THIAMIN DIPHOSPHATE-DEPENDENT ENZYMES AND THEIR CARBOLIGATION ACTIVITY

By

ABHILASH S CHRISTIAN

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Professor Frank Jordan

And approved by

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ABSTRACT OF THE DISSERTATION

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By ABHILASH S CHRISTIAN

Dissertation Director:

Professor Frank Jordan

Thiamin diphosphate dependent enzyme use thiamin diphosphate (ThDP) an active form of vitamin B1, as co-enzyme to carry out reactions that include cleavage of carbon-carbon, carbon-sulfur, carbon-oxygen and carbon-nitrogen bonds. The understanding of its mechanism has opened a new spectrum of green chemistry involving manipulation of enzymes to give side reactions which create chemical bonds instead of breaking them.

Herein, two different ThDP-dependent enzymes and their carboligation reactivity were studied. E1o which is a component of the oxoglutarate dehydrogenase multienzyme complex (OGDHc) and MenD, an enzyme involved in the biosynthesis reaction of vitamin K2. MenD and E1o's ability to carry out carboligation reaction of bulky aromatic and short aliphatic acceptors with substrate was studied using circular dichroism (CD). The observations using CD were made possible due to an inherent property of the activated nucleophile, prochirality. The reaction between the prochiral nucleophile and

the electrophile led to the formation of chiral products which in turn were observed by CD.

To carry out these reactions the plasmid carrying enzyme genes were first over-expressed in *E. coli* cells and purified using immobilized metal affinity chromatography chromatography (IMAC). The enzymatic reactions were carried out using 2-oxoglutarate (substrate), benzaldehyde (acceptor) and propanal (acceptor) giving their respective products 5-hydroxy-4-oxo-5-phenylpentanoic acid and 5-hydroxy-4-oxo-heptanoic acid. The reactions were also performed using a different 2-oxo-acid (substrate), 2-oxo-5-hexenoic acid with benzaldehyde (acceptor) and propanal (acceptor). The results indicated that MenD's activity was limited due to its mechanism at high substrate levels, leading to little to no product formation attributed to its ping-pong bi-bi mechanism. Moreover, MenD despite having very little sequence similarity to E1o follows a similar reaction mechanism. This opens new opportunities for MenD's substrate and acceptor spectrum, and its saturation inhibition issues which can be addressed by knocking out or substituting part of its active site residues, or the hydrophobic chains supporting it, using site-directed mutagenesis.

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Last but not the least, I would like to thank my family for standing by my side and supporting me throughout the course of time.

DEDICATIONS

This dissertation is dedicated to my family who have been a cornerstone of my success. Without their constant support I would not have been able to reach my goals.

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CHAPTER 1

INTRODUCTION

1.1 Thiamin diphosphate (ThDP) dependent enzymes

ThDP is the catalytically active form of vitamin B1, used as a cofactor by enzymes involved in a variety of pathways. ThDP-dependent enzymes are involved in a range of reactions, C-N, C-O, C-S bond synthesis and also the breakdown and formation of carbon-carbon bonds adjacent to a carbonyl group. All these reactions involve ThDP as cofactor [1]. Unlike other cofactors such as NADH (Nicotinamide adenine dinucleotide), ThDP remains bound to the enzyme throughout the catalytic cycle. The mechanism involves activation of ThDP by forming a C2-carbanion or ylide which acts as a nucleophile adding to the α-carbonyl of the α-keto-acid (substrate) [2-6]. When this ylide attacks an α-keto-acid it forms a tetrahedral intermediate that loses carbon dioxide to form the 'activated aldehyde' [7-9]. The deprotonation of the activated ylide is made favorable by the enzyme's active site conformation, where the N'-imino group is positioned next to the thiazolium ring [10]. This enzyme conformation is supported by the bulky hydrophobic residue located beneath the ring system observed in x-ray structures of all ThDP-dependent enzymes [11-15].

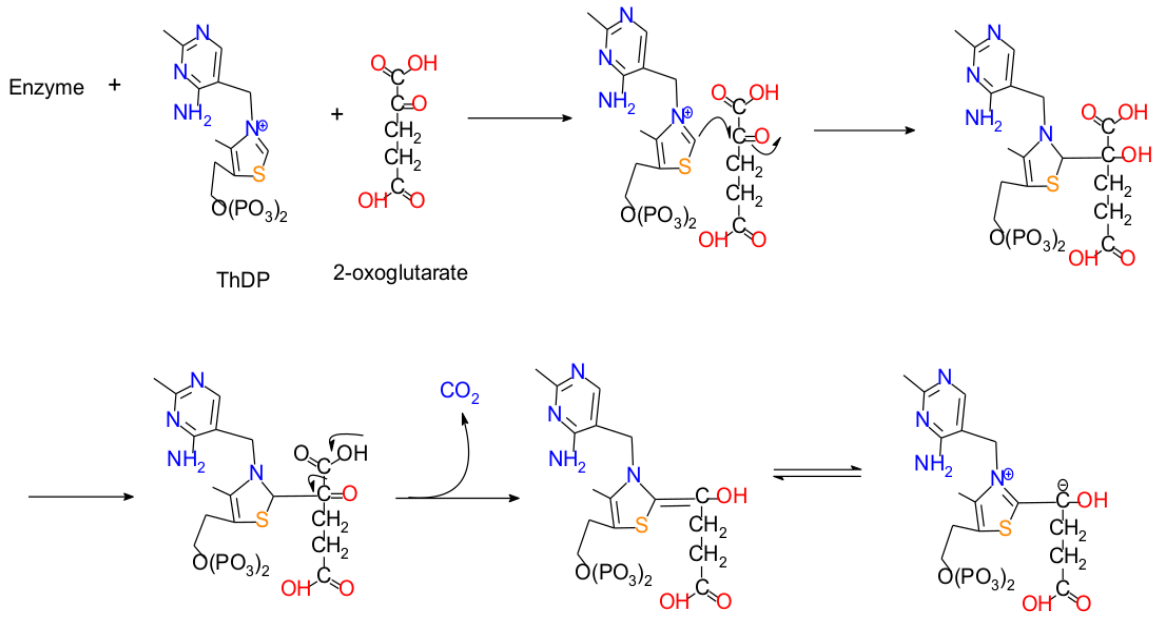

Figure 1.1 The decarboxylation step of ThDP-dependent enzymes with the formation of an activated aldehyde.

Herein, we have studied two different ThDP-dependent enzymes and their carboligation reactivity. E1o, a ThDP-dependent oxoglutarate decarboxylase (105 kDa, EC 1.2.4.2), and MenD, an enzyme involved in the menaquinone biosynthetic pathway responsible for the conversion of isochorismate to SEPHCHC (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate) (61 kDa, EC 2.2.1.9) [16,17]. Both enzymes have previously been known and extensively studied, including their crystal structure and reaction mechanism [18,19].

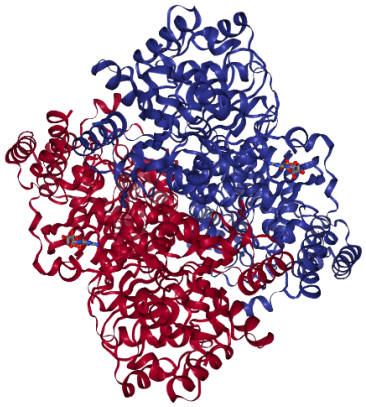

Figure 1.2 Crystal structure of ThDP-dependent E1o [18].

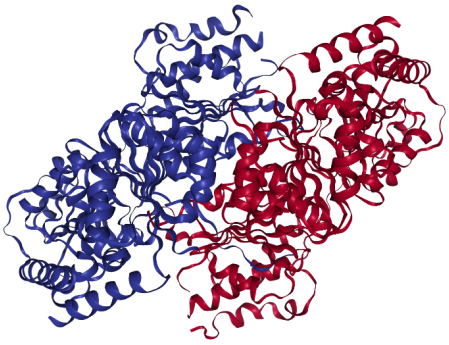

Figure 1.3 Crystal structure of ThDP-dependent MenD [19].

1.2 Thiamin diphosphate (ThDP) dependent 2-oxoglutarate decarboxylase

The 2-oxoglutarate decarboxylase (E1o) is part of the oxoglutarate dehydrogenase complex (OGDHc) a multienzyme complex of *E. coli*, part of the citric acid cycle, also known as the TCA cycle (tricarboxylic acid cycle). The primary focus of the citric acid cycle is to convert the stored energy of carbohydrates, fats and proteins into ATP (adenosine triphosphate). The OGDHc catalyzes the formation of succinyl coenzyme A (CoA) according to Equation 1.1.

