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ABSTRACT

ESCHERICHIA COLI 2-OXOGLUTARATE DEHYDROGENASE MULTIENZYME COMPLEX: E1 AND E2 SUBSTRATE SPECIFICTY, E1 CARBOLIGASE ACTIVITY, AND E2 INTERCHAIN SUCCINYL TRANSFER

by
Da Jeong Shim

Escherichia coli (*E. coli*) 2-oxoglutarate dehydrogenase multienzyme complex (OGDHc) contains three components: a thiamin diphosphate (ThDP) dependent 2-oxoglutarate dehydrogenase (E1o), a dihydrolipoylsuccinyl transferase (E2o), and a dihydrolipoyl dehydrogenase (E3). The first two components carry out the principal reactions for succinyl CoA formation while the third one reoxidizes dihydrolipoamide to lipoamide. This mechanism is similar to other 2-oxoacid dehydrogenase complexes, including pyruvate dehydrogenases (PDHc) and branched-chain dehydrogenases.

E1o of the *E. coli* OGDHc was engineered to accept unnatural substrates. The natural substrate for E1o is 2-oxoglutarate (2-OG) and the enzyme was engineered to accept substrates lacking the 5-carboxylate group, 2-oxovalerate (2-OV). E1o was subjected to saturation mutagenesis at His260 and His298. The His298Asp E1o variant was identified in the screen to accept the unnatural substrate. In addition, it was discovered that His260 is required for substrate recognition, but His298 could be replaced with hydrophobic residues of similar molecular volume. To interrogate whether the second component would allow synthesis of acyl-coenzyme A derivatives, hybrid complexes consisting of recombinant components of OGDHc (o) and pyruvate dehydrogenase (p) enzymes were constructed, suggesting that a different component is the “gatekeeper” for specificity for these two multienzyme complexes in bacteria, E1p for pyruvate but E2o for 2-OG.

Although His298Asp E1o accepted 2-OV, reconstitution of the variant with E2o and E3 did not generate NADH in the overall reaction using 2-OV. Hence, the reaction may be hindered in the E2o component. E2o consists of an amino-terminal lipoyl domain (E2oLD, 12 kDa), followed by a peripheral subunit binding domain (4 kDa) and a succinyltransferase domain (E2oCD, 28 kDa). There are two possibilities for the failure to form NADH. Reductive acylation is not occurring in the E2oLD or acyl transfer to CoA is not taking place in E2oCD. His298Asp E1o, E2oLD, and 2-OV form butyrylated E2oLD, which was shown by electrospray ionization mass spectrometry. Therefore, the E2oCD domain necessitates optimization to produce acyl-CoA derivatives.

Succinyl transfer to the CD domain may occur through an intrachain or interchain pathway. The E2oLD and E2o with a lysine to alanine substitution at position 43 (E2oK43A) were created by site directed mutagenesis. It is clearly shown that E2oLD was capable of rescuing the crippled E2oK43A variant by measuring the NADH production in the overall reaction. Therefore, an interchain mechanism is likely between two different E2o subunits.

ThDP-dependent enzymes have the potential to be used for chemical synthesis. These enzymes share a common feature in that they catalyze carboligase reactions. E1o catalyzed carboligation products with a variety of substrates and acceptors that vary in the size and functional groups. Structures of the products were confirmed with NMR. In addition, high *enantiomeric excess* (*ee*) values were found for the products as shown by chiral gas chromatography and CD spectroscopy. Finally, it was shown that E1o is capable of forming stable esters. This is important because when the carboligase reactions produce β-ketoacids, these products are unstable and prone to decarboxylation.

ESCHERICHIA COLI 2-OXOGLUTARATE DEHYDROGENASE MULTIENZYME COMPLEX: E1 AND E2 SUBSTRATE SPECIFICTY, E1 CARBOLIGASE ACTIVITY, AND E2 INTERCHAIN SUCCINYL TRANSFER

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A Dissertation
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Shim, D. J, Nemeria, N. S., Balakrishnan, A., Patel, H., Song, J., Wang, J., Jordan, F. and Farinas, E. T. "Altering the substrate specificity of the *Escherichia coli* 2-oxoglutarate dehydrogenase multienzyme complex," ASBMB Annual Meeting, Anaheim, CA. April 26, 2010.

Shim, D. J, Nemeria, N. S., Balakrishnan, A., Patel, H., Song, J., Wang, J., Jordan, F. and Farinas, E. T. "Altering the substrate specificity of the *Escherichia coli* 2-oxoglutarate dehydrogenase multienzyme complex," Biomolecular Engineering Annual Advances Meeting organized by New York Academy of Science, New York City, NY. April, 2012.

This thesis is dedicated to my mom and dad, who taught me the value of education and my sister and my brother for support throughout my studies. Without their patience, understanding, and support, the completion of this work would not be possible.

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CHAPTER 1

INTRODUCTION

1.1 2-Oxoglutarate Dehydrogenase Multienzyme Complex from *Escherichia coli*

The 2-oxoglutarate dehydrogenase multienzyme complex (OGDHc) catalyzes the rate-limiting step in the citric acid cycle (Figure 1.1) [1, 2], which is the common pathway for the oxidation of fuel molecules, including carbohydrates, fatty acids and amino acids. In addition, neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [3-5], have been linked to inactivation of OGDHc in highly active tissues. OGDHc catalyzes the formation of succinyl coenzyme A (CoA) according to Equation 1.1.

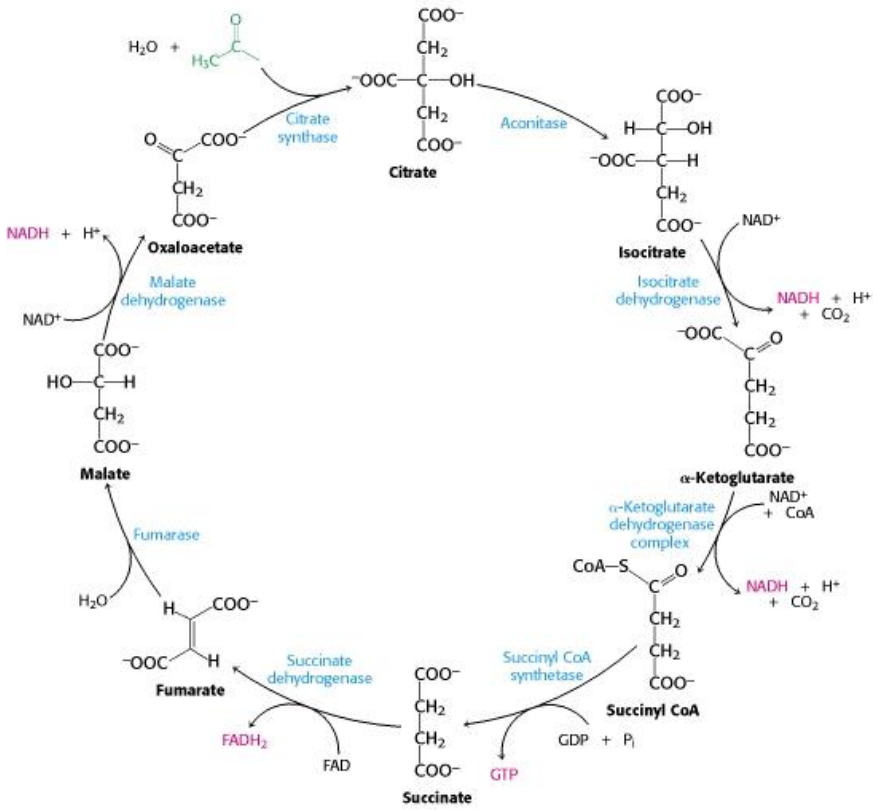


Figure 1.1 Citric Acid Cycle.

Source: Jeremy M. Berg, John L. Tymoczko, and Lubert Stryer "Biochemistry" fifth edition, Freeman, pp. 478. http://www.ncbi.nlm.nih.gov/books/NBK22427/figure/A2403/?report=objectonly.

$$\text{2-oxoglutarate} + \text{CoA} + \text{NAD}^{+} \rightarrow \text{succinyl CoA} + \text{CO}_2 + \text{NADH} \qquad (1.1)$$

The OGDHc is composed of multiple copies of three components (Figure 1.2) [6-9]: (1) A thiamin diphosphate (ThDP) dependent 2-oxoglutarate dehydrogenase (E1o, 105 kDa, EC 1.2.4.2, Figure 1.2 A). (2) A dihydrolipoylsuccinyl transferase (E2o, 45 kDa, EC 2.3.1.6, Figure 1.2 B and C) and (3) A dihydrolipoyl dehydrogenase (E3, 55 kDa, EC 1.8.1.4). The first two components carry out the principal reactions for succinyl-CoA formation while the third one reoxidizes dihydrolipoamide E2 to lipoamide E2. This mechanism is similar to other 2-oxoacid dehydrogenase complexes, including pyruvate dehydrogenase (PDHc) and branched-chain dehydrogenase (BCDHc) (Equation 1.2-1.5).

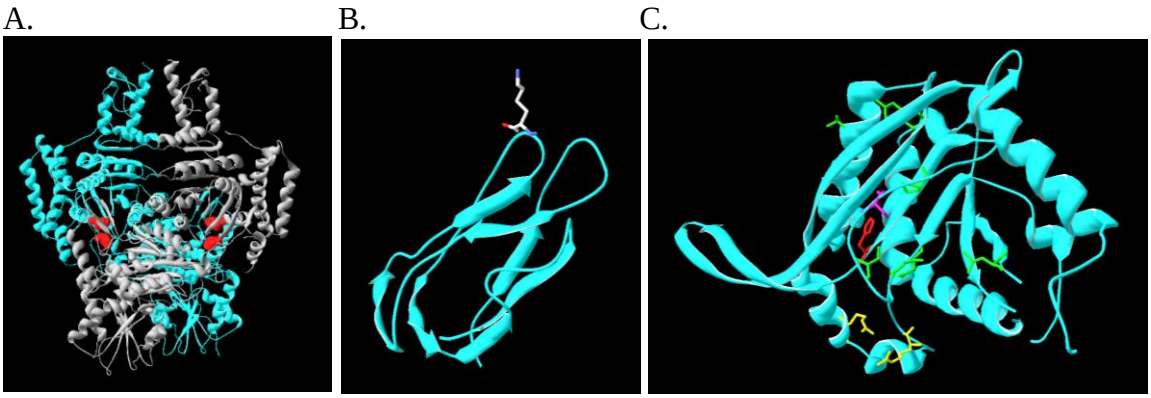
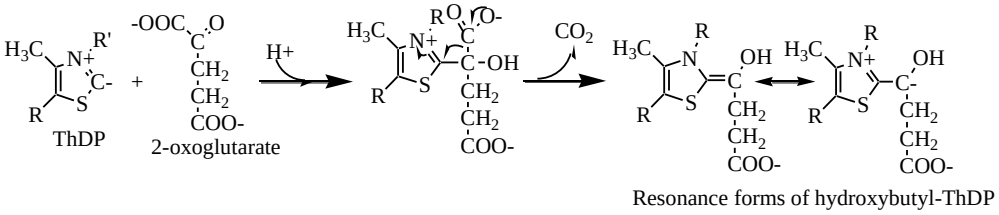


Figure 1.2 Structure of E1o and E2o Components. **A:** Ribbon Diagram of Truncated E1o. Histidine 260 and 298 are Shown as Red Spheres. **B:** Ribbon Diagram of the Lipoyl Domain of E2o. Lys43 is Shown as Sticks. **C:** Ribbon Diagram of E2o Catalytic Domain. The Carboxyl-binding Residue (Ser333) is Shown in Magenta. The Carboxylate-binding Group (His348) is Shown in Red.

