Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

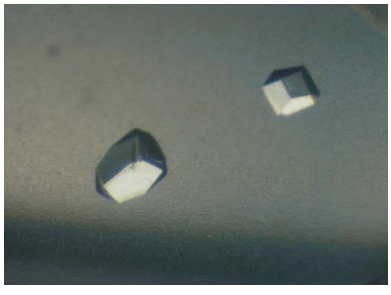
Crystallization and preliminary X-ray analysis of (1*R*,6*R*)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase (MenD) from *Escherichia coli*

Edyta A. L. Sieminska, Andrea Macova, David R. J. Palmer and David A. R. Sanders*

Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5C9, Canada

Correspondence e-mail: david.sanders@usask.ca

Received 19 February 2005
Accepted 7 April 2005
Online 22 April 2005



(1*R*,6*R*)-2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase, also called MenD, participates in the menaquinone (vitamin K_2) biosynthetic pathway. The enzyme is a part of the superfamily of ThDP-dependent enzymes; however, it is the only enzyme known to catalyze a Stetter-like 1,4-addition of a ThDP adduct to the β-carbon of an unsaturated carboxylate. This is the first reported crystallization of the apoenzyme and holoenzyme forms of MenD. The apoenzyme crystals were obtained by sitting-drop vapour diffusion with 70% MPD. However, the crystals were too small to collect diffraction data and a search for better conditions was not successful. Single crystals of the holoenzyme with ThDP and Mn^{2+} as cofactors were obtained by the hanging-drop vapour-diffusion method with 35% ethylene glycol as precipitant. Diffraction data were collected on a cryocooled crystal to a resolution of 2.0 Å at BioCARS, Advanced Photon Source (APS), Chicago, IL, USA. The crystal was found to belong to space group $P2_12_12_1$, with unit-cell parameters a = 106.86, b = 143.06, c = 156.85 Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

MenD (EC 2.5.1.64), or 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase, belongs to the superfamily of thiamin diphosphate (ThDP) dependent decarboxylases, which includes pyruvate decarboxylase, benzoylformate decarboxylase, indolepyruvate decarboxylase, pyruvate oxidase and acetohydroxy acid synthase (AHAS; Bhasin *et al.*, 2003). It is the second enzyme in the menaquinone (vitamin K_2) biosynthetic pathway (Meganathan, 1996), which converts isochorismate and 2-oxoglutarate to SHCHC, pyruvate and carbon dioxide as shown in Fig. 1. Menaquinone, and therefore MenD, is required for the anaerobic growth of bacteria and plays a role in the electron-transport system (Meganathan, 1996). In the widely accepted mechanism of MenD proposed by Emmons *et al.* (1985), ThDP is deprotonated at carbon 2 of the thiazolium ring and the resulting ylide attacks the carbonyl carbon of 2-oxoglutarate. The tetrahedral intermediate formed is decarboxylated, resulting in a carbanion/enamine intermediate which in turn acts as a nucleophile in attacking the β-carbon of isochorismate. The subsequent loss of ThDP from the complex results in the formation of a ketone and the abstraction of the α-proton results in the δ-elimination of pyruvate to give SHCHC and carbon dioxide. Thus, the initial steps of the MenD-catalyzed reaction closely resemble those of its homologues, but it is the only member of this group of decarboxylases known to catalyze the addition of a ThDP adduct to the β-carbon of a second substrate. The closest non-enzymatic analogue of this reaction is the Stetter reaction (Stetter, 1975), which resembles a Michael-type variation of the benzoin condensation (Kerr & Alaniz, 2002). The high-resolution structure of MenD should provide insight into how an enzyme can control the fate of the anionic ThDP adduct.