The OGDHc multienzyme complex is composed of several other catalytic components alongside the 2-oxoglutarate decarboxylase. The catalytic components of OGDHc are (a) E1o, (b) A dihydrolipoylsuccinyl transferase (E2o, 45 kDa, EC 2.3.1.6) and (c) A dihydrolipoyl dehydrogenase (E3, 55 kDa, EC 1.8.1.4) [18,20-22]. The E1o and E2o component of OGDHc are involved in the reaction described in Equation 1.1, the formation of succinyl coenzyme A. Whereas, E3 reoxidizes the E2o component of OGDHc and prevents the complex from getting blocked; it could be the case if the E2o component is not re-oxidized after forming succinyl-CoA, this would prevent it from accepting another 4-hydroxybutyryl from E1o, leading to a block in the entire complex (Figure 1.4).

The E1o component of OGDHc is primarily responsible for substrate specificity and ThDP-dependent decarboxylation step leading to the formation of 4-hydroxybutyryl-ThDP.

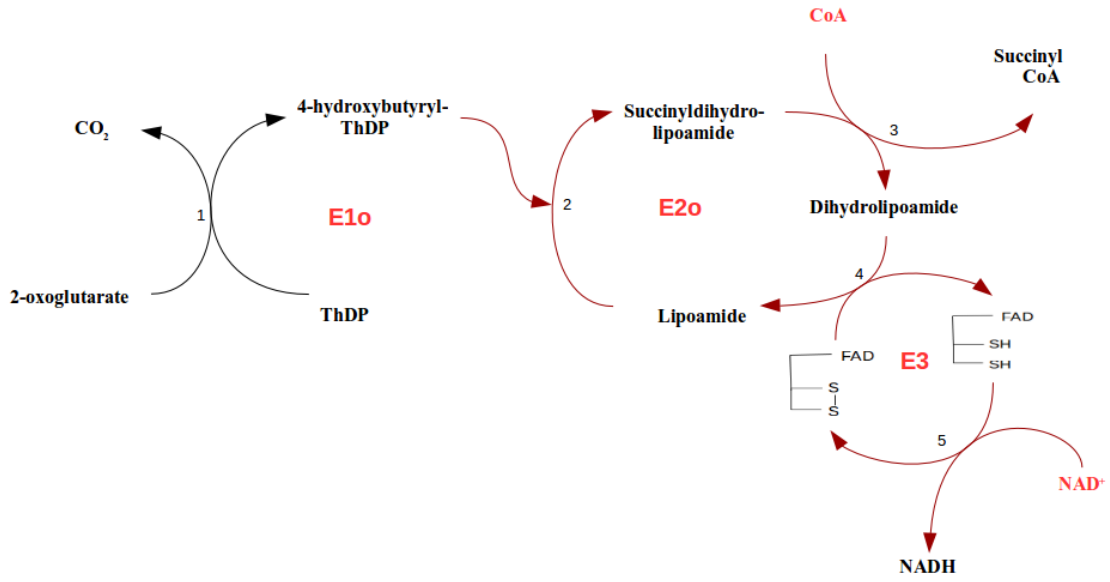

Figure 1.4 OGDHc reaction scheme.

1.3 Thiamin diphosphate (ThDP) dependent MenD

The ThDP-dependent MenD also known as, SEPHCHC synthase. MenD belongs to the decarboxylase superfamily, but is not connected by global sequence similarity to other members of the decarboxylase superfamily [23]. Among all the ThDP-dependent decarboxylases only MenD is able to carry out a "Stetter-like" 1,4 addition reaction [24].

The Stetter reactions involve the transformation of an aromatic aldehyde (substrate) with an α,β-unsaturated carbonyl compound (Michael acceptor) in the presence of an anion (eg. CN^-). MenD uses ThDP cofactor in its reaction to decarboxylate and activate the aldehyde similar to other ThDP-dependent enzymes and then carries forward the 1,4 addition of 2-oxoglutarate to isochorismate yielding SEPHCHC (Figure 1.5).

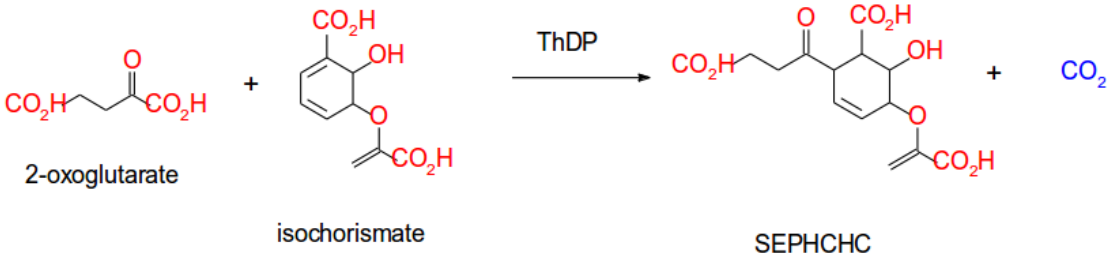

Figure 1.5 MenD's 1,4 addition reaction**.**

Unlike E1o, MenD is not part of any multienzyme complex, it does not depend on other components for its reaction completion, instead it contributes independently in a biosynthetic reaction cascade which is responsible for the synthesis of menaquinone. Moreover, its active site accepts two substrates, isochorismate and 2-oxoglutarate [16,17]. Whereas, E1o only accepts 2-oxoglutarate as its substrate. Since, MenD naturally accepts two different substrates, it comes as no surprise that its reaction mechanism is different from E1o. MenD follows a ping-pong bi-bi reaction mechanism also known as, double displacement reaction [25].

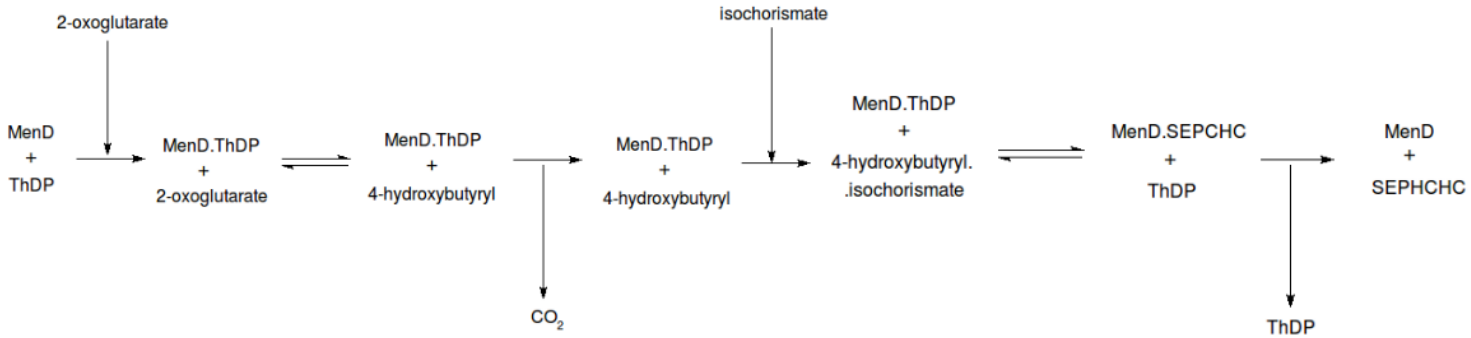

Figure 1.6 MenD's ping-pong bi -bi reaction mechanism.

1.4 Carboligation reactions

The construction of C-C bond with complete stereochemical control is of utmost importance in organic synthesis and enzyme catalyzed reactions are making an important contribution in this regard. With enzyme catalyzed reactions, enzymes control the configuration of the newly formed stereogenic center. Moreover, enzymes also catalyze C-C bond formation as an off-pathway side reaction which can occur either due to the nature of substrate used or the catalytic promiscuity of the enzyme [26-28]. The ThDP-dependent decarboxylases are important in this regard, as they not only decarboxylate the 2-oxo-acid but they also have a C-C bond forming side reaction which is stereochemically controlled [29,30].

With the ThDP-dependent enzymes the C2-atom of thiazolium ring reacts with the carbonyl carbon of 2-oxo-acid forming an intermediate. This intermediate upon decarboxylation leaves behind a highly reactive nucleophile (4-hydroxybutyryl-ThDP enamine) at the enzyme (Figure 1.1). If an electrophilic aldehyde or ketone is introduced to this highly reactive nucleophile it yields a carboligation side reaction forming a new C-C bond between the nucleophile and electrophile [18,29]. This decarboxylation activity with the formation of a reactive C2-α-carbanion/enamine complex is observed in all ThDP-dependent decarboxylases making carbon-carbon bond forming side reactions inherent to all ThDP-dependent decarboxylases [31-33].