The E1o component catalyzes the initial decarboxylation of the 2-oxoglutarate, using ThDP as a cofactor (Equation 1.2), and then reductively succinylates a lipoyl group bound to a lysine residue in E2o (Equation 1.3, Figure 1.2 B). E2o is a succinyl transferase responsible for transferring the succinyl group to CoA (Equation 1.4), and the dihydrolipoyl group left on E2o finally reoxidized to the dithiol ring by the flavoprotein E3, with NAD^+ as the ultimate electron acceptor (Equation 1.5). The mechanism of OGDHc and the role of ThDP are shown in Scheme 1.



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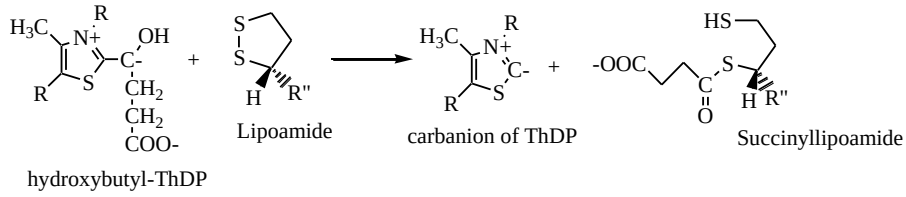

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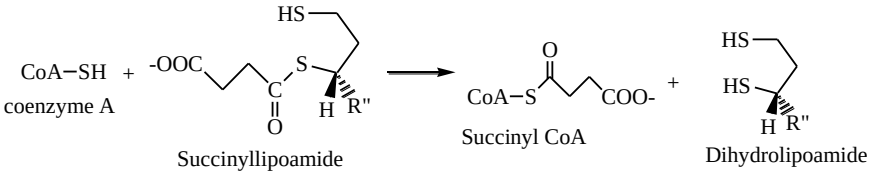

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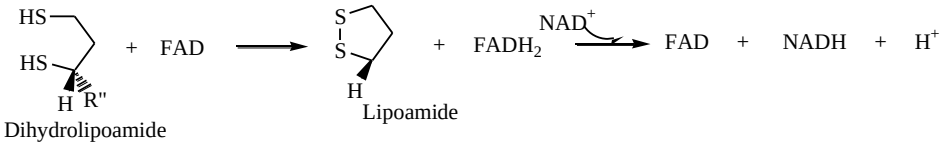

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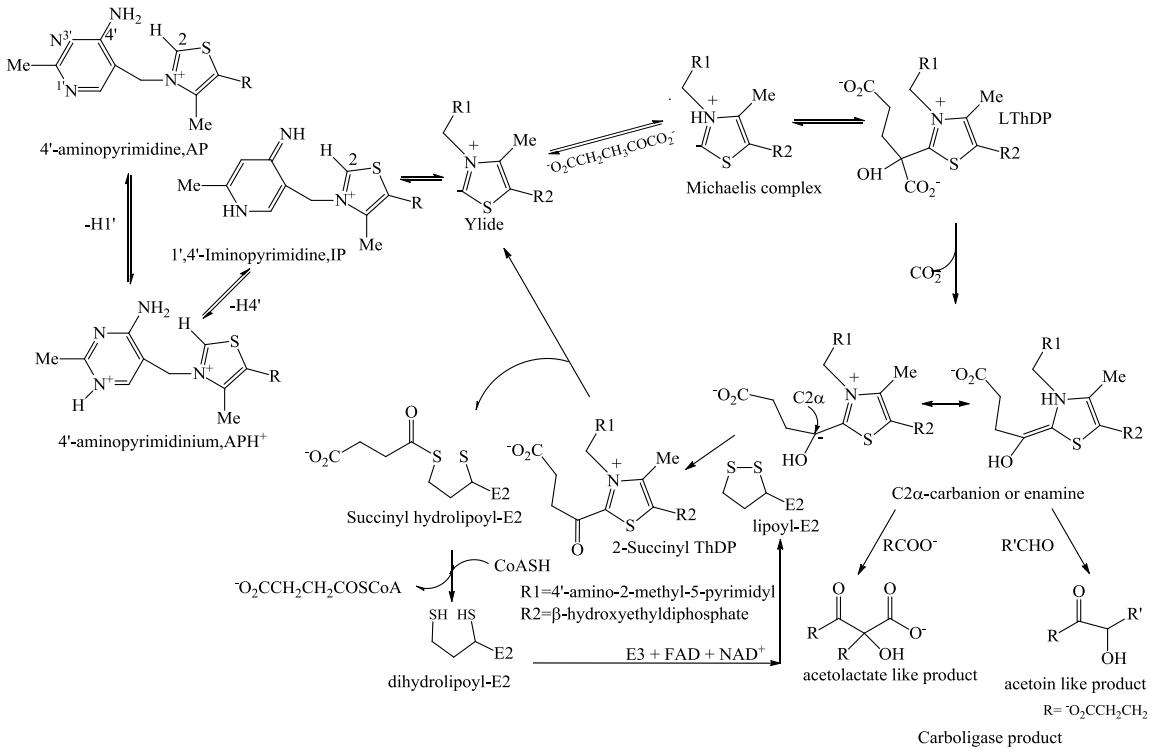


Scheme 1 Mechanism of 2-Oxoglutarate Dehydrogenase Complex of *E. coli*.

The OGDHc is organized about a core consisting of the oligomeric E2o [10-12], which are bound to multiple copies of E1o and E3. The E2o component is a modular protein that is composed of one, two, or three amino-terminal lipoyl domains, followed by an E1o and/or E3-binding domain, and then by carboxyl terminal catalytic domain. The domains are connected by linker segments [13-15]. In *E. coli*, E2o contains only one lipoyl domain, subunit binding domain, and catalytic domain (Figure 1.3). The E2o is organized either as a 24-mer with octahedral symmetry or a 60-mer with icosahedral symmetry depending on the organism. The optimal stoichiometry for the *E. coli* complex has been estimated at 12 E1o: 24 E2o: 12 E3 (3120 kDa) [16]. The E3 component is identical in OGDHc and the pyruvate dehydrogenase complex (PDHc) [17, 18]. PDHc is also part of

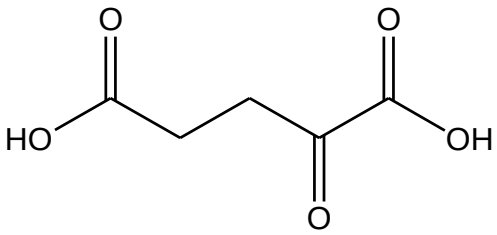
the citric acid cycle. PDHc shares a similar mechanism with OGDHc, but it utilizes pyruvate as a substrate.

Figure 1.3 The *E. coli* E2o Component.

1.2 Substrate Specificity of OGDHc

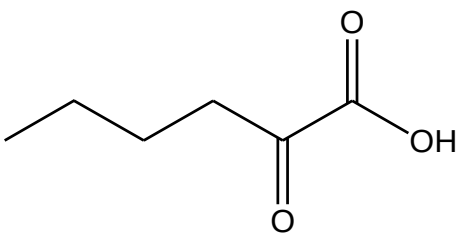
Substrate specificity of OGDHc is regulated by E1o component as well as E2o. E1o is responsible for recognition and decarboxylation of 2-oxoglutarate (2-OG) (Equation 1.2, Figure 1.4 A). The crystal structure for a truncated E1o from *E. coli* (Figure 1.2 A) has been reported [6]. Site directed mutagenesis and kinetic studies indicated that His260 and His298 were positioned to interact with the distal carboxylate of 2-OG (Figure 1.5).

A.



2-oxoglutarate (2-OG)

B.



2-oxovalerate (2-OV)

Figure 1.4 Substrates for OGDHc. **A:** Structure of 2-Oxoglutarate. **B:** Structure of 2-Oxovalerate.

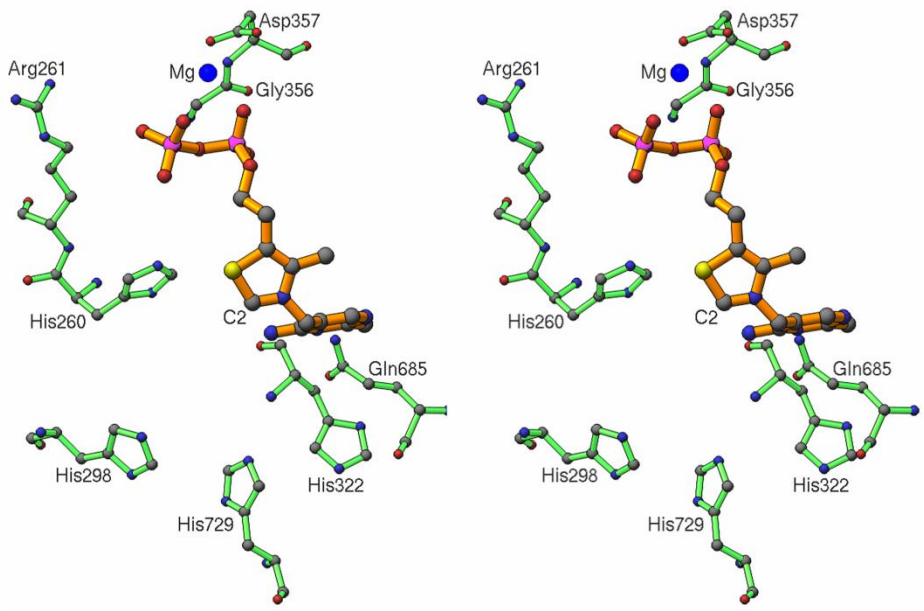


Figure 1.5 Stereo View Showing Histidines and a Few Other Residues near the Active Site of the *E. coli* 2-Oxoglutarate Dehydrogenase Multienzyme Complex E1 Component, Showing Their Proximity to the Reactive Center C2 Atom on Thiamin diphosphate (ThDP). Coordinates for the Protein Atoms were Obtained from the PDB entry 2JGD, which is Described in [6].