MenD is a dimer in solution with a molecular weight of 1.4×10^5 Da for the hexahistidine-tagged protein (Bhasin *et al.*, 2003). The protein is absolutely dependent on the presence of ThDP and a divalent metal ion such as Mg^{2+} or Mn^{2+} for activity, with the maximum reaction rate observed in the presence of Mn^{2+} at pH 8.5 in Tris–HCl buffer. As with most of the family members, MenD also exhibits a lag phase or slow approach to the zero-order steady-state and shows cooperativity with respect to both substrates (Bhasin *et al.*,

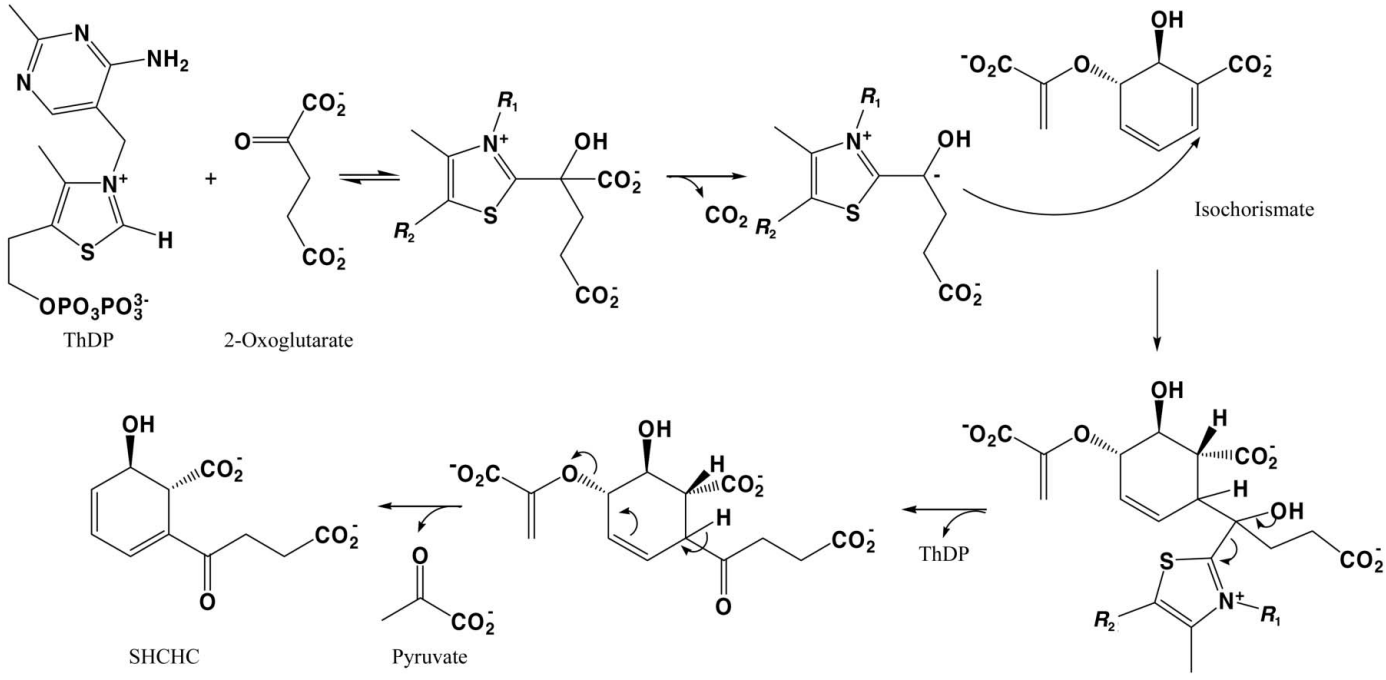


Figure 1
Reaction sequence and proposed mechanism for the synthesis of SHCHC catalyzed by MenD. R_1 = 4′-aminopyrimide; R_2 = $-CH_2CH_2OPO_3PO_3^{3-}$.

2003). Alignment of the MenD sequence with other structurally characterized family members indicates overall divergence in sequence. Although MenD is a member of the ThDP-dependent enzyme family, its sequence homology is only 14% (Bhasin *et al.*, 2003) when compared with its nearest neighbour AHAS (PDB code 1t9d), which also exhibits decarboxylase and carboligase activity (Pang *et al.*, 2002).

