with DHNA and are likely to enhance binding (Fig. 2, A and B). Arg-97 is located at the C

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**Figure 1. Role of Mtb-MenD in the MK biosynthesis pathway.** A, known enzymes in the classical biosynthesis pathway. M^2⁺, a divalent metal ion, commonly Mg^2⁺, but MenD enzymes are also active with Ca^2⁺ and Mn^2⁺; IPP, an isoprenyl diphosphate substrate of variable repeat length; PPI, diphosphate; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; CoASH, CoA; OSB, o-succinylbenzamide; SHCHC, (1R,6R)-6-hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate. The orange X symbolizes feedback inhibition by DHNA. *, MenD is the first committed step, with the step before often carried out by an isochorismate synthase enzyme either nonspecific to the pathway (e.g. EntC in *Mtb*) or specific to the pathway (e.g. MenF in *E. coli*). B, the MenD catalytic cycle showing the two covalent ThDP intermediates. C, the *Mtb-MenD* tetramer (composed of two interfacing dimers, one depicted as green/yellow cartoons and the other as marine blue/blue-white cartoons). DHNA is shown as orange sticks, and intermediate II is shown as yellow sticks. The approximate distance between the bound DHNA and the closest active sites (within the same dimer, shown for a single dimer only for clarity) and between allosteric sites across the dimer-dimer interface are depicted by lines/distance labels. D, close-up view of one MenD dimer from the tetramer. One monomer in the dimer is depicted as blue-white, whereas the other is colored by domain (domain I in magenta, domain II in marine blue, and domain III in dark blue). DHNA is shown as orange sticks, and intermediate II is shown as yellow sticks.
terminus of a long helix that originates in the more distant of the paired active sites, indicating a potential line of communication with that site (Fig. 2, A and B). Arg-277 hydrogen-bonds with two regions of the closest allosteric site across the tetramer (Fig. 2A). These regions contain residues (Arg-107, Asn-117, Gln-118, and Arg-399) known to be important for Mtb-MenD function (28, 30). Arg-303 is located in a region of the three arginine cage residues; Arg-277 hydrogen-bonds with that site (Fig. 2, A and B). Superpositions of the three known MenD structures, from Escherichia coli (Ec), Listeria monocytogenes (Lm), and Bacillus subtilis (Bs) (28, 30–32), on to Mtb-MenD (overall root mean square differences of 1.8–2.4 Å over 406–477 aligned Ca atom positions) suggest limited conservation of the DHNA-binding site. In Ec-MenD and Lm-MenD, few of the allosteric site residues are conserved, and in Ec-MenD, the site is partly filled by the hydrophobic Leu-316.

To test the importance of these three residues for MenD activity and/or DHNA inhibition, we carried out alanine mutagenesis experiments. These confirmed that each of the three arginines is crucial for MenD activity (Table 2); the R303A, R97A, and R277A mutant enzymes had 56, 50, and 18%, of the WT enzyme activity, respectively, when measured under conditions and in the absence of DHNA. In terms of DHNA inhibition, R97A and R303A showed 19- and 6-fold increases in IC_{50}, respectively, compared with WT Mtb-MenD (Table 2), indicating the importance of these arginines for DHNA binding and feedback inhibition. The catalytic activity of the R277A variant was too low to accurately measure an IC_{50} for DHNA. We conclude that these three residues are important for maintenance of WT MenD activity and that this underpins their roles in signal propagation from the allosteric site to the active site of Mtb-MenD.

### Conservation of the “arginine cage” and allosteric site in other MenD enzymes

To explore how widely conserved this binding site and its arginine cage residues may be across the bacterial kingdom, we investigated the sequence and structural relationships among MenD orthologues (Fig. 5, B and C). Superpositions of the three known MenD structures, from Escherichia coli (Ec), Listeria monocytogenes (Lm), and Bacillus subtilis (Bs) (28, 30–32), on to Mtb-MenD (overall root mean square differences of 1.8–2.4 Å over 406–477 aligned Ca atom positions) suggest limited conservation of the DHNA-binding site. In Ec-MenD and Lm-MenD, few of the allosteric site residues are conserved, and in Ec-MenD, the site is partly filled by the hydrophobic Leu-316. In Bs-MenD, the site retains some key elements, including two of the three arginine cage residues; Bs-Arg-96 (equivalent to Mtb-Arg-97) and Bs-Arg-323, adjacent to the Bs-Trp-322 (equivalent to Mtb-Trp-304), which, with rearrangement, could fill a role equivalent to that of Mtb-Arg-277 (Fig. 5B).