E2o substrate recognition occurs in two stages. First, E1o reductively succinylates the prosthetic group lipoic acid that is covalently attached to Lys43 in the lipoyl domain (Equation 1.3, Figure 1.2 B). An important interaction is between the E1 component and the loop region of E2o. Key residues include 7-20 and Thr42 [17]. Second, the catalytic domain (CD) of the E2o component catalyzes the transfer of a succinyl group from the S-succinyldihydrolipoyl moiety to coenzyme A. Computational modeling and sequence alignment studies of all known E2's indicate that in E2o serine residues 330 and 333 are located near the proposed succinyl-binding site. Ser333 may hydrogen bond with the carboxyl group of the substrate. His348 is conserved in all E2's, and it is positioned to interact with the carboxylate oxygen of the succinyl group (Figure 1.6) [7]. The origin of

substrate specificity remains unclear. It will be determined that the contribution of His260 and His298 for substrate specificity. In addition, the "gate-keeper" will be determined. For example, does E1 or E2 control the substrate specificity?

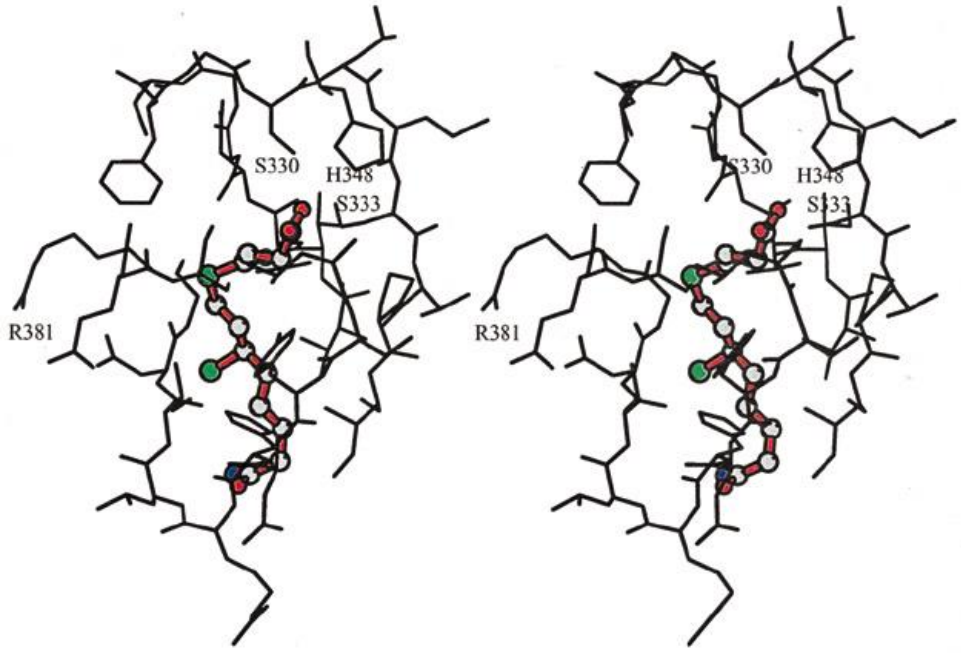


Figure 1.6 The Stereo View of the Substrate Binding Sites on E2o Component [7].

1.3 Applications of OGDHc

1.3.1 Green Chemical Synthesis with OGDHc

Enzyme**s** catalyze reactions under mild conditions in aqueous solutions, and they can also provide tools for environmental friendly chemical synthesis [2, 19-21]. However, biocatalysts often display poor catalytic activity for unnatural substrates. This is especially true for enzymes that have specific functions across many species [22]. In addition, enzymes are readily denatured under typical reaction conditions, which include organic solvents and elevated temperatures. Hence, an efficient strategy is necessary to engineer

enzymes, and semi-rational methods offer an effective approach to engineer and optimize catalytic activity [23].

OGDHc has the potential to synthesize coenzyme A-linked thiol esters, comprising a large class of metabolically relevant compounds participating in many metabolic pathways. Also, thiol esters are important as organic synthetic intermediates by virtue of the 'high energy' bound between the carbonyl group and the sulfur atom, and they can be converted to a number of carboxylic acid derivatives [24]. It can also be used for peptide coupling [25-27], acyl transfer [28, 29], protecting groups for thiols [30], and also as coupling partners in organometallic reactions [31-33]. They are also key intermediates in various biological systems [34] and find broad applications in medicinal chemistry [35].

There are many traditional methods for the formation of thiol esters, such as, the direct coupling of a thiol with the parent carboxylic acid and an activating agent [36, 37], the coupling of a thiol with an acid chloride [38], or reaction of thiol with an acid anhydride [39] and the coupling of thiocarboxylates with arenediazonium salts [40]. However, there are limitations of these methods such as difficulties in handling thiols and thioacids and also the availability of starting materials. Thus, it is necessary to find a novel method for such highly useful organic products.

It is proposed that the synthesis of thiol esters using a multi-enzymatic pathway to convert a racemic amino acid precursor $RCH(NH_2)COOH$, which are readily available, to thiol esters RC(=O)SR', with a broad range of R and R' groups under mild neutral aqueous conditions (Figure 1.7, reaction 1). First, a broad-spectrum racemase (EC 5.1.1.10) will catalyze the stereochemical inversion around the asymmetric carbon atom (Figure 1.7, Reaction 2). Next, a transaminase (EC 2.6.1.1) will catalyze the removal of the amino

group to yield a 2-oxo acid (Figure 1.7, Reaction 3). The product will be decarboxylated by E1 (Figure 1.7, Reaction 4), and E2 transfers to acyl group to a thiol (Figure 1.7, Reaction 5).

E1o will be engineered to accept non-natural substrate. E1o will be converted to accept 2-oxovalerate over 2-oxoglutarate. The substrates are similar, but the carboxylate is substituted by a methyl group (Figure 1.4).

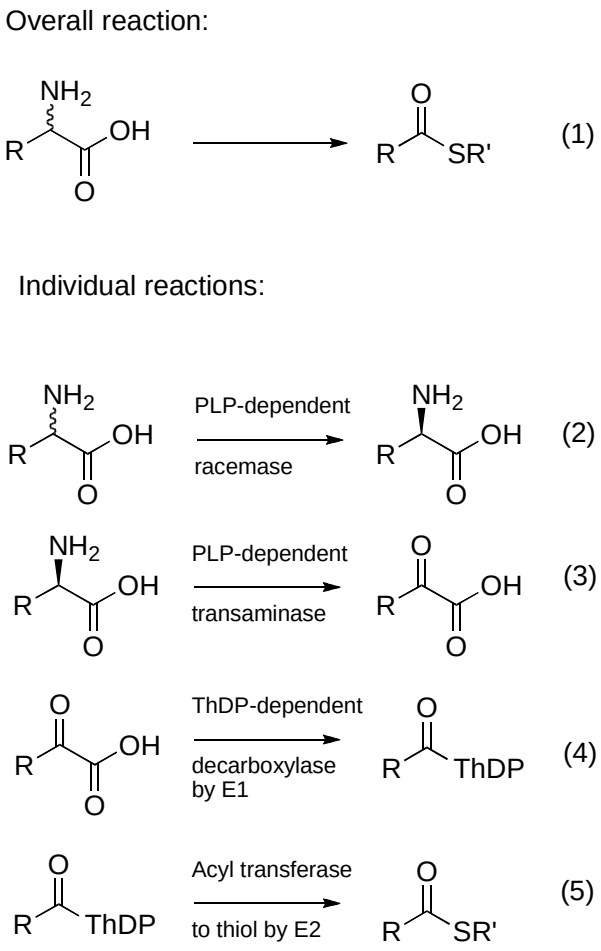


Figure 1.7 A Method to Convert a Racemic Amino Acid Precursor to Thiol Esters.

1.3.2 Carboligase Reactions of OGDHc

The evolutionary pressure for optimized and efficient turnover has resulted in enzymes with tight substrate specificities. However, most enzymes catalyze off pathway side reactions caused by substrate and catalytic promiscuity [41-43].

This promiscuity has become very popular in point of redesigning and optimizing enzymatic activities for industrial application [44, 45]. There are huge challenges to make an existing enzymatic side reactions (low activity) become higher than its original activity or introduce desired new activity without spoiling the original enzymatic activity [46].

The thiamin dependent enzymes, pyruvate decarboxylase and benzoylformate decarboxylase, are well known enzymes for biocatalytic applications owing to their inherent substrate and catalytic promiscuity. In addition to the decarboxylation of 2-oxoacids, ThDP-dependent decarboxylases, including the first components of the 2-oxoacid dehydrogenases catalyze so-called carboligation side reactions to form C-C bonds [47, 48]. Conversion of the substrate 2-oxo acids involves a series of substrate-cofactor conjugates as reaction intermediates (Scheme 1). Initially, addition of the reactive C2 atom of the cofactor thiazolium ring to the carbonyl carbon of 2-oxoacids yields the pre-decarboxylation intermediate C2α-lactyl-ThDP. Subsequent decarboxylation produces the resonating C2α-carbanion/enamine forms of C2α-hydroxyethylidene-ThDP, a central and highly reactive intermediate. A carboligase side reaction takes place when the nucleophilic C2α-carbanion/enamine adds to electrophilic aldehydes or ketones [19, 47].

ThDP-dependent enzymes have the potential to be used for chemical synthesis. Carboligation is the native activity for several enzymes, but it is a side reaction for nearly

all 2-oxo acid decarboxylases [19]. Previously several active site variants were created in yeast pyruvate decarboxylase (YPDC) from *Saccharomyces cerevisiae*, the E1 subunit of *E. coli* PDHc (E1p) [49] and PDHc from *Zymomonas mobilis* [46]. Those enzyme complexes produced different enantiomeric selective carboligase products using amino acid analogs as the substrates [48, 50]. It can be used to synthesize important chemical intermediates such as chiral 2-hydroxy ketones. The 2-hydroxy ketone products produced from this activity afford versatile, exchangeable chemical groups in the immediate vicinity of a chiral center. Valuable potential building blocks for the synthesis of numerous compounds with pharmacological importance include: the tranquilizer and smoking cessation drug bupropion [51], the anti-allergic drug cytoxazon [52], and the multidrug-pump inhibitor 5-methoxyhydnocarpin [53]. Furthermore, carboligase products can be used to treat health issues such as Alzheimer's disease because they can inhibit the citric acid cycle [49, 50]. This research will explore the possibility of using E1o for carboligase reactions with 2-OG and various acceptors.

