Crystal Structure of Mycobacterium tuberculosis MenB, a Key Enzyme in Vitamin K<sub>2</sub> Biosynthesis

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Bacterial enzymes of the menaquinone (Vitamin K<sub>2</sub>) pathway are potential drug targets because they lack human homologs. MenB, 1,4-dihydroxy-2-naphthoyl-CoA synthase, the fourth enzyme in the biosynthetic pathway leading from chorismate to menaquinone, catalyzes the conversion of O-succinylbenzoyl-CoA (OSBB-CoA) to 1,4-dihydroxy-2-naphthoyl-CoA (DHNA-CoA). Based on our interest in developing novel tuberculosisc chemotherapeutics, we have solved the structures of MenB from Mycobacterium tuberculosis and its complex with acetoacetyl-coenzyme A at 1.8 and 2.3 Å resolution, respectively. Like other members of the crotonase superfamily, MenB folds as an (α<sub>3</sub>β<sub>3</sub>)<sub>2</sub> hexamer, but its fold is distinct in that the C terminus crosses the trimer-trimer interface, forming a flexible part of the active site within the opposing trimer. The highly conserved active site of MenB contains a deep pocket lined by Asp-192, Tyr-287, and hydrophobic residues. Mutagenesis shows that Asp-192 and Tyr-287 are essential for enzymatic catalysis. We postulate a catalytic mechanism in which MenB enables proton transfer within the substrate to yield an oxyanion as the initial step in catalysis. Knowledge of the active site geometry and characterization of the catalytic mechanism of MenB will aid in identifying new inhibitors for this potential drug target.

Critical issues in the treatment and control of tuberculosis, a disease that kills more than two million people world-wide every year (1, 2), include the role of this disease as a major opportunistic pathogen in patients with HIV-AIDS and the emergence of multidrug resistance strains of M. tuberculosis (MDRTB) (3–5). Based on the knowledge that menaquinone is the sole quinone in M. tuberculosis and that humans must obtain this vitamin (K<sub>2</sub>) either in their diet or from intestinal bacteria, menaquinone biosynthesis may be an attractive novel target for anti-TB drug discovery. Consequently, we have initiated studies on the putative biosynthetic enzymes in this pathway from M. tuberculosis.

Quinones are lipid-soluble molecules that shuttle electrons between the membrane-bound protein complexes in the electron transport chain. In mammalian cells, the electron transport chain is located in the inner mitochondrial membrane where the membrane-soluble quinone is ubiquinone, a benzquinone (coenzyme Q, Fig. 1). In prokaryotes, the electron transport chain sometimes utilizes menaquinone, a naphthoquinone (vitamin K<sub>2</sub>, Fig. 1), in addition to or instead of ubiquinone. The principal structural variations within the two main classes of quinones occur from the length of the isoprenoid chain. Thus, in mitochondria the ubiquinone has a side chain of 10 isoprene units (CoQ-10) while in Escherichia coli the ubiquinone has a side chain of 8 isoprene units (CoQ-8) (6). E. coli, a facultative anaerobe, utilizes ubiquinone (CoQ-8) under aerobic conditions, but uses menaquinone (MK-8) when grown anaerobically (6, 7). Bacillus subtilis, a Gram-positive aerobe, contains only menaquinone (MK-7) (7). Thus, inactivation of genes encoding the menaquinone biosynthetic enzymes in B. subtilis produces an absolute requirement for menaquinone or a downstream metabolite in the growth media (8, 9).