Carboligation reactions using enzymes can yield potential building blocks owing to the streospecific nature of the enzymatic reactions. This can be exploited to get the desired type of stereogenic centers and enantiomers which find their use in pharmaceuticals, as intermediates of drug synthesis [34]. ThDP-dependent reactions can also be used to understand and treat health problems related to metabolism and/or the citric acid cycle such as, Alzheimers disease [34,35]. Synthesis of pseudoephedrine and non-pseudoephedrine isomers has also be carried out effectively by exploiting this carboligation reactivity [36]. Recently, the enzymes of the TCA cycle were recognized for their role in cellular stress and formation of ROS (reactive oxygen species) which play a critical role in Parkinson's disease [37-39].

CHAPTER 2

PURIFICATION AND ACTIVITY ASSAY FOR THIAMIN DIPHOSPHATE DEPENDENT E1o AND MenD

2.1 Introduction

To proceed with the carboligation reaction it was necessary to obtain pure and active enzyme to catalyze the reaction. Herein, the genes for E1o and MenD were over-expressed in a plasmid and then purified with modifications [24,40-42]. The purified protein was concentrated to carry out analytical scale carboligation reactions. The protein concentration was measured using the Bradford assay [43]. And the presence of active enzyme determined using a formerly established method of using an external oxidizing agent, DCPIP (2,6-Dichlorophenolindophenol).

2.2 Materials

E. coli AG1 cells containing the E1o component of OGDH complex and MenD were obtained from the National Institute of Genetics, Japan. All chemicals and salts, such as KH_2PO_4 , KCl, NaCl , $MgCl_2$, Thiamin hydrochloride, chloramphenicol, Ni-Sepharose resin, LB (Luria broth), ThDP (thaimin diphosphate), benzamidine hydrochloride, methanol, streptomycin sulfate, lysozyme, PMSF

(phenylmethylsulfonylfluoride), IPTG (Isopropyl β-D-1-thiogalactopyranoside), imidazole, BSA (bovine serum albumin), DCPIP (2,6-Dichlorophenolindophenol) were purchased from Sigma-Aldrich.

2.3 Methods

2.3.1 Expression and Purification of E1o

An *E. coli* AG1 frozen stock harboring the E1o plasmid was streaked onto Luria agar plates containing chloramphenicol (50 μg/mL) and incubated at 37 ˚C overnight. A single colony from it was used to inoculate 20 mL LB, containing chloramphenicol (50 μg/mL) and allowed to grow overnight at 37 ˚C with continuous shaking. This overnight culture was then diluted 10X into 800 mL of LB medium at 37 ˚C, containing chloramphenicol (50 μg/mL), thiamin hydrochloride (1.0 mM), and $MgCl_2$ (2.0 mM). The culture was induced with IPTG (0.8 mM) at OD_{595} = 0.5 - 0.8 and incubated at 30 ˚C, overnight with continuous shaking. The cells were centrifuged with 4,400 g at 4 ˚C, washed with KH_2PO_4 (50 mM, pH 7.5) containing NaCl (0.15 M) to remove the excess LB; the pellets were air dried and stored at -20 ˚C. All subsequent steps were carried out at 4 ˚C. The cells were resuspended in KH_2PO_4 (20 mM, pH 7.5) containing NaCl (0.2 M), $MgCl_2$ (1.0 mM), ThDP (0.2 mM), benzamidine hydrochloride (1.0 mM), PMSF (1.0 mM in methanol) and lysozyme (0.6 mg/mL) incubated on ice for 20 min, then

sonicated for 10 min (20 sec pulsar “on” and 20 sec pulsar “off”). The lysate was centrifuged with 30,000 g at 4 ˚C for 30 min. The supernatant was treated with streptomycin sulfate (0.8 % w/v), and centrifuged twice with 30,000 g at 4 ˚C for 30 min. This supernatant was then applied to a Ni-Sepharose 6 fast flow column which had been equilibrated with KH_2PO_4 (20 mM, pH 7.5), containing NaCl (0.2 M), $MgCl_2$ (1.0 mM), ThDP (0.2 mM) and benzamidine hydrochloride (1.0 mM). The lysate was washed with KH_2PO_4 (20 mM, pH 7.5) containing NaCl (0.2 M), $MgCl_2$ (1.0 mM), ThDP (0.2 mM), benzamidine hydrochloride (1.0 mM) and imidazole ranging from (30 mM – 50 mM). The protein was eluted with KH_2PO_4 (20 mM, pH 7.5) containing NaCl (0.2 M), imidazole (150 mM), $MgCl_2$ (1.0 mM), and ThDP (1.0 mM). Fractions with protein were collected and combined, then dialyzed against KH_2PO_4 (20 mM, pH 7.5) containing NaCl (0.35 M), $MgCl_2$ (1.0 mM), ThDP (1.0 mM) and benzamidine hydrochloride (1.0 mM). Next, the protein was concentrated by ultrafiltration with a cutoff of 30 kDa. The purity was confirmed using SDS-PAGE analysis.

2.3.2 Expression and purification of MenD

The cells for MenD were grown similarly to E1o described in (2.3.1) with slight modifications *E. coli* AG1 cells harboring the MenD plasmid were streaked onto Luria agar plates containing chloramphenicol (30 μg/mL) and incubated overnight at 37 ˚C. A single colony was picked and incubated in 20 mL of LB media containing

chloramphenicol (30 μg/mL) and allowed to grow overnight at 37 ˚C with shaking. This was diluted 10X into an 800 mL LB media and incubated at 37 °C with constant shaking, containing chloramphenicol (30 μg/mL), thiamin hydrochloride (1.0 mM), and $MgCl_2$ (1.0 mM). The cultures were induced with IPTG (0.5 mM) when the culture attained an OD_{595} between 0.5 - 0.8. After induction the cultures were allowed to grow overnight with constant shaking at temperature reduced to 25 ˚C. The cells were later centrifuged and collected with 4,400 g at 4 ˚C, washed with KH_2PO_4 (50 mM, pH 7.5) and KCl (0.15 M). The pellets were air dried and stored at -20 ˚C prior to sonication. The cells were resuspended in the sonication buffer containing KH_2PO_4 (50 mM, pH 7.5), KCl (0.3 M), ThDP (0.5 mM), $MgCl_2$ (2.0 mM), benzamidine hydrochloride (1.0 mM) and PMSF (1.0 mM in methanol) with lysozyme (0.6 mg/mL). The sonication and treatment with streptomycin sulfate (0.5 % w/v) was carried out similarly to E1o (2.3.1). The supernatant collected at the end was applied to a Ni-Sepharose 6 fast flow column equilibrated with sonication buffer without lysozyme and PMSF. The loaded protein was subsequently washed with increasing concentrations of imidazole (30 - 50 mM) containing, KH_2PO_4 (50 mM, pH 7.5), KCl (0.3 M), ThDP (0.5 mM), $MgCl_2$ (2.0 mM), benzamidine hydrocloride (1.0 mM) and eluted using buffer containing imidazole (150 mM). Fraction from the elution were collected and dialyzed against a dialysis buffer similarly to (2.3.1) with modified KH_2PO_4 (50 mM) concentration. The protein was concentrated using ultrafiltration with a cutoff of 30 kDa.

2.3.3 Estimation of protein concentration

The protein concentrations were estimated using the Bradford assay [43]. Bovine serum albumin (BSA) (2 mg/mL) and Bradford reagent (1X) were used to construct a standard curve of protein concentrations with spectra measured at 595 nm. The unknown protein's OD were plotted against the established standard curve providing an estimate of its concentration. This method was used to estimate protein concentration for both E1o and MenD.

The Bradford assay using the same principle was also developed for microplate with a reaction volume of 270 µL. For the microplate based assay, Bradford reagent (1X) and BSA (1 mg/mL) were used to construct a standard protein concentration curve at 595 nm.