1.4 Importance of Recombinant 2-Oxoglutarate Dehydrogenase Complex

In 1985, the activity of OGDHc of *E. coli* was measured by Steginsky et al [54]. Their results showed that OGDHc utilizes pyruvate as a poor substrate, with an activity of 0.083 units/mg protein compared with 22 units/mg protein for 2-OG. Activity with pyruvate could result from the presence of contaminating PDHc or acceptance of pyruvate as a poor substrate 2-OGDHc. They concluded that activity on pyruvate cannot be explained by either of the above, but rather that the 2-OGDHc is a hybrid complex containing a significant amount of the E1p component from *E. coli* PDHc. They separated those two

enzyme complexes using their isoelectric precipitation (PI). There was a problem to separate those two enzyme complexes because they have very similar PIs. These results did not provide a clear origin of substrate specificity. Hence, it is important to use recombinant proteins to ensure there is no contamination. This is necessary to understand the true origin of substrate specificity of the complex.

In this work [55], all of the recombinant E1p, E2p, E1o, E2o and E3 components were prepared as pure enzymes from *E. coli*. Each component will be reconstituted to measure its overall activity so that the origin of substrate specificity can be clearly elucidated.

1.5 Interchain Succinyl Transfer in the E2 Component of the *E. coli* 2-Oxoglutarate Dehydrogenase Complex

The structural core of the complex is provided by E2, with E1 and E3 bound around the periphery [14, 15, 56, 57]. The E2 chains are highly segmented, from the N-terminus, one to three lipoyl domains (LD, approx. 9kDa), a peripheral subunit-binding domain (PSBD, approx. 4kDa) and a catalytic domain (CD, approx. 28kDa) (Figure 1.3) [15, 58]. The E2o chain of 2-OGDHc contains a single LD and an assembly of 24 separate CD arranged with octahedral symmetry forms the core of the complex [7, 17], and it is possible that interchain acyl transfer would take place between an LD of one chain and a CD of another one, with the mobility of the LD provided by flexible alanine and proline rich extended linkers.

Recently, the Jordan group at Rutgers investigated whether the acetyl group is passed from the LD to the CD in an intra- or interchain reaction using E2p from *E. coli*

PDHc, which contains three lipoyl domains [59]. They demonstrated interchain transfer of the acetyl group by providing mass spectrometric and kinetic evidences. These data provided a model for the presence of multiples of three chains in all E2 components, and their assembly in bacterial systems. They created a hybrid single lipoyl domain instead of using the natural three-lipoyl domain E2 component. Although it was shown that the activities of one-LD E2p and three-LD E2p are virtually the same, it is possible that the mutated single LD E2p can affect the results. Therefore, it is better to use native E2 component itself. As previously mentioned, the E2o from *E. coli* has only a single lipoyl domain. This system is simpler and it should be easier to determine if interchain transfer is a possible mechanism.

1.6 Research Objectives in This Dissertation

1. Elucidate the origin of substrate specificity in *E. coli* OGDHc and PDHc.
2. Determine the versatility of *E. coli* E1o for carboligase reactions.
3. Investigate interchain succinyl transfer of the E2o component of *E. coli* OGDHc.

CHAPTER 2

ALTERING SUBSTRATE SPECIFICITY OF THE *Escherichia coli* E1o OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

2.1 Introduction

The factors that govern specificity of 2-oxoglutarate dehydrogenase multienzyme complex (OGDHc) towards its 5-carboxyl substituent will be elucidated. The goal is to synthesize acyl-coenzyme A analogs, comprising a large class of metabolically relevant compounds participating in many metabolic pathways.

According to the x-ray structure of E1o, there were three His residues (His260, His298 and His729) positioned near the thiazolium ring of thiamin diphosphate (ThDP) [6] (Figure 2.1), and substitution of His260 and His298 to alanine drastically reduced the activity. The results suggested that the histidine side chains interacted with the distal carboxylate of 2-oxoglutarate (2-OG) [6].

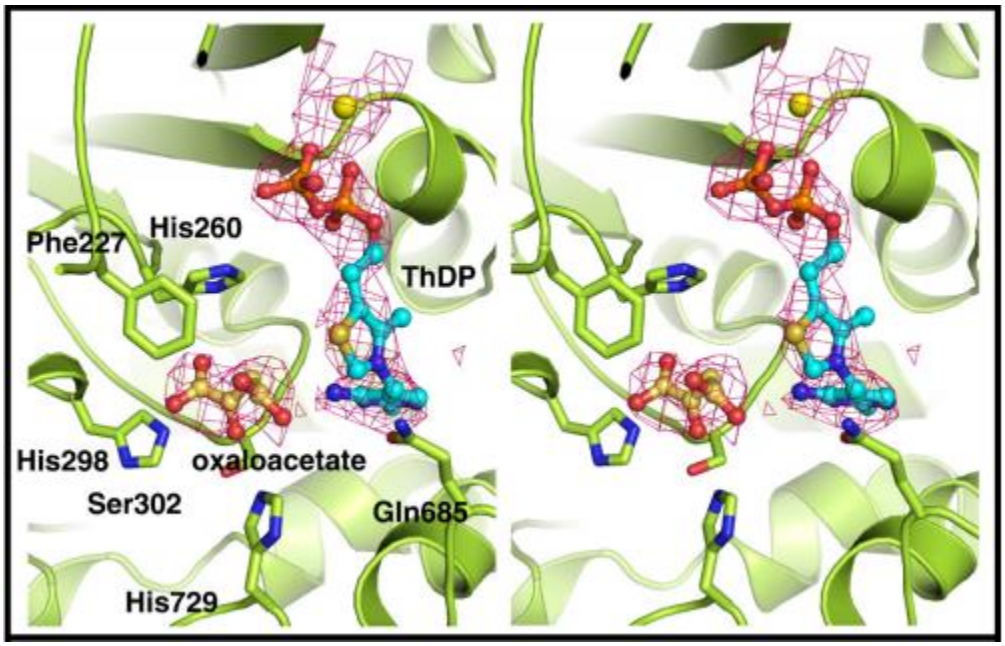


Figure 2.1 Active Site of E1o Showing Mg^{2+}, ThDP, and the Inhibitor Oxaloacetate [6].

Saturation mutagenesis libraries of His260, His298, and His260/His298 were created and screened for activity towards 2-OG, and an unnatural substrate, 2-oxovalerate (2-OV), in which a nonpolar methyl group replaced the charged carboxylate (Figure 2.2).

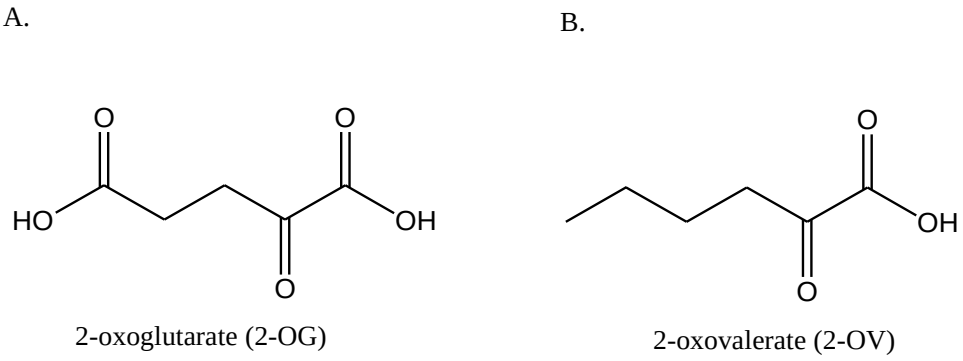


Figure 2.2 Substrates for OGDHc. **A:** Structure of 2-Oxoglutarate. **B:** Structure of 2-Oxovalerate.

Several E1o variants were isolated, some with the ability to decarboxylate 2-OV. The E1o variants created by the His260 and His298 substitutions were shown to be functionally competent according to their ability to produce a ThDP-bound pre-decarboxylation intermediate [60]. Next, it was determined whether the second E2o component would allow synthesis of acyl-coenzyme A derivatives. Hybrid complexes consisting of recombinant components of the *E. coli* 2-oxoglutarate (o) and pyruvate dehydrogenase (p) enzymes were constructed (the E3 component is common to both) and it was demonstrated that E1p imparts specificity for acetyl-CoA formation from pyruvate, but E2o controls specificity for succinyl-CoA formation by OGDHc in Gram-negative bacteria.

2.2 Materials

Thiamin diphosphate (ThDP), dithiothreitol, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from U.S. Biochemical Corp. NAD^+, coenzyme A (CoA), and 2, 6-dichlorophenolindophenol (DCPIP) were from Sigma-Aldrich. Restriction enzymes were purchased from Invitrogen. Primers were procured from Fisher.

E. coli strain JW0715 containing the plasmid pCA24N encoding the OGDHc-E1 (E1o) component and AG1 cells containing pCA24N plasmid encoding OGDHc-E2o [both ASKA clone (-)] were obtained from National Bio Resource Project (NIG, Japan).

2.3 Methods

2.3.1 Site Directed Saturation Mutagenesis

Site-directed saturation mutagenesis libraries were constructed using a modified QuikChange procedure for His260, His298, and the doubly substituted His260/His298 variants. A typical 50 μL reaction contained 10x PfuUltra buffer, dNTP (0.2 mM) and PfuUltra DNA polymerase (1 unit), pCA24N encoding the E1o subunit, and randomization primers are shown in Table 2.1.

Table 2.1 List of NNS Primers

No	Site	5' NNS Primers 3'
1	H260X-F	CGGGATGGCGNNSCGTGGTCGTC
2	H260X-R	GACGACCACGSNNCGCCATCCCG
3	H298X-F	CGTGAAATACNNSATGGGCTTCTCG
4	H298X-R	CGAGAAGCCC ATSNNGTATTTCACG

The PCR reaction consisted of 18 cycles at 95 ℃ for 30 s, 55 ℃ for 1 min and 68 ℃ for 10 min. After the PCR, DpnI was added to the reaction mixture and then PCR product was transformed into *E. coli* DH5α cells. In order to randomize both positions, the template was the PCR product from H298 randomization and primers were H260X-F and H260X-R (Figure 2.3).