Several pieces of data support the notion that M. tuberculosis, like B. subtilis, utilizes only menaquinone in the electron transport chain. Firstly, studies classifying bacteria based on their quinone content identified only menaquinone and not ubiquinone in M. tuberculosis (6, 10, 11). Secondly, Goldman and co-workers (12, 13) isolated the NADH oxidase system from M. tuberculosis and demonstrated that it catalyzed the reduction of naphthoquinones. Lastly, the M. tuberculosis genome contains homologs of most of the E. coli men genes (see below and genolist.pasteur.fr/TubercuList/, Ref. 14). Conversely, homologs of some of the key ubiquinone biosynthetic genes are absent. For example, there is no homolog of chorismate pyruvate-lyase (UbiC), the enzyme that converts chorismate into 4-hydroxybenzoate (15, 16), the first committed step in ubiquinone biosynthesis.

The biosynthesis of menaquinone has been most heavily studied in E. coli (reviewed in Ref. 16) and, to a lesser extent, in Bacillus subtilis (9, 17, 18) and Mycobacterium phlei (19–29). The proposed pathway from E. coli, where the primary menaquinone has a 40 carbon isoprene chain (MK-8), is shown in Fig. 2. Chorismate, derived from the shikimate pathway, is initially converted into isochorismate by MenF, an isochorismate synthase (30) and then to 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) by the action of MenD, a thiamin-dependent enzyme (31, 32). SHCHC is then dehydrated by MenC to give the aromatic compound O-succinylben-
our long standing interest in the enzymology of enoyl-CoA
clustered in one region of the genome (Fig. 3). Devolving from
glutamic acid residues in prothrombin (45). However, humans
clotting, for example being involved in the
menaquinone, this compound plays an essential role in blood

MenB also catalyzed the hydrolysis of DHNA-CoA to DHNA.

Following the MenC reaction, OSB is then converted into 1,4-
dihydroxynaphthoyl-CoA synthase, MenB (27, 38, 39). It was previously thought that
cyclization reaction catalyzed by 1,4-dihydroxynaphthoyl-CoA
(37), the naphthoquinol skeleton of DHNA is generated via a

from DHNA by the actions of MenA, which catalyzes the pre-

Finally, in the last two steps of the pathway, menaquinone is synthesized
from DHNA by the actions of MenA, which catalyzes the pre-

Based on the premise that menaquinone biosynthesis is es-

Materials—M. tuberculosis genomic DNA was obtained from the TB
Research Materials Facility at Colorado State University.

Cloning, Overexpression, and Purification of MenB from M. Tuberculosis—Rv0548c, the gene encoding the putative 1,4-dihydroxynaphthoyl-CoA synthase (MenB) from M. tuberculosis, was obtained by PCR
from genomic DNA and cloned into the pET-15b plasmid (Novagen). Use of the NdeI and XhoI restriction sites placed the menB gene in-frame with an N-terminal His tag sequence. Protein expression was performed using BL21(DE3)/pLyS3 cells. Following growth in 800 ml of
LB containing 0.2% ampicillin and 25 mg/ml of kanamycin, an
isopropyl-1-thio-β-D-galactopyranoside for 12 h at 37 °C, cells were harvested
by centrifugation (5,000 rpm for 5 min at 4 °C), resuspended in
10 ml of His-binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM
Tris-HCl, pH 7.9) and lysed by 5 passages through a French Press cell
(12,000 psi). Cell debris was removed by centrifugation at 35,000 rpm
for 90 min at 4 °C, and the clarified supernatant was passed through a
column containing 3 ml of His-bind resin (Novagen). The column was
washed with 50 ml of His-binding buffer and 30 ml of wash buffer (60
mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) before MenB was
subsequently eluted using 20 ml of elution buffer (0.5 mM imidazole, 0.5
M NaCl, 20 mM Tris-HCl, pH 7.9). Fractions containing MenB were
pooled and the imidazole immediately removed by chromatography on
G-25 resin using 20 mM NaH2PO4, 0.1 M NaCl, pH 7.0, as the elution
buffer. The concentration of MenB was determined by measuring the
absorption at 280 nm using an extinction coefficient of 41,370 M−1 cm−1
calculated from the primary sequence.