A multiple-sequence alignment of 35 different bacterial, archaeal, and plant MenD amino acid sequences using MAFFT (33) (Fig. S2) shows that conservation across domain II is generally low and the three key DHNA-binding arginines (Arg-97,
Arg-277, and Arg-303) are present only in closely related species, such as other Mycobacteria (e.g. Mycobacterium canetti) and Rhodococcus (overall 66% sequence identity to Mtb-MenD). A further six of the 35 sequences showed conservation for two of the three arginines (Arg-97 with either Arg-277 or Arg-303) or had Arg-97 and an arginine adjacent to the Trp-304–equivalent residue as observed for Bs-MenD.

Discussion

**Biological significance of a regulatory role for DHNA**

Our study reveals the downstream metabolite DHNA as a negative allosteric regulator of Mtb-MenD and a possible self-regulating signal in the pathogen *M. tuberculosis*. As far as we are aware, this is the first report of allosteric regulation of the menaquinone biosynthesis pathway by a downstream metabolite. The ability of DHNA to act as a regulatory signal in the pathogen *M. tuberculosis* is in line with both the biological significance of DHNA and the importance of regulating menaquinone levels within the bacteria. As the last nonprenylated soluble metabolite in the MK biosynthetic pathway, DHNA sits at the point where the pathway moves from an aqueous cytosolic location to a lipophilic membrane-immersed one (25) and has the potential to provide feedback on the catalytic status of MenA (and perhaps the downstream MK pool).

DHNA is also the first metabolite in the pathway with a complete (and CoA-free) redox-capable napthoquinol ring (34) and has the capacity in its own right to catalyze redox reactions (35). It may thus act as a signal of redox status, with excessive levels exerting toxicity if the redox balance within the cell is disrupted. DHNA has also been shown to act as a virulence factor in the intracellular pathogen *L. monocytogenes*, where it promotes cytosolic survival, and may be a sensor of cytosolic stress (36). In plants, phyloquinone biosynthesis enzymes share homology to those of classical bacterial MK biosynthetic enzymes, and 1,4-naphthoquinones derived from DHNA act in roles mediating plant-plant, plant-insect, and plant-microbe interactions (37).

How might binding at a site remote from the active site impact catalysis?

*Mtb*-MenD is a complex enzyme, characterized by significant conformational changes and disorder-order transitions that take place when DHNA binds.

Figure 2. The DHNA-binding site is connected to the active site. A, the DHNA-binding sites, viewed looking down onto the top of one of the Mtb-MenD dimers with distances between the allosteric and active sites marked in black and a close-up of the DHNA binding site nearest to an occupied active site. The binding site is surrounded by residues from domain II (pale white blue sticks) and domain I (residues 95–97 shown as pink sticks) from one monomer in the dimer, and the site is capped by residues 111–116 of the active site loop 105–125 of domain I from the neighboring monomer in the dimer (pale green sticks). DHNA is shown in orange sticks. B, a side view of the same Mtb-MenD dimer showing a close-up of the network of residues between the allosteric site and its closest active site (colored the same as A). Hydrogen bonds linking the two sites, from the DHNA-binding region through to key intermediate II binding residues, are shown with broken lines. Arg-277, which links two important active-site regions (105–125 loop and 399–401), is highlighted in boldface. DHNA is shown in orange sticks, and intermediate II is shown in yellow sticks.
place during the catalytic cycle (13). In its apo (cofactor-free)-state there are substantial regions of disorder. Cofactor-bound structures, including the two covalent intermediates, are more ordered and are also asymmetric, with only two of the four active sites occupied per tetramer (13). Our work has highlighted that the allosteric site is affected by, and can influence, the active site of Mtb-MenD in all of these functional states.

The DHNA-binding site is separated from the closest active site (20 Å distant) primarily by two sections of polypeptide, residues 399 – 402 from domain III and residues 112 – 120 from the flexible domain I active-site loop contributed by the other subunit of the dimer (Fig. 2B). This latter region also closes off the DHNA-binding cleft, packing against the DHNA molecule. Both regions contribute key residues to the active site; the 399 – 402 region provides Arg-399 (Fig. 2B), critical for α-ketoglutarate recognition, and Ala-402, whose carbonyl oxygen hydrogen-bonds to the ThDP AP ring. Residues 112 – 120 carry two key active-site residues, Gln-118, essential for catalysis, and Asn-117, which binds to the α-ketoglutarate moiety when intermediate I is formed. Whereas the conformation of the backbone of the 399 – 402 region does not seem to alter significantly in our snapshots across the catalytic cycle (or in the presence or absence of DHNA), the 105 – 125 domain I loop does in ways that highlight the connectivity between the allosteric site and the Mtb-MenD catalytic machinery.