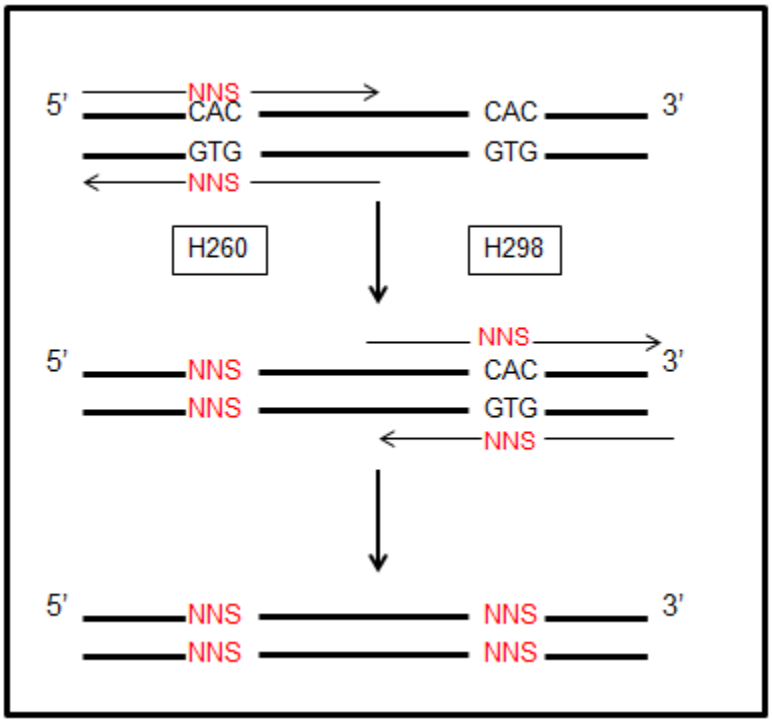


Figure 2.3 PCR Strategy for Creating Site Directed Saturation Mutagenesis for H260X, H298X, and H260X/H298X.

2.3.2 E1o Specific Assay

The E1o specific activity assay was adapted to measure activity in whole cell lysates in 96-well microtiter plates (Figure 2.4). The plasmids containing the mutant genes for H260X, H298X or H260X/H298X were transformed into DH5α competent cells and plated onto LB agar plates containing chloramphenicol (100 μg/mL). Single colonies were

picked into 96-well microtiter plates containing 200 μL LB medium and chloramphenicol (100 μg/mL). The first column contained wild-type E1o as control. The plates were incubated overnight at 37 ℃ in the shaker at 250 rpm. The master plates were duplicated using 96-slot pin multi-blot replicator and it was stored at 4 ℃. The duplicate plates contained 200 μL LB medium supplemented with chloramphenicol (100 μg/mL), $MgCl_2$ (2 mM), thiamin hydrochloride (1 mM) and IPTG (1 mM). The resulting plates were incubated overnight at 37 ℃ with shaking at 250 rpm. Next, the plates were centrifuged (1600 g) and the medium was discarded. The cell pellets were resuspended with 200 μL NaH_2PO_4 (100 mM, pH 7.0) containing ThDP (1 mM), $MgCl_2$ (2 mM), lysozyme (1 mg/mL), and DNaseI (0.1 μg/mL). The plates were incubated at 37 ℃ for 30 min and centrifuged (1600 g). The supernatant was assayed for enzymatic activity (Figure 2.4).

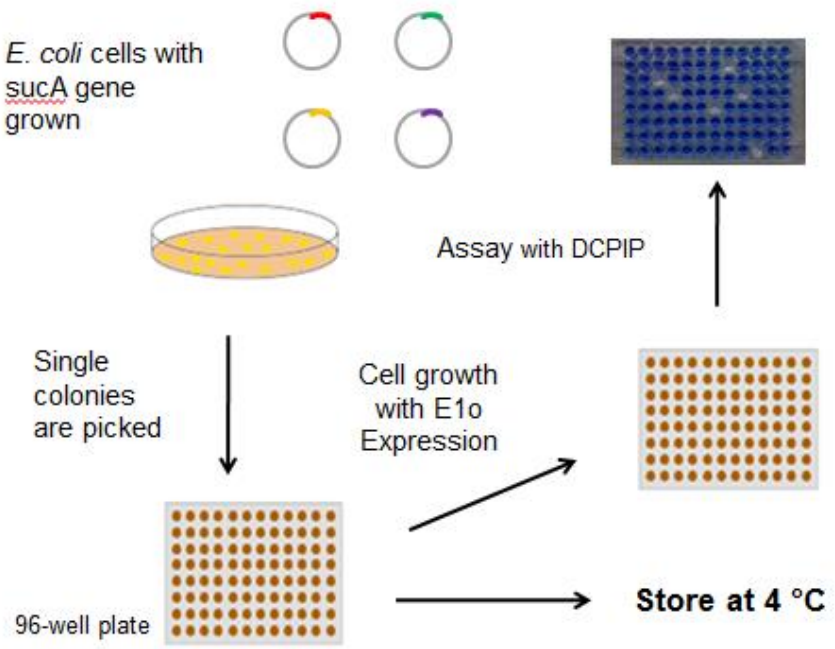


Figure 2.4 E1o Specific Screen for Alter Substrate Specificity.

E1o-specific activity was assessed using the external oxidizing agent 2,6-dichlorophenolindophenol (DCPIP) (Figure 2.5); its reduction was measured at 600 nm. The assay mixture contained: 110 μL NaH_2PO_4 (100 mM, pH 7.0), DCPIP (0.1 mM), $MgCl_2$ (2.0 mM), ThDP (1.0 mM), and 2-oxoglutarate (2.0 mM, 2-OG) or 2-oxovalerate (4.0 mM, 2-OV). The reaction was initiated by the addition of 40 μL of supernatant, and active variants were identified from the disappearance of the blue color upon DCPIP reduction. The endpoint absorbance was measured using the microtiter plate reader at 600 nm at 30 ℃. A rescreen was performed to eliminate false positives. Each positive clone was retrieved from the master plate and it was streaked on LB agar plates containing chloramphenicol (100 μg/mL). Single colonies from each clone were picked into a column of wells in a microtiter plate, and one column was reserved for the wild-type E1o. The rescreen was similar to the E1o assay described above. The positive clones for 2-OG or 2-OV were sequenced using ABI BigDye Terminator chemistry on an ABI 3130xl genetic analyzer (Molecular Resource Facility, University of Medicine and Dentistry of New Jersey, Newark).

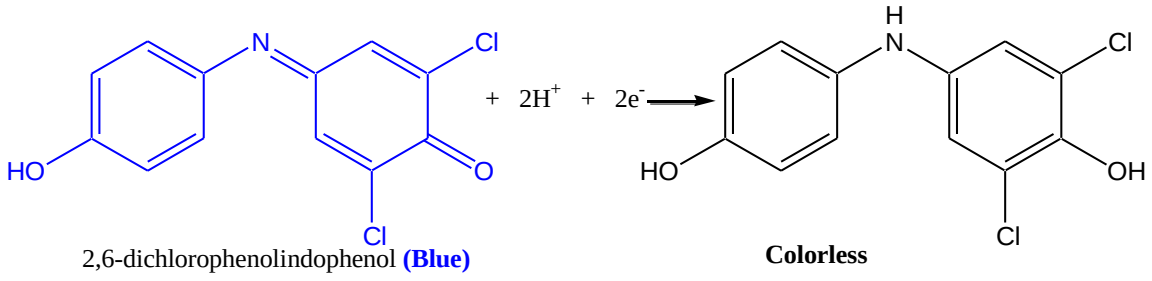


Figure 2.5 E1 Specific Colorimetric 2,6-Dichlorophenolindophenol Reduction Reaction.

2.3.3 Expression and Purification of Wild Type E1o and Its Variants

An E. coli AG1 frozen stock harboring the E1o plasmid was streaked on LB agar plates containing chloramphenicol (100 μg/mL) and incubated at 37 ℃ overnight. A single colony was used to inoculate 20 mL LB containing chloramphenicol (100 μg/mL). The overnight culture was used to inoculate 1 L of LB medium containing chloramphenicol (100 μg/mL), thiamin hydrochloride (1.0 mM), and $MgCl_2$ (2.0 mM). The culture was induced with IPTG (1.0 mM) at OD_{595} = 0.5-0.8 and incubated at 30 ^{o}C with shaking overnight. The cells were centrifuged at 4400 g at 4 ℃, and the pellet was stored at -20 ℃.

All subsequent steps were carried out at 4 ℃. The cells were resuspended in 20 mM KH_2PO_4 (pH 7.0) containing NaCl (0.1 M), $MgCl_2$ (2.0 mM), ThDP (1.0 mM), benzamidine hydrochloride (1.0 mM), PMSF (1.0 mM), lysozyme (0.6 mg/mL) and incubated on ice for 20 min. The cells were sonicated for 4 min (10 s pulsar "on" and 10 s pulsar "off") using the Sonic Dismembrator Model 550 (Fisher Scientific). The lysate was centrifuged at 30,000 g at 4 ℃ for 30 min. The supernatant was applied to a Ni Sepharose 6 Fast Flow Column (GE Healthcare) that was equilibrated with KH_2PO_4 (20 mM, pH 7.4), which contained NaCl (0.1 M), $MgCl_2$ (2 mM), ThDP (1 mM), benzamidine hydrochloride (1 mM). The enzyme was eluted with KH_2PO_4 (20 mM, pH 7.4) containing NaCl (0.5 M), imidazole (150 mM), $MgCl_2$ (2.0 mM), and ThDP (1.0 mM). Fractions with enzyme were combined, dialyzed against KH_2PO_4 (20 mM, pH 7.4) containing $MgCl_2$ (2.0 mM), ThDP (1.0 mM) and benzamidine hydrochloride (1.0 mM). Next, the enzyme was concentrated by ultrafiltration with a cutoff of 30 kDa. The purity was confirmed by SDS-PAGE. The E1o variants were overexpressed and purified using the procedure developed for wild-type E1o. Wild-type E1o and E1o variants were stored at -20 ℃.

2.3.4 Expression and Purification of Wild-type E2o

A single colony on the plate was used to inoculate 16 mL LB containing chloramphenicol (30 μg/mL). Next, the overnight culture was inoculated into LB (800 mL) containing chloramphenicol (30 μg/mL) and lipoic acid (0.3 mM), and cells were grown at 37 ℃ to an OD_{650} = 0.6 - 0.7. The E2o expression was induced by IPTG (0.1 mM) for 5 h at 37 ℃. Cells were collected, washed with KH_2PO_4 (20 mM, pH 7.0) containing NaCl (0.15 M) and stored at -20 ℃.