Coupled Assay for MenB—OSB-CoA, the substrate for MenB, is unstable and decomposes relatively rapidly to OSB ariodlactone (Fig. 4). Consequently, we used a coupled assay with MenE, the preceding
enzyme in the pathway, to synthesize OSB-CoA in situ. The menE gene from E. coli was amplified by PCR and ligated into a pET-15b plasmid
in-frame with an N-terminal His tag sequence using the NdeI and XhoI
restriction sites. Following overexpression in BL21(DE3)/pLyS3 cells, MenE was purified by His tag affinity purification on a Ni-ni-
lar protocol as described for MenB. The concentration of MenE was
determined as described for MenB, using an extinction coefficient of 104,770
M−1 cm−1. Coupled assays contained OSB (130 μM), ATP (130 μM), CoA
(2–130 μM), MenE (2 μM), and MenB (0.14 μM). The formation of the
MenB product was monitored at 392 nm using a CARY-100 spectropho-
tometer. Reactions were initiated by the addition of MenB following
preincubation of the other reactants for 10 min to allow complete
conversion of OSB to OSB-CoA. All kinetics were performed at 25 °C.
Product formation was also monitored using reversed-phase HPLC.

Reaction mixtures were analyzed using a Vydc C18 analytical column
and running a gradient of 0–40% buffer B over 40 min at 1 ml/min.
Buffer A was 50 mM NH4CH3COO, pH 5.9 while buffer B was 100% methanol.

Crytalization and Structure Solution—Wild-type MenB was crys-
tallized using the hanging drop vapor diffusion method, equilibrating a
mixture of 1λl of protein solution and 1λl of reservoir solution containing
0.5–2% dioxane, 1.2–1.3 M (NH4)2SO4, and 0.1 M MES pH 6.5
an excess of reservoir solution. Crystals were soaked in reservoir
solution containing 30% glycerol and subsequently cryo-cooled in
liquid nitrogen. Data were collected at beamline X26C at the National
Synchrotron Light Source at Brookhaven National Laboratory,
equipped with an ADSC Quantum 4 detector. Diffraction data were
indexed, integrated, and scaled using the HKL software (50). Crystals
belong to space group P21 with a = 90.4 Å, b = 139.0 Å, c = 142.0 Å, and β = 97.3°. and contain 2 (αl)p hexamers per asymmetric unit (asu).
Dynamic light scattering suggests that MenB is a hexamer in solution.
The self-rotation function of the diffraction data is compatible with two
hexamers of 32 point symmetry in the asu. The structure was solved by

1 The abbreviations used are: OSB-CoA, O-succinylbenzoyl-coenzyme A; AA-CoA, acetacetyl-Coenzyme A; DHNA-CoA, 1,4-dihydroxy-2-naphthoyl-CoA; rms, root mean square; asu, asymmetric unit; NCS, non-crystallographic symmetry; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography.

Fig. 1. Oxidized and reduced forms of ubiquinone and menaquinone.
molecular replacement using the programs BEAST (51) and COMO (52) for the rotation and translation search, respectively and the structure of the hexamer of enoyl-CoA hydratase (PDB code 1DUB) as a search model. The presence of two hexamers per asu allowed 12-fold density averaging with the program DM (53) to extend phases from 4 Å to 1.8 Å resolution. The main chain of various loops and the C-terminal residues 270–314 were rebuilt using the program O (54). Side chains from enoyl-CoA hydratase were replaced with those from MenB using Swiss-Pdb Viewer (55) and fitted into the electron density using the program O. 12-fold non-crystallographic symmetry (NCS) restraints were maintained throughout the entire refinement process using REFMAC (56, 57). The tightness of constraints was chosen to minimize the free $R$-value. The average root mean square deviation of equivalent main chain and side chain atoms of NCS-related subunits is 0.05 Å and 0.30 Å, respectively.