The clearest effect of DHNA appears to be to induce asymmetry in apo-MenD and lock in place flexible domain I elements of the active site into conformations similar to those seen when ThDP is bound. The domain I flexible loop region is connected in several ways to the allosteric site, via Arg-277 from the Arg cage as well as direct contacts with the DHNA ring and other parts of the allosteric site (i.e. Asp-306 to Thr-114). There are also connections from the allosteric site via Arg-277 to domain III components of the active site (i.e. Arg-399) (Fig. 3B).
No domain III ordering/conformational changes are observed, however, and the flexible domain III loop 471–486 does not adopt its closed form until ThDP is bound. Due to the asymmetry in these domain I and III regions, they are candidates for any order-disorder transitions that might play a role in orchestrating the half-of-sites occupancy.

DHNA binding could thus impact any communication between the active sites, as well as many points along the catalytic cycle. The two domain I regions most affected have roles ranging from direct binding to substrates and covalent intermediates to more indirect roles positioning other elements in the active site. As substrate binding, formation of intermediates, and product release (both CO₂ and the final SEPHCHC) all depend on flexibility in the active site (13), DHNA binding, by limiting active-site flexibility, has the power to affect catalysis.

A key player in this scenario is likely the invariant Gln-118, even conservative mutations of which abolish SEPHCHC synthase activity (38). In unoccupied active sites, Gln-118 is disordered or has high B factors. In occupied sites, however, it is ordered but undergoes side-chain movements that enable specific interactions that are critical to several key steps of the catalytic cycle. These include stabilizing the active tautomer of the AP ring and hydrogen-bonding to both the incoming isochorismate substrate and then the resultant intermediate II. Gln-118 also interacts with the “CO₂-like” formate ion that likely models the location of the carboxyl group that is removed during formation of intermediate I (13, 30).

**Regulatory variation in the ThDP-dependent enzyme superfamily**

Our results demonstrate that Mtb-MenD is an allosterically regulated ThDP-dependent enzyme, inhibited by direct binding of a downstream metabolite (DHNA) from the biosynthetic pathway in which it participates. Although feedback inhibition of this type has not previously been demonstrated for ThDP-dependent enzymes, there is precedence for various mechanisms of allosteric regulation in the wider superfamily. Some enzymes, such as acetohydroxyacid synthase, have an entirely independent regulation (acetyl-CoA) to a shallow pocket on the enzyme surface and allosterically inhibited by binding of a regulatory protein (GarA) (40–42). Similarly, pyruvate decarboxylase (43), phenylpyruvate decarboxylase (17, 18), and oxalyl-CoA decarboxylase (21) have all been shown to be positively regulated by the direct binding of small molecules to domain II.

Structural comparisons of DHNA-bound Mtb-MenD reveal striking parallels with pyruvate decarboxylase, which is allosterically activated by its pyruvate substrate (43). The pyruvate decarboxylase allostERIC site has previously been described as a “switch point” in domain II (43) and has a very similar structural context to the DHNA-binding allosteric site we observe in Mtb-MenD (Fig. 5A). The locations of the allostERIC sites map to each other; DHNA overlays with the substrate bound in the pyruvate decarboxylase allostERIC site. The two activation loops in pyruvate decarboxylase, for example, feature similar features to this site (43). Domain II, however, is also highly conserved across this superfamily (Fig. 5B).
vate decarboxylase (residues 288–304 and 104–113) that undergo conformational change upon allosteric regulator binding are equivalent to regions in Mtb-MenD that are affected by DHNA binding (i.e. residues 277–312 and residues 105–116). Moreover, the substrate-binding residues Asn-117 and Gln-118 in Mtb-MenD, which are positioned in the active site when the 105–125 loop is ordered, align with two active-site histidines (His-114 and His-115) in pyruvate decarboxylase (43) that also rearrange when the allosteric site is occupied.