2.3.4 DCPIP activity assay for demonstrating decarboxylation

The activity of decarboxylase was assayed using an external oxidizing agent DCPIP with measurements obtained at 600 nm, 30 ºC. Upon reduction, DCPIP undergoes a color change which is measured spectroscopically; the color change is from blue to colorless. For the activity assay with E1o, KH_2PO_4 (20 mM, pH 7.5) with $MgCl_2$ (2.0 mM) and ThDP (0.2 mM) were used as the medium which contained DCPIP (0.1 mM) and the substrate. The standard substrate for both E1o and MenD was

2-oxoglutarate (2.0 mM). The reactions were initiated using 10 μg of E1o, reaction volume were maintained at 1,000 μL. For reaction with MenD the media was modified to contain KH_2PO_4 (20 mM, pH 7.5), $MgCl_2$(2.0 mM), ThDP (0.5 mM) with DCPIP (0.08 mM) and substrate, reactions were initiated using 40 μg of MenD. All reactions were performed against a negative control which did not contain enzyme.

DCPIP activity assay was also developed on microplate for the E1o's activity. The reaction medium used for DCPIP assay in cuvette was modified to be used for the microplate assay. To determine the optimal DCPIP and ThDP concentrations, the concentration of KH_2PO_4, substrate and enzyme were kept constant, while the concentrations of DCPIP and ThDP were varied to determine the highest activity in a 200 μL reaction medium at 30 ˚C. The final reaction media which gave maximum activity contained KH_2PO_4 (20 mM, pH 7.5) with ThDP (0.5 mM), $MgCl_2$ (2.0 mM), DCPIP (0.1 mM) and 2-oxoglutarate (2.0 mM). This medium was used to determine the concentration of optimal E1o to obtain a linear curve within 1 minute of initiation, the reactions were initiated using E1o concentrations ranging from 10 μg to 50 μg and the best curve fitting for activity was determined.

2.4 Results and Discussion

2.4.1 Purification of E1o

The purification and expression of E1o was checked for each batch after concentrating the protein. The purity of protein was determined using SDS-PAGE. No extra bands except for the desired protein (Figure 2.1) confirmed the purity of the isolated protein. The concentration of protein obtained varied from batch-to-batch, with concentrations ranging from 11 mg/mL of E1o to 25 mg/mL. For best results, the protein was concentrated up to the 1,000 µL mark in the ultrafiltration tube.

Figure 2.1 Purified and concentrated E1o on SDS-PAGE (12%) with a protein ladder (left). Protein volumes 10 µL, 5 µL, 3 µL and 1 µL from right to left.

2.4.2 Purification of MenD

MenD was obtained at higher yields compared to E1o. Unlike E1o, MenD's concentration had minimum protein precipitation giving high yield of protein per batch. The purity of batches was determined in the manner similar to E1o using SDS-PAGE, as shown in Figure 2.2.

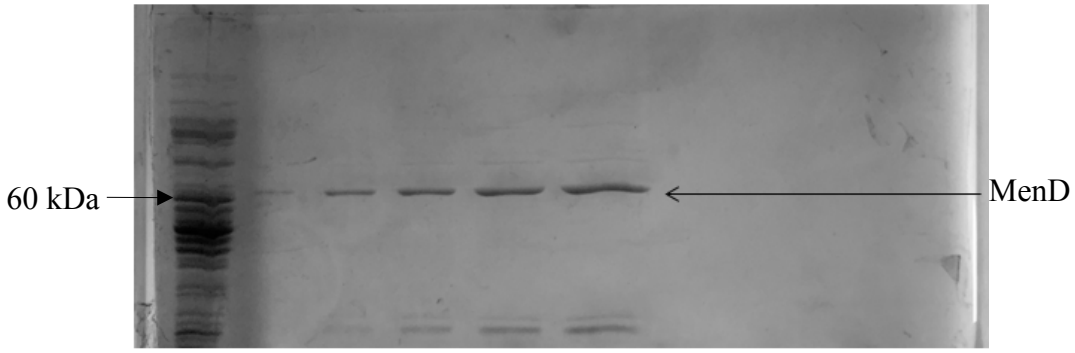

Figure 2.2 Purified and concentrated MenD on SDS-PAGE (7.5 %) with a protein ladder (left). Protein volumes 10 µL, 5 µL, 3 µL and 1 µL from right to left.

2.4.3 Bradford curve to estimate protein concentration

The standard Bradford curve was constructed on a Varian 300 spectrophotometer using BSA at different concentrations and Bradford reagent. The same standard curve was used to determine the protein concentrations of MenD and E1o. A standard curve was constructed for each batch to avoid errors from the instrument or reagents used.

The range of concentration assay was between 0.1 mg to 0.8 mg of protein, the final reaction volume 1,000 µL (cuvette). To estimate the unknown concentration, the OD_{595} from the unknown protein sample was entered into the equation, "y = mx+c." Giving "x," unknown protein concentration.

The Bradford assay was also developed for microplate on the Molecular Devices SpectraMax M2 spectrophotometer. For microplate assay, BSA (1 mg/mL) was used, with the range of assay between 5 µg to 35 µg and a final reaction volume of 270 µL.

2.4.4 DCPIP activity assay for demonstrating decarboxylation

The activity for both E1o and MenD were measured by monitoring the reduction of DCPIP dye at 600nm. One unit of activity is defined as the amount of DCPIP reduced by the enzyme (µmol/min/mg of enzyme).

The activity assay were carried out at 30 ˚C on Varian 300 spectrophotometer, with a final reaction volume of 1,000 µL (cuvette), as described in (2.3.4).

Enzyme	DCPIP Activity (µmol/min/mg of enzyme)
E1o	0.34872
MenD	0.03446

Table 1: DCPIP activity of E1o and MenD**.**

The microplate activity assay were performed at 30 ˚C on the Molecular Devices SpectraMax M2 spectrophotometer with a final reaction volume of 200 µL, as described in (2.3.4).

Concentration of E1o (mg)	DCPIP Activity (µmol/min/mg of E1o)
0.010 mg	0.01333
0.020 mg	0.00557
0.040 mg	0.00628

Table 2: Establishing enzyme concentration for E1o's DCPIP microplate assay.

A good fit for the activity curve was observed with E1o concentration of 40 µg. For concentration below 40 µg, the reduction of DCPIP was slow giving a plateau over longer time periods. For concentrations above 40 µg the reduction was rapid, leading to an increase of error margin.

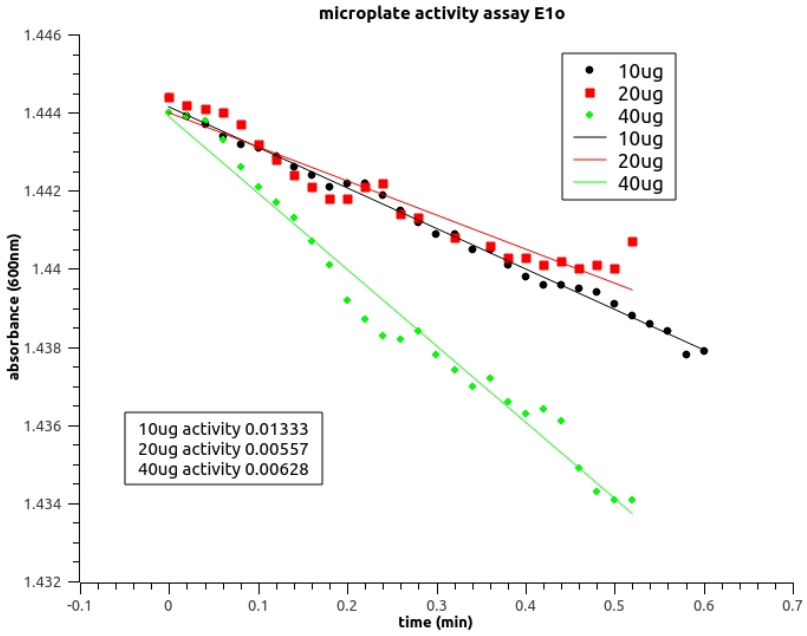

Figure 2.3 E1o's activity assay with DCPIP on microplate.

CHAPTER 3

CARBOLIGATION REACTION OF E1o AND MenD

3.1 Introduction

The ThDP-dependent enzymes have the potential to be used for carboligation reactions and chemical synthesis [31,45]. The carboligation side reactions are an outcome of the ThDP-dependent enzyme decarboxylating the substrate, when it is bound to the thiazolium ring. This decarboxylation step not only leads to the formation of an activated aldehyde, but it also helps activate the prochiral carbon of the substrate.