MenB crystals were soaked with AA-CoA at a concentration of 2.2 mM. Crystals were cryo-protected and data were collected as described above. The soaked crystals were isomorphous to the unsoaked MenB crystals, allowing difference Fourier methods to be used for the calculation of electron density maps. AA-CoA was modeled into the density and refined at an occupancy of 0.75.

Preparation and Kinetic Assays of MenB Mutants—QuikChange mutagenesis (Stratagene) was used to generate the R133A, Y287F, D185N and D192N MenB mutants. Following DNA sequencing, the mutant enzymes were overexpressed and purified using the method described for wild-type MenB. Subsequently, the coupled assay with MenE was used to assay each of the mutant MenB proteins.

RESULTS

Enzymatic Activity of M. tuberculosis MenB—Wild-type MenB was assayed using a coupled assay in which OSB-CoA, the substrate for MenB, was generated in situ by the action of MenE on OSB, ATP and CoA (Fig. 2). Sufficient MenE was included in reaction mixtures so that OSB-CoA was formed from OSB and CoA rapidly enough to preclude significant decomposition of OSB-CoA to the spirodilactone prior to the addition of MenB. In the presence of MenB, UV-visible absorption scans revealed the time-dependent formation of a species with $\lambda_{max}$ at 392 nm that corresponded to a new peak upon HPLC analysis eluting at 39.7 min, while OSB, ATP, and CoA eluted at 6.7, 5.1, and 21.6 min, respectively. The product peak obtained by HPLC was characterized by two absorption bands with $\lambda_{max}$ values at 260 and 392 nm, consistent with the presumption that it was 1,4-dihydroxy-2-naphthoyl-CoA (DHNA-CoA). Assuming that only CoA absorbed at 260 nm, the observed product absorbance ratio $A_{260}/A_{392}$ of 4 allowed the estimation of an extinction coefficient at 392 nm of 4,000 M$^{-1}$ cm$^{-1}$ for the product. Using this extinction coefficient, wild-type MenB had $k_{cat}$ and $K_m$ values of 14.9 min$^{-1}$ and 7.3 $\mu$M, respectively, assuming that the concentration of OSB-CoA in the reaction mixture was equal to the limiting MenE substrate (CoA). To confirm that the product of the MenB-catalyzed reaction was indeed DHNA-CoA, a reaction mixture in 20 mM ammonium acetate (pH 7) was analyzed using ESI-MSMS before and after the addition of MenB. Mass spectrometry (M$^+$H$^+$) revealed the MenB-dependent formation of a single species with a mass of 954.1 Da consistent with the
mass expected for DHNA-CoA (M’H for C_{32}H_{42}N_{7}O_{19}P_{3}S = 954.14691 Da).

Crystal Structure of MenB—MenB crystals belong to space group P2₁ with unit cell dimensions: a = 90.4 Å, b = 139.4 Å, c = 142.0 Å, and β = 97.3°. Initial phases were calculated by molecular replacement using enoyl-CoA hydratase (Ref. 58, PDB code 1DUB) as a search model, and the structure of MenB was refined at 1.8 Å. The model has good overall stereochemistry with 91% of all residues in the most favorable regions of the Ramachandran diagram and 9% in additionally allowed, generously allowed, and disallowed regions of the Ramachandran diagram, as defined by the program PROCHECK (59).