Despite these similarities, key differences exist. The allosteric effect in pyruvate decarboxylase is activation, not inhibition, and, in keeping with this, the effector of Mtb-MenD is not the

Figure 5. Conservation of the allosteric site. A, location of the DHNA-binding site in Mtb-MenD compared with the substrate-modified allosteric site in PDC (right). Domain I of each structure is shown in magenta (Mtb-MenD) and pink (PDC), domain II in white/blue (Mtb-MenD) and gray (PDC), and domain III in deep blue (Mtb-MenD) and light blue (PDC). ThDP/substrate (green) or ThDP intermediate (yellow), DHNA (orange), and modified Cys-221 (green) are shown as sticks and labeled. B, overlay of the allosteric site of Mtb-MenD (pink, light gray, and green) with the equivalent regions in Bs-MenD (gray). The Bs-MenD site shows some conservation, but with no clear Arg-303 equivalent. Bs-Arg323 could, if the side chain rearranged, take up a similar position to Mt-Mt-Arg277. C, representative sequence alignment of domain I and domain II regions of MenD enzymes that contain the Arg allosteric residues (yellow boxes) generated by ESPript (60). The three sequences with representative structures in the PDB are named in red. The Bs-Arg323 position is shown in a green box. Residues similar to Arg in each position (i.e. His or Lys) are colored in orange or green.
substrate but a downstream metabolite. In addition, whereas the asymmetric dimers of pyruvate decarboxylase undergo large quaternary conformational changes (44), no significant alterations in quaternary structure have been observed for any MenD structures reported to date. The enzymes also catalyze different reactions, with different sized substrates and different dynamics in their catalytic cycles (18, 43). Nonetheless, these parallels do point to the wider use of domain II as a regulatory domain for allosteric regulation of ThDP-dependent enzymes, a phenomenon now also seen in MenD.

Limited conservation of the allosteric binding site in other MenD enzymes

Although active sites are generally highly conserved in enzymes catalyzing the same reaction, allosteric sites are often organism-specific, being under less pressure to be stringently conserved. Our studies suggest that there is limited strict conservation of key elements of the allosteric site across bacterial MenD enzymes. This suggests either that regulation of the pathway by DHNA is limited to a small subset of bacteria or that DHNA or related molecules may still bind in this region despite the absence of some key residues found in Mtb-MenD. Indeed, unlike Mtb and other Gram-positive bacteria that have menaquinone as their sole isoprenoid quinone, Gram-negative bacteria like E. coli utilize ubiquinone and menaquinone at different times in their growth (45). Hence, there may be different needs for regulation of this pathway in different bacteria, requiring adaptations of this allosteric site.

Importantly, however, the presence of a site with a powerful ability to regulate enzyme activity is of immediate value as a species-specific antimicrobial target. The field of allosteric inhibition is a growing one (46). Allosteric inhibitors have been recently exploited to inhibit M. tuberculosis growth through inhibiting Mtb Argl (47) and Mtb TrpB (48). Whereas allosteric inhibitors in general do not completely inhibit their targets (46), they exhibit greater specificity compared with the active-site inhibitors (48). Moreover, allosteric sites show greater propensity to bind more hydrophobic molecules, often correlated with more desirable cell permeability properties (48). The latter seems to be the case for Mtb-MenD, where the allosteric site accommodates a more hydrophobic ligand compared with the large and hydrophilic substrates that bind in the active site. Our discovery of a potent inhibitor of Mtb-MenD, acting through a site remote from the active site, has important implications for future development of selective antitubercular drugs targeting the essential MK biosynthesis pathway.

Experimental procedures

Strains and plasmids

The ORF encoding MenD (Rv0555) from M. tuberculosis H37Rv was previously cloned into the pYUB28b vector, which contains a short N-terminal hexahistidine tag (13). MenD mutants were generated using the pYUB28b-MenD construct and oligonucleotide primers (Table S1, Integrated DNA Technologies) with iProof™ high-fidelity DNA polymerase (Bio-Rad). The PCR products were then treated with DpnI and ligated using T4 DNA ligase (Roche Applied Science), before being transformed into E. coli TOP10 cells. The mutations were verified by DNA sequencing.