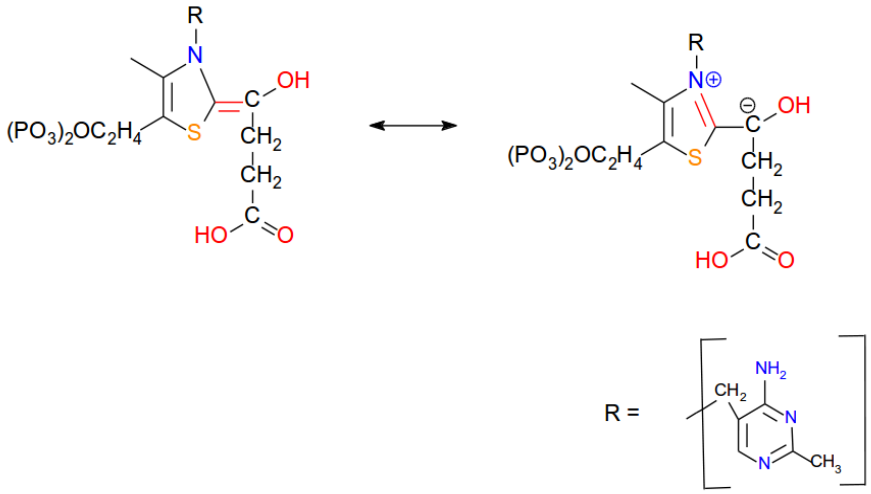

Figure 3.1 The resonance form of ThDP bound 4-hydroxybutyryl between imine (right) and enamine (left) forms.

The highly reactive 4-hydroxybutyryl complex (activated aldehyde) bound to the thiazolium ring resonates between its imine and enamine forms (Figure 3.1). If an electrophilic acceptor is introduced to this thiazolium 4-hydroxybutyryl enamine complex it leads to the formation of a new chiral center. This newly formed chiral center's stereochemistry is determined by the enzyme's active site. The orientation of the substrate and acceptor within the enzymes active site determines the phase of attack for the electrophilic acceptor, giving either the (R) or (S) enantiomer as a major reaction product.

Herein, carboligation reactions were performed for E1o and MenD using propanal and benzaldehyde as acceptors and 2-oxoglutarate as substrate. MenD is known to handle a variety of aliphatic and aromatic acceptors with formation of new carbon-carbon bonds [42]. Carboligation reactions with MenD were performed using a different 2-oxo-acid substrate, 2-oxo-5-hexenoic acid to check for the possibility of an off-pathway side reaction using an unnatural substrate.

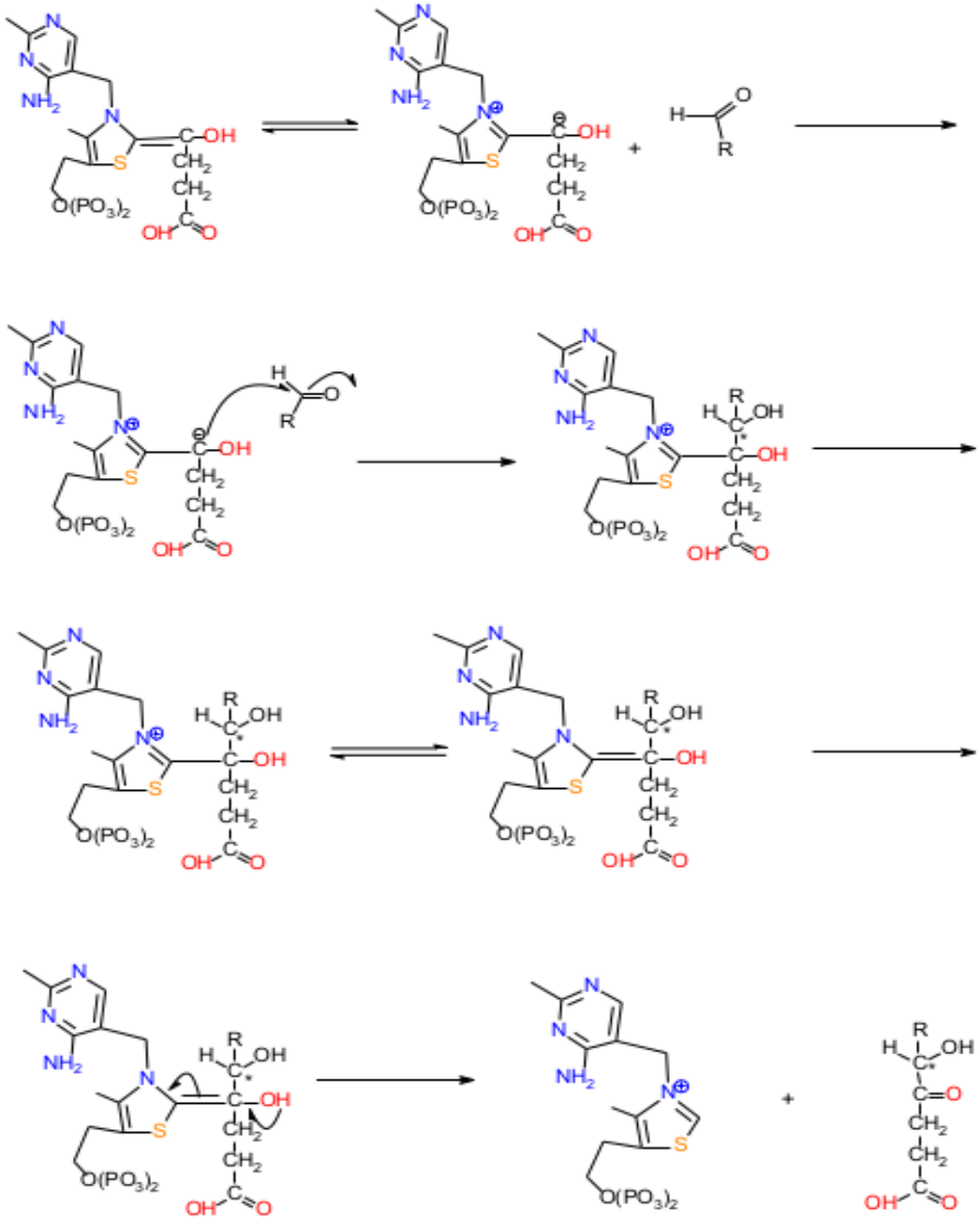

Figure 3.2 Schematic representation of the formation of a new chiral center between an activated aldehyde and electrophilic acceptor.

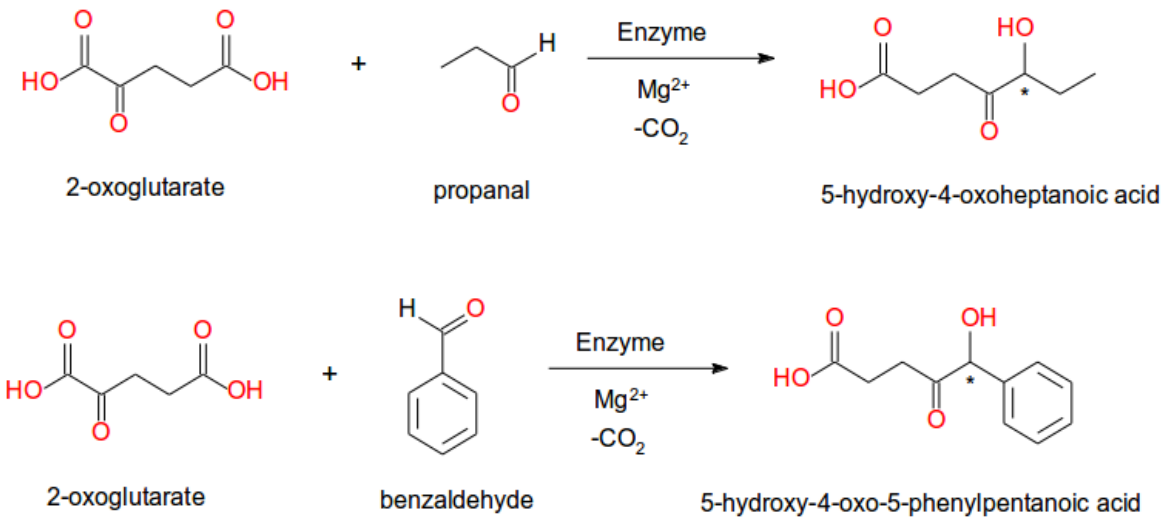

Figure 3.3 Reaction between the substrate and acceptor with the formation of a new chiral carbon.