### Table I
Data collection and refinement statistics

|                                | Native | Acetoacetyl CoA |
|--------------------------------|--------|-----------------|
| **Space Group**                | P2₁    | P2₁             |
| **Unit cell dimensions**       | a, b, c (Å) | 90.4, 139.4, 142.0 | 90.4, 139.4, 142.0 |
| **Resolution limits (Å)**      | 90.0, 97.3, 90.0 | 90.0, 97.3, 90.0 |
| **Completeness (%)**           | 50.0–1.80 Å | 50.0–2.30 Å |
| **R_{free}**                   | 97.9   | 99.7           |
| **Ramachandran Statistics**    | 0.073 (0.506) | 0.116 (0.551) |
| **Refinement**                 | 19.6 (1.8) | 9.8 (2.0)       |
| **Number of observed reflections** | 1,210,072 | 503,676       |
| **Number of unique reflections** | 312,176 | 146,353       |
| **Number of protein/cofactor atoms** | 25,847 | 26,111       |
| **Number of waters**           | 2,673  | 443            |
| **R_{free} (R_{free})**        | 0.195 (0.218) | 0.203 (0.243) |
| **Bond lengths (Å)**           | 0.018  | 0.015          |
| **Bond angle (°)**             | 1.6    | 1.5            |
| **Ramachandran Statistics**    | 91.09/0.00/0.00/0.0 | 91.4/8.6/0.0/0.0 |

AUH protein, in which the C terminus protrudes away from the core domain and covers the active site of an adjacent subunit within the trimer (Fig. 6C). MenB is the first structurally characterized superfamily member that uses its C terminus to cross over the trimer-trimer interface, forming part of the active site of a subunit on the opposing trimer (Fig. 6, A and B). Regardless of the way the C termini of these proteins fold, helices α9 and α10 are in equivalent positions with respect to the active site, and contain residues that are conserved across the superfamily and interact with the CoA moiety of their respective substrates. A minor variation is a helix in methylmalonyl CoA decarboxylase (equivalent to α9 in MenB) that is pushed out toward the trimer interface.

A smaller difference between MenB and other crotonase superfamily structures concerns the disordered region spanning residues 108–125 in MenB located near its active site. Throughout the crotonase superfamily, this region shows variability in sequence and structure (Fig. 7). In enoyl-CoA hydratase, this region has been predicted to be important in controlling the relative reaction rate of varying substrates by reorienting itself depending on the size of the substrate’s alkyl chain (67). In MenB the substrate does not contain an alkyl chain, and the function of this region is unclear.

MenB in Complex with Acetoacetyl-CoA—Initially, MenB

![Fig. 5. Two orthogonal views of the MenB hexamer. A, top view. The 3-fold axis is perpendicular to the paper. B, side view. The 3-fold axis is parallel to the paper. The three subunits of the upper trimer are colored in green, orange, and violet; the three subunits in the lower trimer are colored in yellow, blue, and red. This figure and Figs. 6, 8, and 9 have been generated with the programs MOLSCRIPT (71) and RASTER3D (72).](http://www.jbc.org/content/42355/1/42355/F5.large.jpg)
structures were soaked with DHNA-CoA, the product of the MenB reaction (Fig. 2). Difference density was observed only for the CoA moiety in the active site, but not for the DHNA moiety. We then soaked MenB crystals with AA-CoA and solved the structure of the complex at 2.3 Å resolution. Both DHNA-CoA and AA-CoA contain a β-keto-thioester (the (C=O)CH2(C=O)S fragment), but AA-CoA lacks the bicyclic aromatic ring of DHNA. The structure reveals that AA-CoA binds to MenB in a similar orientation as seen in enoyl CoA hydratase and other crotonase family members. The binding cleft is lined on one side by the residues C-terminal of Asp-277 of a subunit located on the opposing trimer (Fig. 8).