Protein expression and purification

WT and mutant MenD constructs were expressed in M. smegmatis mc^2 4,517 cells (49) and purified using immobilized metal affinity chromatography (IMAC) and size-exclusion chromatography (SEC), as described previously (13). In brief, cells were lysed in 20 mM HEPES, pH 8.0, 150 mM NaCl, 5 mM MgCl_2, 20 mM imidazole, 5% glycerol, and 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) using a Microfluidics cell disruptor (Newton, MA). The recombinant Mtb-MenD protein was purified by IMAC with 5-ml HisTrap HP columns (GE Healthcare) using an imidazole gradient of 20–500 mM over 90 ml. The eluted protein solution was then concentrated and further purified by SEC (in buffer containing 20 mM HEPES, pH 8.0, 150 mM NaCl, 5 mM MgCl_2, 5% glycerol, and 1 mM TCEP) using a Superdex 200 10/30 column. The average yield was between 0.5 and 1.5 mg of purified protein/liter of M. smegmatis expression culture. The mutant proteins behaved similarly to WT protein, as they also purified as tetramers in SEC and showed similar thermal melt temperatures in differential scanning fluorimetry analysis. The protein solution was kept at −80 °C for subsequent use (with added 5% glycerol). For NMR experiments, the protein was either purified directly in 50 mM phosphate, pH 7.5, with 50–100 mM NaCl or buffer-exchanged into this after purification.

NMR spectroscopy assay

The activity of Mtb-MenD was monitored using a coupled reaction with E. coli isochorismate synthase (Ec-MenF), which converts chorismate to isochorismate (the substrate for Mtb-MenD). Ec-MenF was expressed and purified as described previously (13). Initial NMR samples were prepared with 2 mM chorismate, 1 mM α-ketoglutarate, 200 μM ThDP, 25 μM Ec-MenF, and varying concentrations of DHNA (in 20 mM potassium phosphate, pH 7.5, 50 mM NaCl, 1 mM MgCl_2, 1 mM 2-mercaptoethanol, 10% (v/v) D_2O, and 0.25 mM 3-(trimethylsilyl)propionic acid (TSP)). Samples were incubated at 25 °C, and one-dimensional 1H NMR spectra were monitored until the reaction reached equilibrium, with an estimated 47:53 ratio of isochorismate to chorismate based on peak integrals. Mtb-MenD (5 μM) was then added, and one-dimensional 1H NMR spectra were recorded at 100-s intervals for up to 90 min. Reaction rates were estimated by monitoring the decrease in peak integral for isochorismate and chorismate and the increase in peak integrals for SEPHCHC, relative to the peak for the TSP internal standard (δ 0 ppm). Due to peak overlap in other regions, an endopryruvyl methylene proton peak was monitored with a chemical shift of 5.25, 5.23, and 5.20 ppm for isochorismate, chorismate, and SEPHCHC, respectively. NMR spectra were collected on an Avance AVIII-HD 500-MHz spectrometer (Bruker) with suppression of the water signal using excitation sculpting (50). Data were processed using the software package TopSpin 4.0.6 (Bruker).

UV-visible spectroscopy assay

Mtb-MenD activity was monitored by the decrease in isochorismate absorbance at 278 nm (ε_278 = 8,300 M⁻¹ cm⁻¹ (29))
at 25 °C. To produce isochorismate, 10 μM Ec-MenF was incubated in a 3-mL reaction for 1 h at room temperature with 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, and at least 3 mg of chorismic acid. Ec-MenF was then removed from the mixture using a Vivaspin concentrator with a 10-kDa cut-off. The mixture was stored in small aliquots at −80 °C prior to use.

Isochorismate was quantified prior to kinetic assays, using the following reaction. 1 μM Mt-MenD (monomer concentration) was incubated with 100 μM ThDP and 100 μM α-ketoglutarate for 30 min at 25 °C in MenD kinetic assay buffer (100 mM Tris, pH 8, 100 mM NaCl, and 5 mM MgCl₂). The reaction was initiated with 30 μL of a mixture of chorismate/isochorismate. The quantity of isochorismate used was back-calculated using Beer’s law. No background catalytic rate was observed when either isochorismate or α-ketoglutarate were absent from the assay mixture. All assays were performed using a Cary 400 UV-visible spectrophotometer and quartz cuvettes with a final reaction volume of 800 μL.

Inhibition assays for WT Mt-MenD contained 0.6 μM Mt-MenD, 300 μM ThDP, and various concentrations of DHNA (0–10 μM) in a reaction buffer of 100 mM Tris, pH 8, 100 mM NaCl, and 5 mM MgCl₂. After preincubation for 30 min at 25 °C, 2 μM isochorismate was added, and the reaction was initiated by the addition of 300 μM α-ketoglutarate. Three technical replicates were conducted at each DHNA concentration. Solutions of DHNA were prepared immediately prior to the inhibition assays as gradual oxidation of DHNA was observed when solutions were stored. The IC₅₀ of DHNA for each mutant Mt-MenD was determined using the same conditions as those for WT Mt-MenD, except that the DHNA concentrations varied from 0 to 50 μM. Initial rate data were fitted to the four-parameter logistic Hill equation with GraphPad Prism.