The E2o was purified, as was E2p [61] with some modifications. The precipitation of E2o by ammonium sulfate was replaced by ultracentrifugation at 117000 g for 4 h. E2o was purified using Sephacryl S-300 HR gel-filtration column equilibrated with 20 mM KH_2PO_4 (pH 7.2) containing 0.50 mM EDTA, 0.20 M NaCl, 1 mM DTT and 1 mM benzamidine HCl. Fractions containing E2o were collected and protein was precipitated by ultracentrifugation as described above. The pellet was dissolved in 20 mM KH_2PO_4 (pH 7.2) containing 0.50 mM EDTA, 1 mM DTT, 1 mM benzamidine HCl and 0.50 mM NaCl (the concentration of NaCl was increased from 0.2 M to 0.5M to keep protein soluble). E2o was stored at -80 ^{o}C. Expression and purification of wild type E3 was carried out according to Wei et al. [62].

2.3.5 Activity and Related Measurements

The E1-specific activity of wild type E1o and its variants was measured by monitoring the reduction of DCPIP at 600 nm using a Varian DMS 300 or DMS 500 spectrophotometer. The assay medium contained in KH_2PO_4 (20 mM, pH 7.0): $MgCl_2$ (2 mM), ThDP (0.2 mM), DCPIP (0.1 mM) and substrate [2-OG (2 mM), pyruvate (25 mM) or 2-OV (45 mM)]

at 30 ℃. The reaction was initiated by adding the enzyme. One unit of activity is defined as the amount of DCPIP reduced (μmol/min/mg of E1o).

The overall activity of the OGDHc was determined from the substrate-dependent reduction of NAD^+ at 340 nm after reconstitution for 20 min at 25 ℃ of E1o with E2o and E3 at a mass ratio of 1:1:1 (mg/mg/mg) in 20 mM KH_2PO_4 (pH 7.2) containing 0.15 M NaCl. The assay medium contained: 0.1 M Tris-HCl (pH 8.0), $MgCl_2$ (1.0 mM), ThDP (0.2 mM), dithiothreitol (2.6 mM), NAD^+ (2.5 mM), and substrate [2-OG (2 mM) or pyruvate (25 mM) or 2-OV (45 mM)]. The reaction was initiated by the addition of CoA (0.13 mM). The initial steady state velocities were determined from the progress curves recorded at 340 nm and 30 ℃. One unit of activity is defined as the amount of NADH produced (μmol/min/mg of E1o). The reaction conditions for the hybrid E1o-E2p-E3 and E1p-E2o-E3 complexes were the same. It should be noted that the E3 component is common to PDHc and OGDHc from *E. coli* [17, 18].

2.3.6 Circular Dichroism (CD) Measurement

CD experiments were carried out on a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK) at 30 ℃ by using the pyruvate and 2-OG analogs sodium acetylphophinate (AcP^-) [63], methyl acetylphosphonate (MAP^-) [64], propionyl-phosphinate (PP^-), succinylphosponate (SP^{2-}) and succinylphosphonate methyl ester ($SPME^-$) (Appendix). E1o and its variants (concentration of active centers = 20 -24 μM) in 20 mM KH_2PO_4 (pH 7.0) containing NaCl (0.15 M), $MgCl_2$ (2.0 mM) and ThDP (0.2 mM) were titrated with substrate analogs (0.5 – 2000 μM). The difference CD spectra were obtained by subtracting the CD spectrum of E1o or its variants in the absence of substrate analogs. The values of K_d were determined as described earlier [65].

2.3.7 Sample Preparation for Fourier Transform Mass Spectrometric Analysis (FTMS)

Electro-spray ionization FTMS was performed with an Apex-ultra 70 hybrid FTMS instrument from Bruker Daltonics (Billerica, MA). The E1p-E2o-E3 or E1o-E2p-E3 hybrid complexes were reconstituted by the same method as above. Sequentially, the complexes were incubated for 10 min in the reaction medium containing: Tris-HCl (0.1 M, pH 8.0), $MgCl_2$ (1.0 mM), ThDP (0.2 mM), dithiothreitol (2.6 mM), NAD^+ (2.5 mM), CoA (0.13 mM) and 2-OG (2 mM) or pyruvate (2 mM) at 25 ^oC. To quench the reaction and precipitate protein an acidic quench solution consisting of 12.5% trichloroacetic acid/1 M HCl was added. After the acid quench, protein was removed by centrifugation at 15,700 *g* for 15 min. For control, acetyl-CoA, succinyl-CoA and CoA stock solutions were prepared in deionized water and diluted to 0.13 mM in acidic quench solution. Then, 50 μL of each sample was mixed with 50 μL of solvent containing 0.1 % (v/v) formic acid and 50 % MeOH (v/v) in deionized water. These samples were then injected into FTMS at 2 L/min; 20 scans were acquired with a netflow rate of 1.0.

To further confirm the ability of E1o to accept pyruvate and use it in reductive acetylation of E2p, a mixture of E1o + pyruvate + lipoyl domain E2p (N-terminally His-tagged and purified to homogeneity on a Ni-Sepharose column) was used. Then quenched the mixture was quenched into acid quench solution and ran the ESI-FTMS on the product. Mass corresponding to reductively acetylated dihydrolipoyl domain E2p or succinylated dihydrolipoyl domain E2p was observed.

2.4 Results

2.4.1 Screening

Results of screening revealed that of the 352 colonies screened for His260 substitutions, 7 were found positive with 2-OV and 61 with 2-OG. Of the 7 colonies found positive for 2-OV, DNA sequencing revealed that all were wild-type E1o. Of the 8 colonies screened positive with 2-OG, 7 were identified as wild-type E1o and one was His260Glu. This immediately suggests that His260 is crucial for substrate binding. At position His298, 440 colonies were screened for 2-OG activity and 350 for 2-OV activity. DNA sequencing identified the His298Thr and His298Leu substitutions active with 2-OG, and the His298Asp and His298Val substitutions active with 2-OV. Screening for dual His260/His298 substitutions with 2-OG (1232 colonies) and with 2-OV (1672 colonies) revealed several active variants: His260/His298Thr (with 2-OG), His260/His298Asp, His260/His298Thr, and His260Glu/His298Asn (with 2-OV) (Table 2.2).

Table 2.2 Mutations from NNS Libraries

Screening	Number of colonies analyzed	Number of positive colonies	Number of sequenced colonies	Wild –type identified	Substitutions
His260 + 2-OG	352	61	7	6	Glu
His260 + 2-OV	352	6	0	0	none
His298 + 2-OG	440	154	6	3	Thr(2), Leu
His298 + 2-OV	352	65	3	0	Gly, Asp, Val
His260/His298 + 2-OG	1232	65	8	7	His260/Thr298
His260/His298 + 2-OV	1672	306	7	0	His260/Gly298(2) His260/Asp298(3) His260/Thr298(1) Glu260/Asn298(1)

2.4.2 The E1-specific activity

The E1-specific activity was unaffected when E1o was reconstituted into E1o-E2o-E3 or E1o-E2p-E3 complexes. Similar E1-specific activity was found with 2-OG, pyruvate or 2-OV using E1o by itself or assembled in the OGDHc or hybrid (E1o-E2p-E3) complex (Table 2.3 A). The activity of E1o was 24% towards pyruvate and 19% towards 2-OV compared to 2-OG. The E1-specific activity in complex reconstituted from E1o and the E2o and E3 components (36% with pyruvate and 21% with 2-OV) remained similar to that with E1o by itself. Reconstitution of E1o in the hybrid complex with E2p and E3, led to an E1-specific activity of 34% with pyruvate and 23% with 2-OV. This indicated that assembly into the OGDHc or hybrid complex does not affect significantly the E1-specific rates (Table 2.3 A). These results gave important evidence that pyruvate and 2-OV were substrates for E1o, as the DCPIP reduction assay clearly indicates that decarboxylation has taken place. No overall activity was detected with pyruvate or 2-OV for either the OGDHc or the hybrid E1o-E2p-E3 complex. The E1o-E2p-E3 hybrid complex exhibited detectable activity (2.2%) with 2-OG. In contrast to E1o, E1p displayed activity only with pyruvate. In the DCPIP assay, E1p by itself showed no activity towards 2-OG or 2-OV. Furthermore, there was no activity for 2-OG or 2-OV for E1p reconstituted with either E2p + E3 or E2o + E3. Similar results were obtained in the overall activity assay (Table 2.3 B).

Table 2.3 A: E1-specific and Reconstituted Complex Activity with E1o

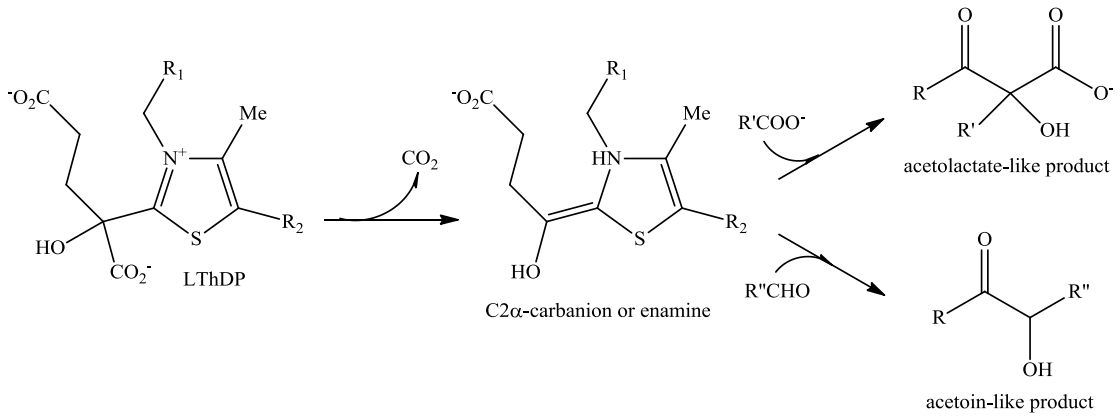
Substrate	DCPIP activity (μmol.min^{-1}.mg^{-1})			Overall activity (μmol.min^{-1}.mg^{-1} E1o)	
	E1o 	E1o-E2o-E3 	E1o-E2p-E3 	E1o-E2o-E3 	E1o-E2p-E3 
2-oxoglutarate	0.340 ± 0.007 (100%)	0.372 ± 0.018 (109%)	0.404 ± 0.013 (119%)	16.6± 0.44 (100%)	0.364 ± 0.010 (2.2%)
pyruvate	0.082 ± 0.004 (24%)	0.122 ± 0.012 (36%)	0.116 ± 0.011 (34%)	no activity detected	no activity detected
2-oxovalerate	0.064±0.001 (19%)	0.071 ± 0.034 (21%)	0.079 ± 0.014 (23%)	no activity detected	no activity detected
Product detected by FTMS	Not measured			Succinyl CoA (2-OG)	Succinyl-CoA (2-OG)