MenB binds to AA-CoA through hydrogen bonds, salt bridges, and hydrophobic interactions (Fig. 8). The adenine moiety is sandwiched between Phe-299 and Ala-60. In the unliganded structure, the distance between these residues varies significantly in the 12 copies of the asu (the average Ca-Ca distance is 15.0 Å, with values ranging from 14.5 Å to 15.7 Å). In the AA-CoA MenB complex, this distance is smaller and less variable (the average distance is 14.5 Å, with values ranging from 14.2 Å to 14.7 Å). Superposition of the unliganded and AA-CoA bound structure shows that the C-terminal helix α10 (residues 290–301) is displaced by up to 1.1 Å in the direction of the substrate binding pocket (Fig. 9A). In both the AA-CoA-bound and the unliganded structure, the C-terminal residues have the highest mobility as indicated by high B-factors (in the unliganded structure, the average B-factor for all atoms in residues 271 through 314 is 53.4 Å², compared with 30.9 Å² in the remainder of the molecule). A comparison of the MenB/AA-CoA structure with the enoyl CoA hydratase/AA-CoA structure reveals that the adenine is sandwiched tighter in the enoyl CoA hydratase with a distance between the Ca atoms of Phe-279 and Ala-60 (corresponding to Phe-299 and Ala-60 in MenB) of 13.2 Å and a distance of 4.1 Å between Phe-279 and C5 of the adenine base. In MenB, Phe-299 is positioned too far away from the adenine (the distance varies from 4.2 to 5.3 Å in the 12 active sites present in the asu, with an average value of 4.8 Å) to interact productively. However, the phenylalanine is strictly conserved, suggesting that in the MenB complex with OSB-CoA, Phe-299 moves closer to the adenine.

The hydrogen bonds to AA-CoA involve only main chain atoms of MenB. The thioester oxygen of the mercaptoethylamine unit hydrogen bonds to the nitrogen atoms of the strictly conserved residues Gly-105 and Gly-161, whose equivalent residues Ala-98 and Gly-141 in enoyl CoA hydratase form what is referred to as the oxyanion hole (49). Furthermore, Gly-105 O and Gln-107 N interact with N6 and N1 of the adenine ring, respectively. The conserved positively charged residues Arg-58 and Lys-302 are in close proximity to the negatively charged phosphates of CoA. The indole ring of Trp-57 engages in hydrophobic interactions with the dimethyl group of the pantothenate unit. The electron density observed for the acetoacetyl moiety is weak except for the thioester carbonyl. This apparent flexibility of the acetoacetyl moiety might be related to the differences between the real substrate/product and AA-CoA.

Apart from the movement of the C-terminal helix described above, the main chain of MenB undergoes no significant change upon binding of AA-CoA; the rms deviation of all Ca atoms is 0.21 Å. Nevertheless, there is a notable change in the side chain conformation of Tyr-287, which points away from the binding pocket in the structure of unliganded MenB. Upon binding of AA-CoA, the side chain of Tyr-287 swings toward Ser-190 in the active site pocket in 2 out of 12 subunits, maintains the conformation observed in the unliganded structure in 3 subunits and becomes disordered (with difference density for the side chain observed in both locations) in the remaining 7 subunits. The two conformations are shown in Fig. 8. A conformational change of the Tyr-287 side chain toward Ser-190 places the phenol oxygen group in closer proximity to the active site.

Location and Properties of the Active Site Pocket—Where does the aromatic ring of MenB’s substrate OSB-CoA bind? We have mapped the conservation of MenB sequences of different organisms on the structure of M. tuberculosis MenB (Fig. 9B). The highest degree of conservation on MenB’s surface is observed in a deep pocket next to Gly-161. This pocket lies in between a loop in extended conformation (residues 190–192) and three helices (Fig. 8). Residues Leu-134, Ile-136, and Leu-137 of one helix as well as Gly-280, Thr-283, and Tyr-287 from a helix of the neighboring subunit form a hydrophobic lining on one side of the pocket, while the other side of the pocket contains the charged residue Asp-192. A calculation of the electrostatic surface potential of MenB shows that the bottom of the pocket is negatively charged, while the sides are positively charged (Fig. 9C). The positive charge is not due to basic residues, but rather due to the dipole of the helix spanning residues 161 to 168. This positive charge is located near the strictly conserved Gly-161 of the oxyanion hole. The loop that is disordered in the MenB crystals is located above the active site pocket. If ordered upon substrate binding, this loop could potentially obstruct access to the pocket while the reaction proceeds. If residues of this loop come into close proximity with the substrate, they could also play a role in binding or catalysis. However, only Arg-110, Gly-111, and Gly-114 of this region are strictly conserved. Finally, comparison of the MenB structure...
with that of enoyl-CoA hydratase reveals that Asp-185 in MenB occupies a similar position to Glu-164 in enoyl-CoA hydratase. This is significant since Glu-164 is a key catalytic residue in the reaction catalyzed by enoyl-CoA hydratase (68). While it is intriguing to speculate that Asp-185 in MenB plays a similar role to that of Glu-164, we note that Asp-185 is not conserved in the MenB family, and is sometimes replaced by a glycine (Fig. 7).