Crystallization, soaking, and freezing

APO_DHNA structure—The WT MenD apo-enzyme was concentrated to 15–20 mg/mL in buffer A (20 mM HEPES, pH 8.0, 150 mM NaCl, 5% glycerol, 1 mM TCEP, 5 mM MgCl₂) and crystallized using 96-well sitting-drop format in a variant of the MORM crystallization screen (0.1 M MOPS/HEPES, pH 7.5, 20% glycerol, 8% PEG 4000, 0.02 M CA mix (where CA mix is a mixture of sodium formate, ammonium acetate, sodium citrate tribasic dihydrate, sodium oxamate, and potassium sodium tartrate tetrahydrate)). Crystals grew within 1–7 days and were soaked in 10 mM DHNA solution (5% DMSO, 95% 0.1 M MOPS/HEPES, pH 7.5, 25% glycerol, 10% PEG 4000, 0.02 M CA mix) solution overnight before being flash-frozen in liquid nitrogen.

ThDP, intermediate I or II + DHNA structures—The WT MenD apo-enzyme was concentrated to 15–20 mg/mL in buffer A, thiamine diphosphate was added to a final concentration of 1 mM, and the protein was crystallized as for the apo-enzyme. Crystals grew within 1–7 days and were soaked as follows: for ThDP_DHNA, soaked in 5 mM DHNA (0.1 M MOPS/HEPES, pH 7.5, 25% glycerol, 14% PEG 4000, 0.01 M CA mix) for 30 min; for IntL_DHNA, soaked in 1 mM α-ketoglutarate (in 0.1 M MOPS/HEPES, pH 7.5, 25% glycerol, 14% PEG 4000, 0.01 M CA mix) for 1–10 min followed by the addition of DHNA (final concentration 3.3 mM) to the α-ketoglutarate soak drop for a further 20 min; for IntII_DHNA, isochorismate (final concentration 100 μM) was added to an α-ketoglutarate soak drop for less than 1 min, followed by the addition of DHNA (final concentration 3.3 mM) for a further 20 min. All crystals were then flash-frozen in liquid nitrogen.

Other soaks into Mt-MenD crystals were undertaken using solutions of crystallization mother liquor containing varying concentrations of menaquinones (MK-1, MK-2, MK-2 H2, MK-3) or (1R,6R)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC). MK-1 was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). MK-2, MK-2 H2, and MK-3 were kindly provided by Prof. Debbie Crans (Colorado State University) as characterized previously (51). SHCHC was produced enzymatically using MenH and used as a crude mixture after removal of the enzyme.

Data collection, structure determination, and refinement

All diffraction data were collected using the macromolecular crystallography beamline MX2 at the Australian Synchrotron (52). The data were indexed and processed using XDS (53), re-indexed using POINTLESS (54), and scaled with SCALA (54) from the CCP4 program suite (55). Analyses of merged CC₁₂ correlations between intensity estimates from half-data sets were used to influence high-resolution cutoff for data processing (56). This established method is less conservative than other resolution cutoff criteria, resulting in the higher Rmerge values (Table 1).

The structures of the DHNA-soaked crystals were solved by molecular replacement using Phaser (57), with 5ERY (13) as a search model, and a dimer of a previous Mt-MenD structure (Protein Data Bank (PDB) code 5ESU (13)) was used as a search model for the ThDP/intermediate structures. The final models of all structures were then completed with iterative rounds of manual building using COOT (58) and refinement using Refmac5 (59) and Phenix (59). After building, additional density corresponding to DHNA, ThDP, intermediate I or II, and α-ketoglutarate, as appropriate, was modeled using available PDB dictionary restraints (omit maps of the ligand density are given in Fig. S1). Water molecules were identified by their spherical electron density and appropriate hydrogen bond geometry with the surrounding structure. Unless otherwise stated, all protein structure images were generated using PyMOL (PyMOL Molecular Graphics System, version 1.5, Schrödinger, LLC).

Accession codes

The structures presented in this paper have all been deposited in the PDB with the following codes: 6O04, 6O0G, 6O0J, and 6O0N.

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