Table 2.3 B: E1-specific and Reconstituted Complex Activity with E1p

Substrate	DCPIP activity (μmol.min^{-1}.mg^{-1})			Overall activity (μmol.min^{-1}.mg^{-1} E1p)	
	E1p 	E1p-E2p-E3 	E1p-E2o-E3 	E1p-E2p-E3 	E1p-E2o-E3 
2-oxoglutarate	No activity detected	No activity detected	No activity detected	No activity detected	No activity detected
pyruvate	0.751 ± 0.01 (100%)	0.638 ± 0.07 (85%)	0.702 ± 0.01 (93%)	27.57 ± 1.01 (100%)	No activity detected
2-oxovalerate	No activity detected	No activity detected	No activity detected	No activity detected	No activity detected

To provide further evidence that pyruvate is indeed a substrate for E1o, these studies were conducted. (1) The carboligase side reactions commonly accompany

ThDP-catalyzed decarboxylations. These reactions involve nucleophilic addition of the enamine to the carbonyl carbon of reactant or product, resulting in the formation of acetoin-like or acetolactate-like ligated products (Scheme 2).



Scheme 2 Mechanism of Carboligase Reaction.

Observation of carboligase products provides strong confirmation that decarboxylation of substrate had taken place. On addition of pyruvate to E1o, a negative CD band developed at 300 nm, indicating formation of optically active (*R*)-acetolactate, (Figure 2.6). The negative circular dichroism (CD) band at 300 nm was still present after removal of protein; both the sign of the band and its location are similar to those observed with the Glu636Ala variant of E1p ([50], confirmed on that enzyme both by CD and NMR).

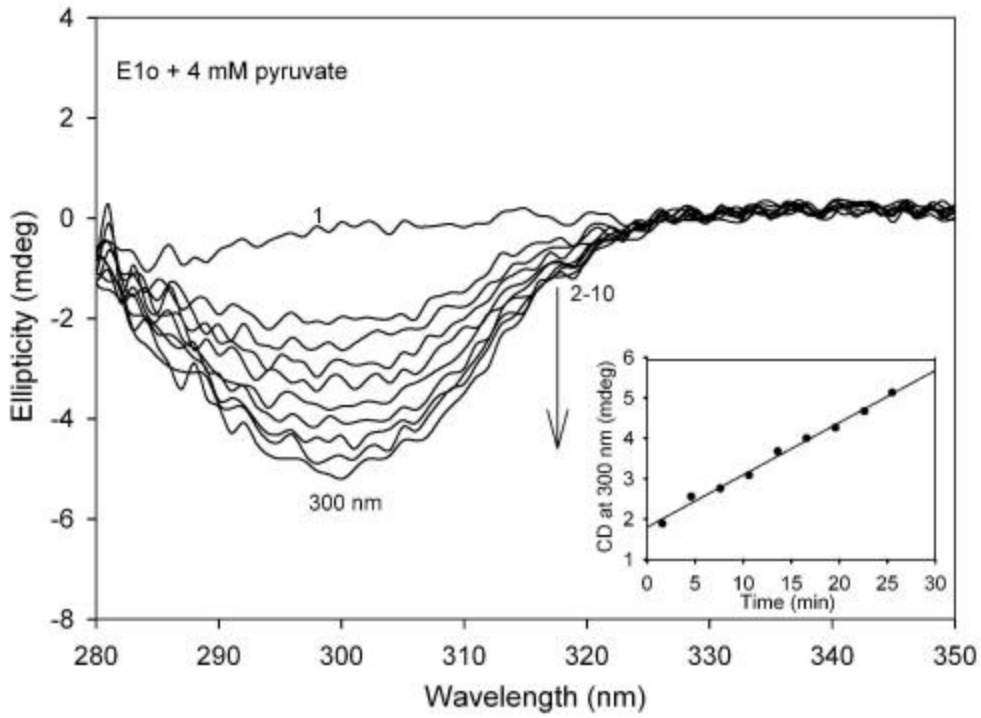


Figure 2.6 Near-UV CD Spectra of E1o in The Presence of Pyruvate. The E1o (13.2 μM Active Center Concentration) in 20 mM KH_2PO_4 (pH7.0) Containing 2.0 mM $MgCl_2$ and 0.20 mM ThDP (curve 1). CD Spectra of E1o after Addition of 4 mM Pyruvate at Different Times of Incubation (curves 2-10). Inset: The Dependence of the CD at 300 nm on Time of E1o Incubation with 4 mM Pyruvate.

Next, it was next demonstrated that the pyruvate decarboxylated by E1o could reductively acetylate lipoyl domain derived from E2p (LD-E2p), which could be detected by Fourier Transform Mass Spectrometry (FTMS). The rate constant for reductive acetylation of LD-E2p by (E1o + pyruvate) was 0.0056 + 0.001 s^{-1}, compared to 51.7 + 5.4 s^{-1} for (E1p + pyruvate) under the same conditions. The reductive acetylation of the didomain comprising lipoyl and subunit binding domains of the E2p was also demonstrated (not shown). Formation of succinyl-CoA by E1o-E2o-E3 and 2-OG was confirmed by both the isotopic pattern and m/z ratio (868.14) of a succinyl-CoA standard in FTMS (Figure 2.7). Next, E1o-E2p-E3 was reacted with 2-OG to produce detectable

amounts of succinyl-CoA (Table 2.3 A), displaying a similar isotopic distribution with the standard spectrum of succinyl-CoA (Figure 2.8).

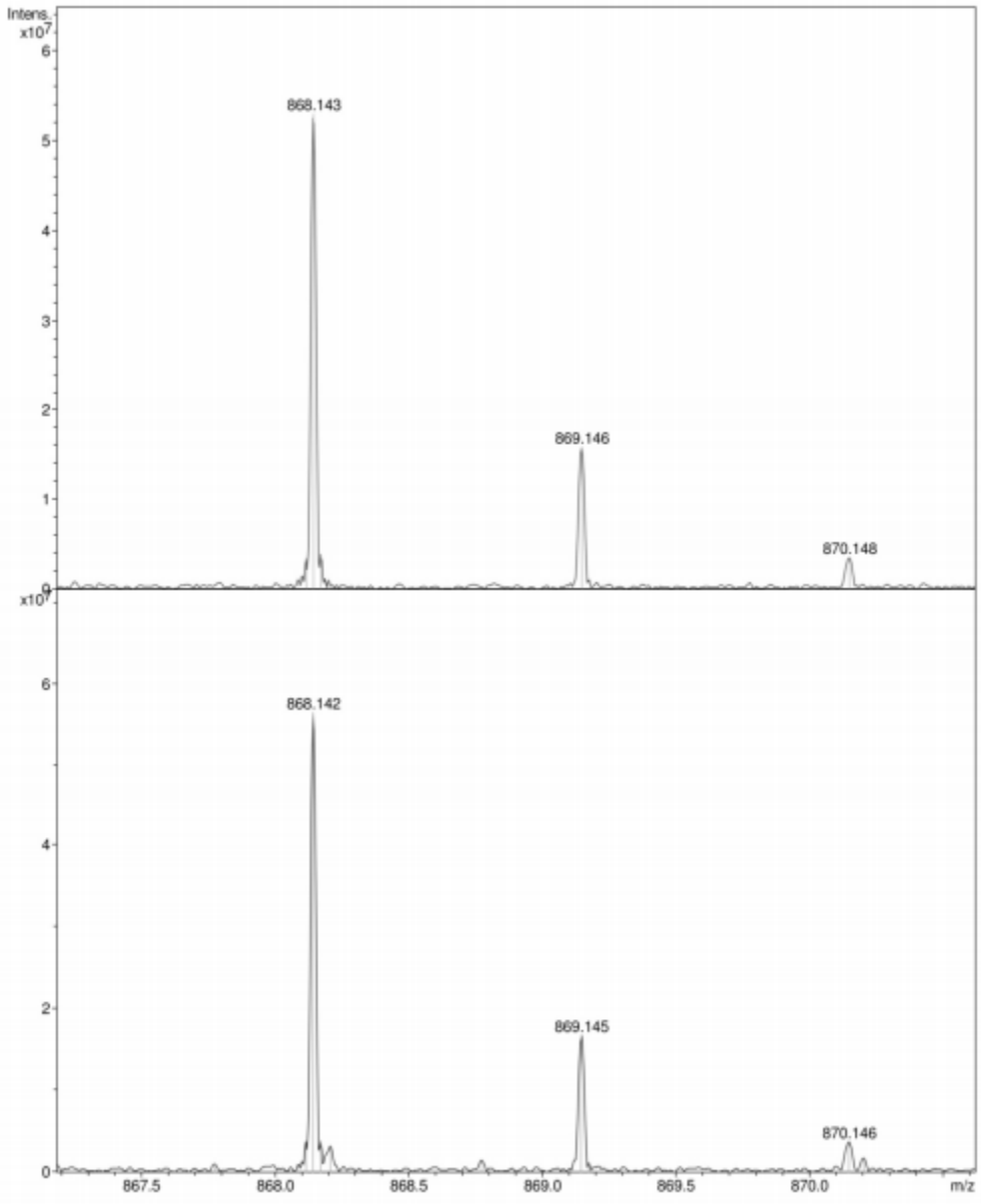


Figure 2.7 Mass Spectrum of Succinyl-CoA Standard (top) and as Produced by E1o-E2o-E3 with 2-OG (bottom).