Active Site Mutants Demonstrate Residues Important for Catalysis—The reaction catalyzed by MenB involves the formation of a carbon-carbon bond through an intramolecular Claisen-like condensation (formally a Dieckmann condensation). Enzymatically catalyzed Claisen condensations play an important role in fatty acid biosynthesis (69), and require that at least one of the reacting carboxylates is activated by formation of a thioester with CoA. For the reaction to proceed, the thioester has to be deprotonated, leading to an enolate intermediate. This reactive species acts as a nucleophile in a substitution at the second carboxylate, yielding a β-keto ester. Enzymatic catalysis proceeds by stabilizing the enolate anion (through hydrogen-bond donors or charged groups) and providing a good leaving group for the carboxylate (through prior reaction to a thioester with another CoA or with an active site cysteine).

The MenB active site contains only a few potential proton donors and acceptors that are strictly conserved and sufficiently close to Gly-161 (i.e. close to the enolate anion) to directly interact with the reactants. The obvious candidates are Ser-190 and Asp-192, in addition to Asp-185, although the latter residue is not conserved in the MenB family. Interestingly, there are no nearby histidines or cysteines, residues that are implicated in the condensation reactions of fatty acid biosynthesis (69). An additional candidate is Tyr-287. Its hydroxyl group points away from the active site in the unliganded structure of MenB. Upon ligand binding, however, the side chain of Tyr-287 becomes disordered in the majority of the subunits and is positioned close to the active site Ser-190 in two others (Fig. 8).

We have prepared the following point mutants of MenB to study the effects of active site residues on structure and catalytic activity of MenB: R133A, D185N, D192N, and Y287F. All mutants expressed well and were soluble. We obtained crystals of Y287F and R133A. Preliminary diffraction data (results not shown) indicate that the overall fold of the mutants has not changed in comparison to the wild type enzyme. All four MenB mutants, R133A, Y287F, D185N, and D192N, showed no activity when assayed under the same conditions as those used for wild-type MenB. R133A exhibited 20% wild-type activity when

FIG. 7. Sequence conservation of MenB. The first nine sequences are MenB sequences from nine different bacterial species. The last three are members of the enoyl CoA hydratase superfamily. Highlighted residues are discussed in the text. Residue numbers above the alignment refer to the M. tuberculosis sequence. The solid bar above residues 108–125 delineates the disordered region of MenB. For 1EF8 (methylmalonyl CoA decarboxylase) and 1DUB (enoyl CoA hydratase), residues in capital letters are structurally homologous to MenB, while lowercase letters are not. BadI is the 2-ketocyclohexanecarboxyl CoA hydrolase of the benzoate degradative pathway in R. palustris. This figure was generated with ALSCRIPT (73).