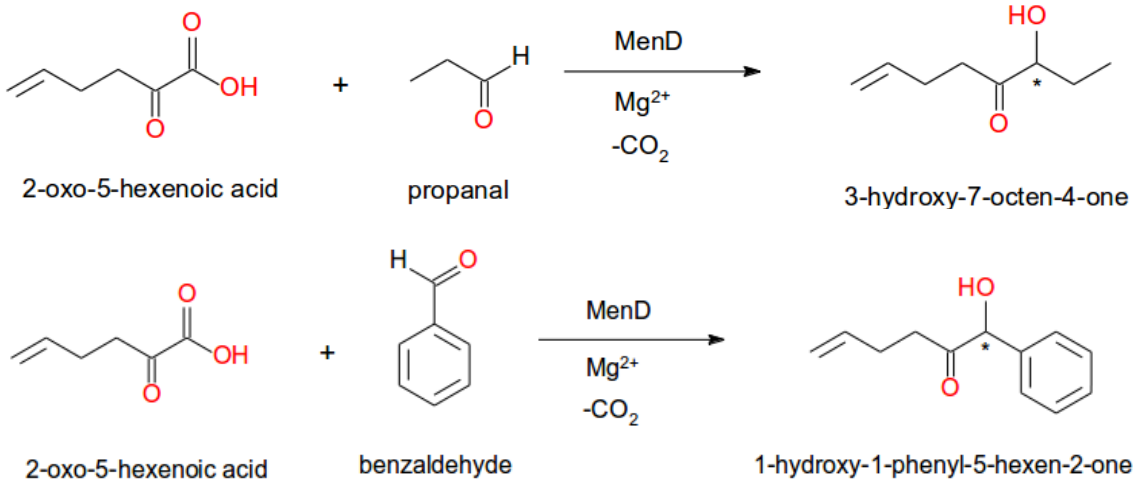

Figure 3.4 MenD's expected reaction with 2-oxo-5-hexenoic acid as substrate.

3.2 Materials

DCPIP (2,6-Dichlorophenolindophenol), DMSO (dimethyl sulfoxide), 2-oxoglutarate ($C_5H_6O_5$), $MgCl_2$, KH_2PO_4, propanal (C_3H_6O), benzaldehyde (C_7H_6O), thiamin diphosphate (ThDP), formic acid (HCOOH), deuterated chloroform ($CDCl_3$) and anhydrous sodium sulfate (Na_2SO_4) were purchased from Sigma-Aldrich.

2-oxo-5-hexenoic acid was synthesized and provided by Szotak group.

3.3 Methods

3.3.1 Determination of enzyme stability in DMSO

The stability of the enzyme was determined using a modified DCPIP activity assay. Enzyme stability determination were made indirectly by monitoring its decarboxylation activity using the reduction of an external oxidizing agent DCPIP (0.1 mM) at 600 nm, 30 ˚C. The enzyme buffer was prepared using KH_2PO_4 (20 mM, pH 7.5), ThDP (0.5 mM), $MgCl_2$ (2.0 mM) and 2-oxoglutarate (2.0 mM) containing different concentrations of DMSO (5% v/v – 30% v/v). The reaction were initiated using an enzyme concentration of 40 μg with reaction volumes kept constant at 1,000 μL.

3.3.2 Carboligation reaction on an analytical scale

The carboligation reaction were performed overnight at room temperature with constant shaking in vials. The reactions were performed in the modified DCPIP activity buffer which contained KH_2PO_4 (50 mM, pH 8.0), ThDP (0.1 mM), $MgCl_2$ (2.0 mM), DMSO (5 % v/v), 2-oxoglutarate (30 mM, 10 mM), 2-oxo-5-hexenoic acid (30 mM) and acceptors (propanal and benzaldehyde) (20 mM). The carboligation reactions were initiated using 700 μg of E1o and 500 μg of MenD. The final reaction volume was fixed at 1,200 μL. After completion of the reaction, the product formed was confirmed by obtaining a CD spectrum of the products.

3.3.3 Circular Dichroism

The CD spectra were obtained on an Applied Photophysics Chiralscan CD Spectrometer. After completion of the analytical scale carboligation reaction the entire reaction volume was spun down with 4,400 g at room temperature. Subsequent steps included treating the supernatant with formic acid (50% v/v) to precipitate any remaining protein. This supernatant was used for the CD spectrum measurements, the measurements were made against a blank containing reactants without enzyme to remove any background noise. The spectra were recorded in a cell with path length of 1 cm, over a wavelength range of 260 – 400 nm at 30 ˚C.

3.3.4 Extraction of products

After completion of the analytical scale carboligation reaction, the products were extracted from the reaction buffer (aqueous) into deuterated chloroform (organic) for further analysis using NMR. The reaction volume was treated with 500 µL formic acid (50 % v/v), if needed to precipitate any excess protein. Deuterated chloroform was added to this aqueous solution gradually to match the volume of the aqueous solution (1,200 µL). This mixture was vortexed and homogenized followed by addition of anhydrous sodium sulfate salt (18 mg). The organic layer from previous step was transferred to another vial containing deuterated chloroform (300 µL) and the procedure was repeated. The final volume of deuterated chloroform containing the product was transferred into an NMR tube for characterization and identification.

3.4 Results and Discussion

3.4.1 Enzyme stability in DMSO

The acceptors used for carboligation had poor solubility in an aqueous reaction buffer. To overcome the solubility issue the medium was modified to contain DMSO. Since, ThDP-dependent enzymes naturally exist in an aqueous environment their performance is better in an aqueous environment compared to an organic one. In organic

environment their activity was reduced and even tend to cease at higher concentrations of DMSO. To overcome this issue, the relative enzyme activity using DCPIP was monitored against different DMSO concentrations and an optimal balance between enzyme activity and DMSO was obtained which would be used for the carboligation reactions.

DMSO (% v/v)	MenD activity (µmol/min/mg)	E1o activity (µmol/min/mg)
0	0.03411	0.34872
5	0.03045	0.31036
10	0.02821	0.28539
15	0.01974[a]	nr
20	nr	nr
30	nr	nr

Table 3: Enzyme activity at various DMSO concentrations.

([a] reported with uncertainty) and (nr: not reported due to inconsistent result)

An optimal balance between DMSO and enzyme activity was achieved at DMSO concentration of 5 % (v/v). For concentrations ranging 15 % or above, the enzyme activity was difficult to maintain and record due to an increase in viscosity caused by the high amount of DMSO and phase separation. Moreover, over prolonged period of incubation at concentrations of 10 % and above a significant reduction in the enzyme activity for MenD was observed (Table 4).

DMSO (%, v/v)	MenD activity (0 min) (µmol/min/mg)	MenD activity (30 min) (µmol/min/mg)
5	0.03045	0.02391
10	0.02821	0.01763
15	0.1974[a]	0.01585[a]

Table 4: MenD activity with incubation at various DMSO concentrations. ([a] reported with uncertainty)

3.4.2 Carboligation reaction of E1o and MenD

The carboligation reactions were carried out according to (3.3.2) and the presence of a chiral product in excess was confirmed using CD spectroscopy. The results for carboligation between substrate and acceptors using E1o and MenD are summarized in Table 5.

The readings were measured against blank which contained the reactants without the enzyme, to remove any background caused by the reactants.

Enzyme	Substrate	Acceptor	Θobs (mdeg)
E1o (700 µg)	2-oxoglutarate[a]	Propanal[b]	-54
		Benzaldehyde[b]	-16
MenD (500 µg)		Propanal[b]	-19
	2-oxoglutarate[c]	Benzaldehyde[b]	-9
	2-oxoglutarate[a]	Benzaldehyde[b]	Nd
	2-oxo-5-hexenoic acid[a]	Propanal[b]	Nd
	2-oxo-5-hexenoic acid[a]	Benzaldehyde[d]	Nd

Table 5: CD results obtained after carboligation reactions. ([a] 30 mM substrate, [b] 20 mM acceptor and [c] 10 mM substrate) **(**nd: not detected)

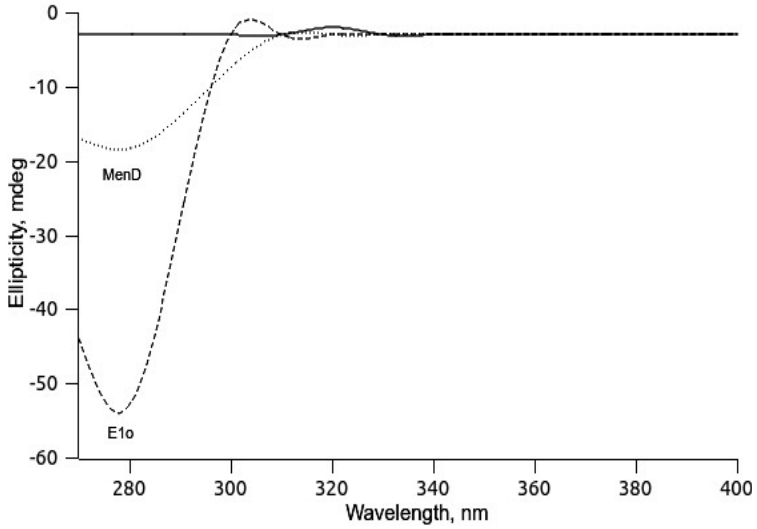

Figure 3.5 CD spectra of 2-oxoglutarate and propanal.