2. Protein overexpression and purification

2.1. Cloning and overexpression

The *menD* gene was PCR-amplified from *Escherichia coli* K12 MG1655 genomic DNA using primers with *Xho*I and *Bam*HI restriction sites and subsequently ligated into the pET14b vector (Novagen). The resulting construct was named pMD14 and used to transform *E. coli* BL21(DE3) cells. Growth took place on LB plates in the presence of ampicillin (100 µg ml^{-1}). Single colonies were selected and grown overnight in LB medium. The starter culture was used to inoculate a 4 l culture. The cells were grown overnight at 310 K. No induction with isopropyl-β-D-thiogalactopyranoside (IPTG) was required in order to obtain large quantities of the N-terminal hexahistidine fusion protein. The cells were harvested by centrifugation (20 min, 8000*g*, 277 K) and the pellets were stored at 193 K until further use.

2.2. Purification

MenD protein was purified as described by Bhasin *et al.* (2003) with minor modifications. The thawed cells were lysed by sonication on ice in chilled sonication buffer (20 m*M* Tris–HCl pH 8.0, 1 m*M* AEBSF, 20 µg ml^{-1} DNase and 20 µg ml^{-1} lysozyme). Sonicated material was centrifuged (20 min, 18 000*g*, 277 K) and the lysate precipitated using 10%(*v*/*v*) ammonium sulfate. The lysate was further filtered with a PALL Acrodisc Syringe Filter and applied directly onto a 5 ml Chelating Sepharose FF column from Amersham Biosciences, which had been charged with ten column volumes of 50 m*M* $NiSO_4$. Excess $NiSO_4$ was removed by washing the column with five column volumes of H_2O and five column volumes of binding buffer (5 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). The injection of the sample was followed by five column volumes of binding buffer and ten column volumes of wash buffer (60 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). The elution of protein was achieved by running a gradient over 15 column volumes starting with 100% wash buffer and ending with 70% wash buffer and 30% strip buffer (0.1 *M* EDTA, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). 10 ml fractions were collected and those showing >98% purity were pooled and dialyzed against a crystallization buffer containing 20 m*M* Tris–HCl, 200 m*M* NaCl and 10% glycerol pH 8.0. About 400 mg of highly pure protein was obtained from 4 l culture, concentrated to 40 mg ml^{-1} and stored at 193 K until use.

2.3. Selenomethionine variant

The pMD14 construct was also used to transform methionine-auxotrophic *E. coli* B834(DE3) cells. Growth took place on LB plates in the presence of ampicillin (100 µg ml^{-1}). Single colonies were selected and grown overnight in LB medium (15 ml) as a starter culture. The starter culture was centrifuged for 20 min at 4150*g* and 277 K. The supernatant was removed and the pellet was resuspended in 10 ml minimal media [7.5 m*M* $(NH_4)_2SO_4$, 8.5 m*M* NaCl, 55 m*M* KH_2PO_4, 100 m*M* K_2HPO_4, 20 m*M* glucose, 1 m*M* $MgSO_4$, 1 mg l^{-1} $CaCl_2$, 1 mg l^{-1} $FeSO_4$, 2 mg l^{-1} of the microelements $ZnSO_4$, $MnCl_2$ and $CuCl_2$, 20 mg l^{-1} biotin and 1 mg l^{-1} thiamine) and used to inoculate 1 l of the same media enriched with 50 mg L-selenomethionine. The culture was grown at 310 K for 24 h and the cells were harvested by centrifugation (20 min, 8000*g*, 277 K). The purification procedure was followed as for the native protein, with one modification being the addition of 1 m*M* Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as a reducing agent to all buffers used in the purification and storage of the variant protein. 3 mg selenomethionine MenD were obtained and stored in the crystallization buffer at 193 K. Selenomethionine incorporation was determined by PS MALDI MS–TOF performed on an Applied BioSystems Voyager-

DE STR mass spectrometer (Plant Biotechnology Institute, Saskatoon). The shift in weight observed between the native protein and the variant was 473 Da, confirming 100% incorporation of selenomethionine into MenD, which was predicted to contain ten methionine residues.