FIG. 8. Interactions between MenB and aceetoacetyl CoA. The substrate analog AA-CoA is shown in ball-and-stick representation. Yellow and green Co traces indicate MenB residues from two different subunits of the hexamer. Selected side chains are shown in all-bonds representation and are labeled (numbers of residues belonging to the green subunit are primed, e.g. Tyr-287'). Main-chain hydrogen bonds of Gly-104 through Asp-107 and Gly-161 with AA-CoA are indicated by red dashed lines. Tyr-287' is shown as observed in the free MenB structure (cyan) and in the second conformation observed in the AA-CoA complex (gray).
used at a concentration 30-fold higher than that used for the wild-type enzyme. However, no enzymatic activity was observed for D192N and Y287F, even at 5 μM enzyme concentration. The conservative exchanges in the mutants make it highly unlikely that the global structure of MenB is influenced by the mutations; our preliminary crystallographic data supports this statement. Therefore, our mutational data indicate that Asp-192 and Tyr-287 are either necessary for substrate binding or for enzymatic catalysis. Because the mutants are completely inactive rather than impaired, kinetic assays fail to distinguish between these two possibilities.

**DISCUSSION**

The structural homology of MenB with the crotonase family suggests that catalysis by MenB involves formation of a CoA thioester enolate, which is stabilized by the oxyanion hole characteristic of this class of enzymes. To understand how MenB catalyzes the ring closure of OSB, we have to identify the proton acceptor responsible for enolate formation and also consider how the benzoate is modified to contain a good leaving group. In addition, a mechanism should address how MenB prevents formation of the spirolactone to which OSB reacts in solution, but rather guides OSB into a conformation that brings the two carboxylates into close proximity.

To study the constraints enforced by the active site of MenB on the conformation of its substrate OSB-CoA, we modeled OSB-CoA into the MenB structure. We placed OSB-CoA such that the thioester of OSB-CoA hydrogen-bonds to Gly-105 N and Gly-161 N as observed in the MenB-AA-CoA complex. With this constraint, OSB is forced to position itself in its productive conformation with the benzoate carboxylate close to the thioester in order to accommodate the aromatic ring into the active site pocket. This positions the hydrophobic ring into favorable contact with Leu-134, Ile-136, and Leu-137 (Fig. 10). It is conceivable that the CoA moiety of the substrate binds first, allowing the OSB moiety to sample different conformations until the productive one is obtained. In this conformation the benzoate ring would bind tightly into the hydrophobic pocket. The flexibility of the loop (residues 108–125) above the active site cleft observed in the crystal structure would allow the OSB moiety of the substrate to approach from above after its CoA

![Fig. 10. The proposed mechanism for the reaction catalyzed by MenB.](image-url)
moity has already been tethered to MenB.

How might Asp-192 and Tyr-287 function in MenB to help formation of the enolate anion and provide a good leaving group for the benzoyl? It is possible that these residues interact with the keto group of the substrate making them necessary for substrate binding rather than catalysis. In this respect, it is interesting to note that both Tyr-287 and Asp-192 are conserved in the ring cleavage enzyme BadI. This 2-ketocyclohex-
ane carboxyl-CoA hydrolase is part of the benzoyl degradation pathway in Rhodopseudomonas palustris (70). It essentially catalyzes the back-reaction of the ring closure catalyzed by MenB; however, its product does not have an additional keto functionality like the substrate of MenB.

Based on our findings, we suggest a plausible mechanism that will be investigated in future experiments (Fig. 10). In the reaction catalyzed by MenB, the aliphatic OSB carbonyl is activated by formation of a CoA ester. However, unlike the standard enzymatic Claisen reaction, the electrophile in the reaction is presumably the carbonyl group of the unactivated aromatic OSB carbonyl. Based on model building, we propose a mechanism in which the active site positions the OSB succinyl α-carbon in close proximity to the aromatic carbonyl as a prelude to nucleophilic attack. The carbonyl group is then ideally positioned to abstract the α-proton resulting in a protonated carbonyl and the required resonance-stabilized carbanion. This step is rapid given the observation that the enzyme activated by formation of a CoA ester. However, unlike the standard enzymatic Claisen reaction, the electrophile in the reaction is presumably the carbonyl group of the unactivated aromatic OSB carbonyl. 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Crystal Structure of \textit{Mycobacterium tuberculosis} MenB, a Key Enzyme in Vitamin K\textsubscript{2} Biosynthesis

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