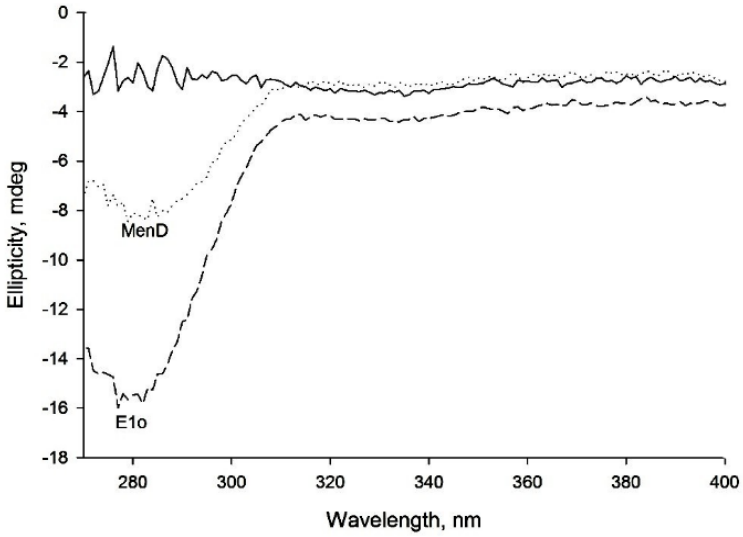

Figure 3.6 CD spectra of 2-oxoglutarate and benzaldehyde.

The CD was able to detect significant amount of enantiomer at 278 nm. For reactions between propanal as acceptor and 2-oxoglutarate as substrate using E1o, the majority of the product obtained was in its (R)-configuration. The same was observed for reactions with MenD.

When reactions between benzaldehyde as acceptor and 2-oxoglutarate as substrate were catalyzed using MenD, the product obtained was in its (R)-configuration. But, the best results were obtained when the substrate concentration were kept lower than the acceptor concentration, if the substrate concentration were increased the enzyme suffered inhibition and decreased reactivity. The reactions between benzaldehyde as

acceptor and 2-oxoglutarate as substrate were also performed using E1o and the amount of substrate used did not inhibit E1o unlike with MenD. This inhibition caused was likely due to the high Km of MenD for 2-oxoglutarate compared to its aromatic substrate. So, an increased concentration of 2-oxoglutarate led the enzyme towards substrate inhibition. (Km value for 2-oxoglutarate for E1o: 0.1 mM and MenD: 1.5 mM; isochorismate for MenD: 0.05 mM, from the literature) [17,46].

For the reactions between 2-oxo-5-hexenoic acid as substrate, propanal as acceptor and benzaldehyde as acceptor using MenD, no enantiomeric product was detected in the CD spectra. These results support the fact that the residues that are responsible for the ThDP-dependent decarboxylase activity are conserved in the family, and mutations induced at the conserved domain and/or the hydrophobic chains supporting the active-site pocket can be used to widen MenD's substrate spectrum and also decrease its substrate inhibition.

3.5 Conclusion

MenD and E1o both successfully carried out the carboligation reactions. For MenD it was observed that its reaction rate suffered when the substrate concentration was increased, suggesting towards a substrate induced inhibition of the enzyme. Such inhibition were not observed with E1o. Substrate inhibition of MenD is likely due to the fact that it has an active site which has, a ping-pong bi-bi mechanism. Wherein, both the

substrate and acceptor need to bind to the active site for the reaction to progress. On the other hand, for E1o the reactions carried out without the other components of OGDHc makes the carboligation reaction a side reaction caused due to the absence of the E2o complex.

MenD offers tremendous possibilities owing to a large active site and a wide acceptor spectrum. The 2-oxo-acid substrate spectrum of MenD can be widened with its inhibition improved by targeting the residues that coordinate with 2-oxoglutarate using site-directed mutagenesis.

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APPENDIX

APPENDIX TO CHAPTER 2

Materials

Tris-HCl, acrylamide, bis-acrylamide, ammonium persulfate (APS), TEMED (tetramethylethylenediamine), glycerol, SDS (sodium dodecyl sulfate) and bromophenol blue were purchased from Sigma-Aldrich.

Methods

SDS-PAGE

The procedure for gel casting was similar to that described in the Mini-PROTEAN Manual. Briefly, separations were done on a Laemmli Buffer System (12 %, 7.5 %), Separating gel was prepared by combining 2.50 mL of 30 % acrylamide/ Bis stock solution in 4.85 mL of deionized water, 2.50 mL of Tris-HCl (1.5 M, pH 8.8), 100 µL of SDS (10 %) and 50 µL of APS (10 %). Polymerization was initiated by adding 5.0 µL of TEMED. The resulting solution was added immediately to the pre-assembled cast, overlaid with 1 mL DI water and allowed to solidify for 20 min at room temperature

The stacking gel was prepared by combining 1.33 mL of acrylamide/ bis stock solution (30 % / 2.67 %) in 6.10 mL of deionized water, 2.50 mL of Tris-HCl (0.5 M, pH 6.8), 100 µL of SDS (10 %) and 50 µL of APS (10 %). Polymerization was initiated by adding 10 µL of TEMED. This solution was poured on the top of the solidified separating gel and allowed to solidify with the sample combs inserted.

Samples were prepared by appropriately diluting the fractions so that maximum sample concentration was less than 3 µg/mL. To 10 µL of this sample, 20 µL of DI water and sample buffer were added and heated for 3 min in boiling water bath. And appropriate amount of this sample was then applied to the gel and allowed to run for 15 min at 80 volts initially and then for 40 min at 160 volts. Subsequently, the gel was stained with staining solution for 20 min and destained for 40 min.

Acrylamide/Bis (30 % T, 2.67 % C)

Acrylamide (29.2 g/100 mL) and N'N'-bis-methylene-acrylamide (0.8 g/100 mL)

Sample Buffer

3.8 mL deionized water, 1.0 mL Tris-HCl (0.5 M, pH 6.8), 0.8 mL glycerol, 1.6 mL SDS (10 % w/v), 0.4 mL 2-mercaptoethanol, 0.4 mL bromophenol blue (1.0 % w/v), 8.0 mL of total volume. Dilute the sample at least 1:4 with sample buffer, and heat at 95 °C for 4 minutes.

5X Electrode (Running) Buffer, pH 8.3

30.3 g Tris-HCl, 144.0 g glycine, 10.0 g SDS Dissolve and bring total volume up to 1,000 mL with deionized water. Do not adjust pH with acid or base. Store at 4 °C. If precipitation occurs, warm to room temperature before use.

Use: Dilute 100 mL of 5X stock with 400 mL deionized water for each electrophoresis run. Mix thoroughly before use.

10% APS

Dissolve 100 mg ammonium persulfate in 1 mL of deionized water.

Kinetic study of MenD using DCPIP

The kinetics of MenD were performed using the DCPIP reduction assay at 600 nm to obtain a Km curve for MenD. The Km was performed to study the binding of substrate (2-ketoglutarate) to MenD, other variables were kept at saturating concentration in media; KH_2PO_4 (20 mM, pH 7.5) with $MgCl_2$ (2.0 mM), ThDP (0.5 mM) and DCPIP (0.08 mM). The substrate 2-oxoglutarate was used in the range of (10 μM to 0.5 mM/500 μM). The activity was measured against blank to avoid any background due to the media, with two negative controls, one without substrate and the other without enzyme. The reactions were initiated using 40 μg of MenD enzyme.

Results and discussion

Kinetic study of MenD using DCPIP

It was possible to note the reduction of DCPIP and obtain a plot for Km for 2-oxoglutarate and MenD. The data are presented in Table A.1.