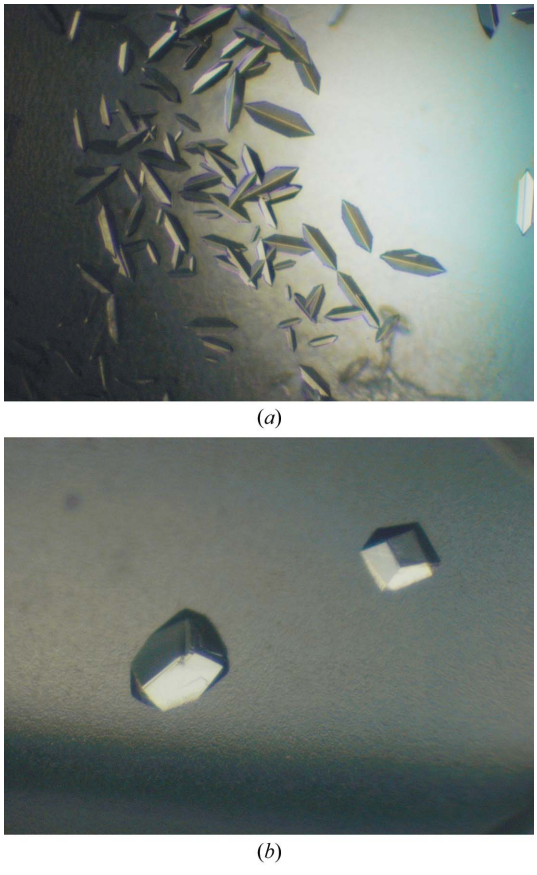
3. Crystallization and data collection

3.1. Crystallization

Initial screening was carried out by the sitting-drop vapour-diffusion method using Crystal Screen and Crystal Screen 2 from Hampton Research. CrystalClear strips (Douglas Instruments) were used to set up 4 µl drops containing 2 µl protein solution and 2 µl well buffer and additives at 293 K. The concentration of protein was 40 mg ml^{-1} for native protein and 30 mg ml^{-1} for the selenomethionine variant.

3.1.1. MenD apoenzyme. MenD apoenzyme single crystals (Fig. 2*a*) were obtained in 1–2 d from conditions consisting of 4 µl sitting drops comprising 1.6 µl well buffer (0.1 *M* HEPES, 70% MPD pH 7.2), 0.4 µl DDAO as an additive and 2 µl protein solution (15 mg ml^{-1} final concentration in the drop).

3.1.2. MenD holoenzyme. MenD holoenzyme crystals (Fig. 2*b*) were initially obtained in 1–2 d from 4 µl sitting drops consisting of 2 µl well buffer [20%(*v*/*v*) ethylene glycol] and 2 µl protein solution (15 mg ml^{-1} final concentration in the drop with 2 m*M* Mn^{2+} and 100 µ*M* ThDP). These conditions were further improved by the hanging-drop vapour-diffusion method in which the final concentration of the protein and cofactors was kept the same, while the concentration of the ethylene glycol in the well buffer was increased to 35%. Crystals were obtained after 7–14 d.



(*a*)

(*b*)

Figure 2
(*a*) Apoenzyme crystals. Maximum dimensions obtained 0.12 × 0. 04 × 0.06 mm. (*b*) Holoenzyme crystals. Maximum dimensions obtained 0.36 × 0.18 × 0.12 mm.

Table 1
Data-collection statistics.

Wavelength (Å)	0.90
Resolution (Å)	65.09–2.10
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 106.86, b = 143.06, c = 156.85, $\alpha = \beta = \gamma$ = 90
Total measurements	824184 (102190)
Unique reflections	137041 (18796)
V_M† (Å^3 Da^{-1})	2.3
Solvent (%)	46.8
$I/\sigma(I)$	14.0 (3.2)
Average redundancy	5.9 (5.4)
Data completeness (%)	96.6 (89.0)
R_{merge}‡ (%)	7.1 (57)

† Assuming a tetramer in the asymmetric unit. ‡ $R_{merge} = \sum I(h)_i - I(h)/\sum I(h)_i$, where $I(h)$ is the measured diffraction intensity and the summation includes all observations.

3.1.3. Selenomethionine variant of MenD holoenzyme. Crystals of the selenomethionine holoenzyme were obtained from 2 µl hanging drops consisting of 1 µl well buffer [30%(*v*/*v*) ethylene glycol] and 1 µl protein (15 mg ml^{-1} final concentration in the drop with 2 m*M* Mn^{2+} and 100 µ*M* ThDP). Crystal growth was observed after 14 d.

3.2. X-ray data collection

The MenD apoenzyme crystals were cryocooled in liquid nitrogen and screened on an in-house Bruker DX8 Proteum Diffractometer (SSSC). Unfortunately, the crystals diffracted poorly (10 Å) and the space group could not be determined.

The drop containing the holoenzyme crystals was covered with 5 µl paraffin oil to prevent cracking and deterioration of the crystals owing to oxidation and screened on the in-house diffractometer for the quality of the diffraction data. The holoenzyme crystals were found to diffract to 2.5 Å and thus were sent to the synchrotron for data collection. Data were collected at 100 K from a cryocooled crystal using an ADSC Q315 detector on beamline 14-BM-C at BioCARS, Advanced Photon Source (APS), Chicago, USA. The data collection was performed with a total oscillation range of 180°, using radiation of 0.90 Å wavelength with an exposure time of 15 s for 1° frames. The crystal-to-detector distance was 200 mm. The crystal diffracted to 2.1 Å resolution with an overall completeness of 96.6%.

The space group was indexed as $P2_12_12_1$ and the data were integrated and merged using *MOSFLM* (Leslie, 1992) and *SCALA* (Collaborative Computational Project, Number 4, 1994). The Matthews coefficient, V_M (Matthews, 1968), was calculated to be 2.3 Å^3 Da^{-1} and the solvent content to be 46.8% assuming four molecules per asymmetric unit and using the predicted molecular weight of 64 261 Da for the His-tagged monomer. Table 1 provides a summary of the collected data. The *B* factor is calculated to be 30 Å^2 and the scale factor is 75.2.

4. Structure solution

4.1. Molecular replacement

All structure-solution trials were performed using the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Molecular replacement was attempted with *PHASER*4.0 (Read, 2001) as implemented in the *CCP*4 suite. The sequence identity between MenD and other ThDP-dependent decarboxylases is very low, ranging between 9 and 14% (Bhasin *et al.*, 2003). AHAS shows

the highest sequence identity (14%); thus, the molecular replacement was performed based on monomer and dimer chain models of AHAS obtained in PDB format from the Protein Data Bank (http://www.rcsb.org/pdb; Pang *et al.*, 2002). Despite extensive testing of various resolution ranges, no solution has emerged using the search models.

Currently, structural studies are under way using the selenomethionine variant of MenD.

We thank the Saskatchewan Structural Science Centre (SSSC) for access to the in-house diffractometer and for technical support. We thank Dr Louis T. J. Delbaere from the Department of Biochemistry, University of Saskatchewan and his group members for data collection at the synchrotron. We thank Dr Andrew Ross and Doug Olson from the Plant Biotechnology Institute for the mass-spectroscopy expertise. We thank Lisa Horton and Jill Cowie for their help with purification and initial screening of crystallization conditions. Use of the Advanced Photon Source was supported by the US Department of Energy, Basic Energy Sciences, Office of Science under contract No. W-31-109-Eng-38. Use of BioCARS Sector 14 was supported by the National Institutes of Health, National Center for Research Resources under grant No. RR07707. Funding for this project was provided by NSERC Discovery grants to DARS and DRJP.

References

Bhasin, M., Billinsky, J. L. & Palmer, D. R. J. (2003). *Biochemistry*, **42**, 13496–13504.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Emmons, G. T., Campbell, I. M. & Bentley, R. (1985). *Biochem. Biophys. Res. Commun.* **131**, 956–960.
Kerr, M. S. & Alaniz, J. R. (2002). *J. Am. Chem. Soc.* **124**, 1028–1029.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Meganathan, R. (1996). *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 642–656. Washington: American Society for Microbiology.
Pang, S. S., Duggleby, R. G. & Guddat, L. W. (2002). *J. Mol. Biol.* **317**, 249–262.
Read, R. J. (2001). *Acta Cryst.* D**57**, 1373–1382.
Stetter, H. (1975). *Angew. Chem. Int. Ed. Engl.* **15**, 639.