Enzyme	DCPIP activity (µmol/min/mg of enzyme)	Km (µM)	kcat (s^{-1})	Vm (slope/min)
MenD	0.0363	50	1.335	0.0227

Table A.1: Activity study of MenD using DCPIP assay**.**

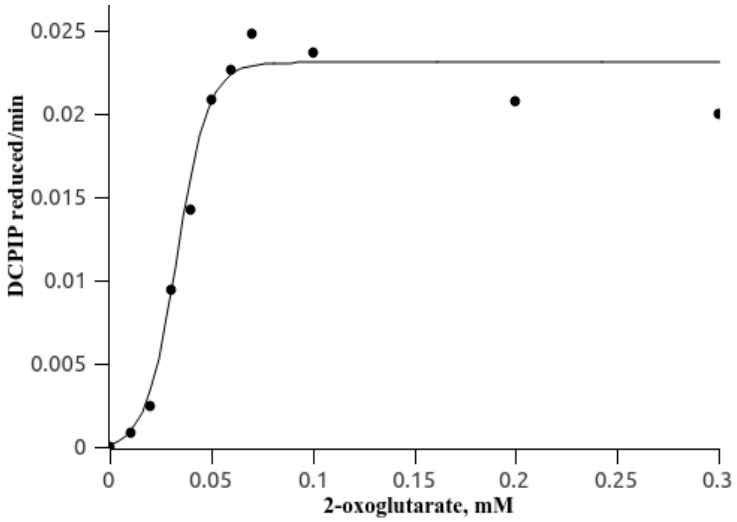

Figure A.1 Km plot of MenD using DCPIP.

The Km values obtained using DCPIP reduction assay were backed by the carboligation reaction. In the carboligation reactions of MenD with benzaldehyde, the product formation was observed (-9 mdeg) when 2-oxolgutarate concentration was at 10 mM (20 µM/µg of enzyme), but when the concentration of 2-oxoglutarate was increased to 30 mM (60 µM/µg of enzyme) no product was observed with CD, suggesting

that substrate induced inhibition maybe involved along with other factors which prevented the product formation at high substrate concentrations.

Bradford curves for estimating protein concentration

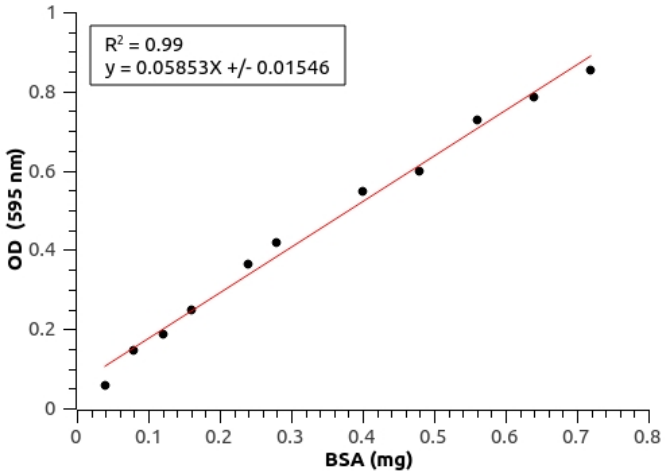

Figure A.2 Standard protein content curve developed in cuvette (2 mg/mL).

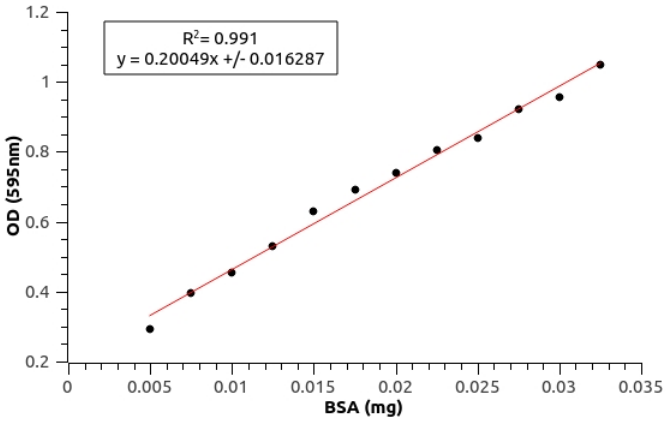

Figure A.3 Standard protein content curve developed in microplate (1 mg/mL).

APPENDIX TO CHAPTER 3

Clustal Omega results of MenD and P32031

MenD's crystal structure is known in its pure form with ligand interacting active-site residues identified [19]. Moreover, MenD has sequence similarity with other proteins of MenD super-family and ThDP-dependent enzymes which contain a central ThDP-binding domain (Rossman fold). It is surprising to know that apart from these families MenD also has sequence similarity with other 2-oxoglutarate binding enzymes. MenD's active site has sequence similarity with the active site of an ethylene forming enzyme from *Pseudomonas savastanoi pv. phaseolicola*, P32031 (2-oxoglutarate dependent ethylene/succinate-forming enzyme) (EC 1.13.12.19), which converts 2-oxoglutarate into ethylene using an oxygen molecule and iron [47]. The sequence similarity of MenD with P32031 and other ketoglutarate binding proteins indicate that MenD's active site can in fact be improved using site-directed mutagenesis.

```
pdb|5MOF|A      ------------------------------------------------------------    0
AUG17016.1      MSVSAFNRRWAAVILEALTRHGVRHICIAPGSRSTPLTLAAAENSAFIHHTHFDERGLGH    60

pdb|5MOF|A      ----------------------------MNHKVHHHHHHNLQTFELPTEVTGCAADISL    31
AUG17016.1      LALGLAKVSKQPVAVIVTSGTAVANLYPALIEAGLTGEKLILLTADRPPELIDCGANQAI    120
                                         : .     .:  * * : * *: .*.*: ::

pdb|5MOF|A      G-----------------------------------------------------------    32
AUG17016.1      RQPGMFASHPTHSISLPRPTQDIPARWLVSTIDHALGTLHAGGVHINCPFAEPLYGEMDD    180

pdb|5MOF|A      ------RALIQAWQKDGIFQIKTDSEQDRKTQEAMAASKQFCKEPLTFKSSCVSDLTYSG    86
AUG17016.1      TGLSWQQRLGDWWQDDKPWLREAPRLESEKQRDW-----FFWR------------QKRG    222
                      : * : **.*  :  ::   :..* ::       * :               *

pdb|5MOF|A      YVASGEEVTAGKPDFPEIFTVCKDLSVGDQRVKAGWPCHGPVP------------WPNNT    134
AUG17016.1      VVVAGRMSA------------EEGKKVALWAQTLGWPLIGDVLSQTGQPLPCADLWLGNA    270
                 *.:*.  :             :. .*.    . ***  * *            * .*:

pdb|5MOF|A      YQKSM-----KTFMEELGLAGERLLKLTALGFELPINTFTDLTRDGWHHMRVLRFPPQTS    189
AUG17016.1      KATSELQQAQIVVQLGSSLTGKRLLQWQAS-----------------------------    300
                  .*       ..    .*:*:***:  *

pdb|5MOF|A      TLSRGIGAHTDYGLLVIAAQDDVGGLYIRPPVEGEKR------------NRNWLPGESSA    237
AUG17016.1      -------------------CEPEEYWIVDDIEGRLDPAHHRGRRLIANIADWLEL----    336
                                   :    :*   :**.                :**

pdb|5MOF|A      GMFEHDEPWTFVTPTPGVWTVFPGDILQFMTGGQLLSTPHKVKLNTRERFACAYFHEPN-    296
AUG17016.1      HPAEKRQPWCVEIPRLAEQAM------QA-----VIARRDAFGEAQLAHRICDYLPEQGQ    385
                   *: :** .  *  .  ::      *      :::  . .      :  * *: * .

pdb|5MOF|A      ---------------FEASAYPLFEPSANER---------------------------    312
AUG17016.1      LFVGNSLVVRLIDALSQLPAGYPVYSNRGASGIDGLLSTAAGVQRASGKPTLAIVGDLSA    445
                              :.**::.  . .

pdb|5MOF|A      -------------------------------------------------IHYGEHFTNMF    323
AUG17016.1      LYDLNALALLRQVSAPLVLIVVNNNGGQIFSLLPTPQSERERFYLMPQNVHF-EHAAAMF    504
                                                                :*: ** : **

pdb|5MOF|A      MRCYPDRITTQ--------------------RINKEN-------RLAHLEDLKKYSDTRA    356
AUG17016.1      ELKYHRPQNWQELETAFADAWRTPTTTVIEMVVNDTDGAQTLQQLLAQVSHL--------    556
                   *    . *                     :*. :        **::..*

pdb|5MOF|A      TGS     359
AUG17016.1      ---     556
```

Figure A.4 Pair-wise peptide alignment of P32031 (5MOF), ethylene forming enzyme and MenD (AUG17016.1) using clustal omega.