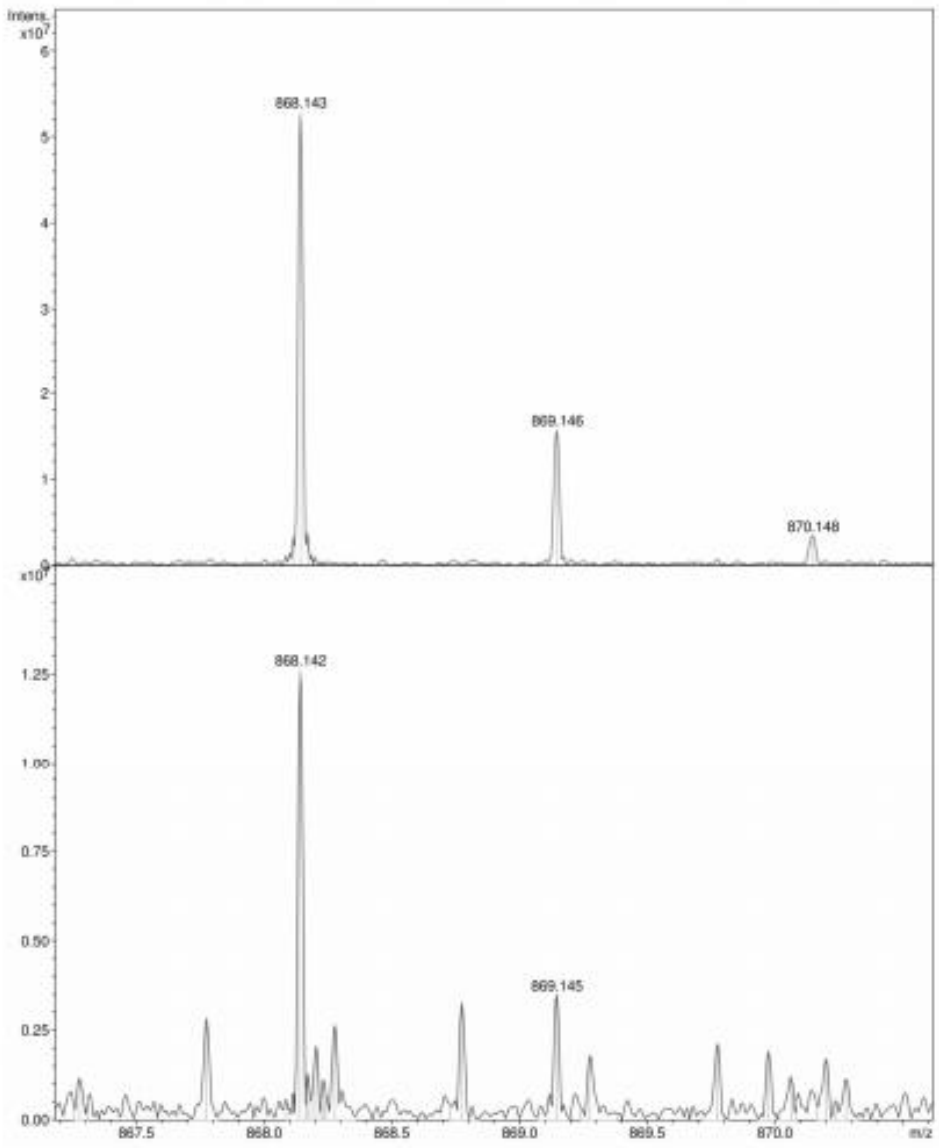


Figure 2.8 Mass Spectrum of Succinyl-CoA Standard (top) and as Produced by E1o-E2p-E3 with 2-OG (bottom).

These additional experiments, in part, were carried out to also address the finding by Frey's group that in the OGDHc isolated from *E. coli*, there is indeed found as much as 10% E1p [54], whose presence would confound our interpretation. It needs to be demonstrated that the pyruvate-decarboxylating activity displayed by E1o was not an

artifact of the presence of E1p. The fact that all of the components of OGDHc were His-tagged and independently overexpressed together in the experiment above rules out any significant contamination from intrinsic E1p components.

2.4.3 Effect of His260 and His298 Substitution of E1o Activity

The effect of His260 and His298 substitutions on E1o activity was examined. Saturation mutagenesis data revealed that His298 could tolerate substitution. The DCPIP activity for the E1o variants with 2-OG was ranged from less than 1% (His298Thr) to 19% (His298Leu) (Table 2.4 A). The K_M for 2-OG increased for some E1o variants, while the catalytic efficiency (k_{cat}/K_M) of all variants with 2-OG was lowered. The catalytic efficiency for the best E1o variants decreased ~7-fold for His298Leu and ~17-fold for His260Glu/His298Asn, and was severely compromised for other variants (Table 2.4 A). Remarkably, the His298Asp and His298Val substitutions converted E1o to 2-OV dehydrogenase with activities comparable to that observed with 2-OG (Table 2.4 B). The His298 substitution in E1o also affected the overall OGDHc activity (Table 2.5). The relative activities according to the E1-specific and overall activity assays were approximately paralleled. Finally, OGDHc did not show any overall activity towards 2-OV, again implying discrimination at the E2o level.

Table 2.4 Effect of His298 and His260/His298 Substitutions on E1-specific Activity of OGDHc

A: 2-Oxoglutarate as Substrate

Substitution	DCPIP activity (μmol.min^{-1}.mg^{-1})	k_{cat} (s^{-1})	K_M (mM x 10^{-3})	k_{cat}/K_M (s^{-1} mM^{-1})
none	0.620 ± 0.03[a]	2.15 ± 0.10	2.61 ± 0.376	824
His298Leu	0.120 ± 0.028	0.415 ± 0.098	3.41 ± 0.237	122
His298Thr	0.0018 ± 0.0001	0.0064 ± 0.0002	4.25 ± 0.160	1.5
His298Asp	nd [b]	nd	nd	nd
His298Val	0.029 ± 0.003	0.099 ± 0.009	165 ± 6	0.60
His260Glu/His298Asn	0.039 ± 0.004	0.134 ± 0.013	2.73 ± 0.469	49.1
His260Glu	0.0091 ± 0.0007	0.032 ± 0.003	383 ± 35.5	0.0084

[a] E1o was from a different preparation than in Table 2. [b] Not detectable above the background.

B: 2-Oxovalerate as Substrate

Substitution	DCPIP activity (μmol.min^{-1}.mg^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
none	0.022 ± 0.002	0.076 ± 0.007	16.3 ± 4.00	0.0047
His298Leu	0.027 ± 0.003	0.094 ± 0.011	6.30 ± 0.64	0.015
His298Thr	0.0086 ± 0.0009	0.030 ± 0.003	15.3 ± 1.00	0.0020
His298Asp	0.357 ± 0.018	1.24 ± 0.062	7.02 ± 0.023	0.18
His298Val	0.160 ± 0.005	0.556 ± 0.018	8.96 ± 0.005	0.062
His260Glu/His298Asn	0.017 ± 0.0008	0.060 ± 0.003	68.4 ± 4.05	0.00088
His260Glu	0.0039 ± 0.0001	0.014 ± 0.0003	0.12 ± 0.034	0.12

.

Table 2.5 Steady-state Kinetic Parameters for the Overall Activity of OGDHc Variants with 2-OG

Substitution	NAD^+ activity ($\mu mol.min^{-1}.mg^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
none	16.6 ± 0.44	57.5 ± 1.57	0.09 ± 3.61	639
His298Leu	3.80 ± 0.21	13.2 ± 0.51	0.27 ± 0.34	48.9
His298Thr	0.26 ± 0.01	0.90 ± 0.02	0.23 ± 0.04	3.91
His298Asp	0.2 ± 0.003	0.79 ± 0.01	0.30 ± 0.03	2.63
His298Val	0.28 ± 0.03	0.98 ± 0.09	0.15 ± 0.01	6.53
His260Glu/His298Asn	0.28 ± 0.01	0.97 ± 0.03	0.22 ± 0.02	4.41
His260Glu	0.40 ± 0.01	1.39 ± 0.02	0.72 ± 0.01	1.93

2.4.4 Analysis of Pre-decarboxylation Intermediate by Circular Dichroism

CD experiments revealed formation of a pre-decarboxylation intermediate analog between E1o and 2-oxophosphonate and 2-oxophosphinate analogs (Figure 2.9). The Jordan group at Rutgers has published extensively on CD detection of ThDP-bound covalent intermediates on enzymes with substrate mimics derived from methyl acetylphosphonate (MAP) and acetylphosphinate (AcP^-), which are analogs of pyruvate (Figure 2.9) [60, 63, 66].

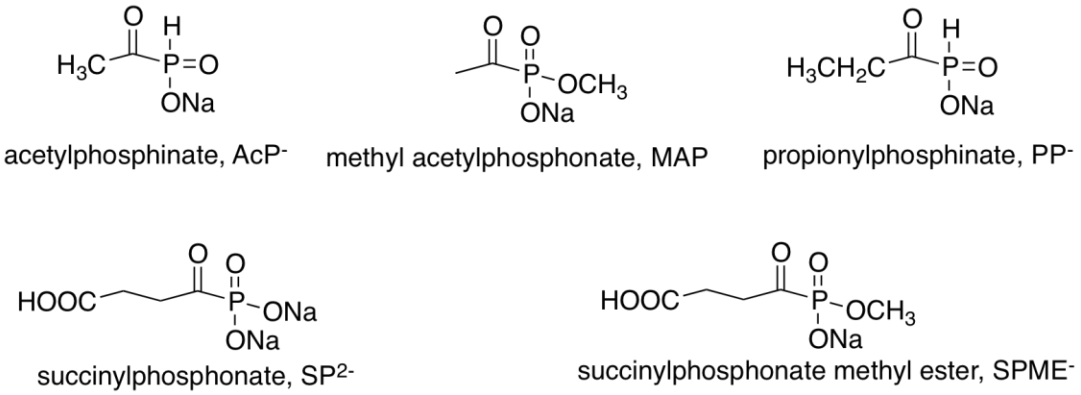


Figure 2.9 Phosphonate and Phosphinate Analogs Used in Experiments.

It had been reported that succinyl phosphonate (SP^{2-}) and its monomethyl phosphonate ester ($SPME^-$), which are analogs of 2-OG, inhibit partially purified OGDHc complex from brain with $S_{0.5,\ SP^-} = 0.12$ mM, which is in the range of values of $K_{M,\ 2\text{-}OG}$ =0.1-0.2 mM reported for OGDHc from different sources [67]. The inhibitory effect of $SPME^-$ was also demonstrated for OGDHc from *E. coli* and pigeon breast muscle [68] and very recently for MenD [69]. MenD is also a thiamin diphosphate-dependent enzyme involved in the biosynthesis of menaquinone in bacteria. Initially, E1o was titrated with AcP^- because this analog was found to bind strongly to a number of ThDP enzymes [60]. CD spectra of E1o titrated with AcP^- revealed the generation of two CD bands: a positive one at 297 nm earlier assigned to the 1',4'-iminopyrimidine tautomer (IP) of the first covalent intermediate and a negative one at 330 nm assigned to a Michaelis complex [66] (Figure 2.10).

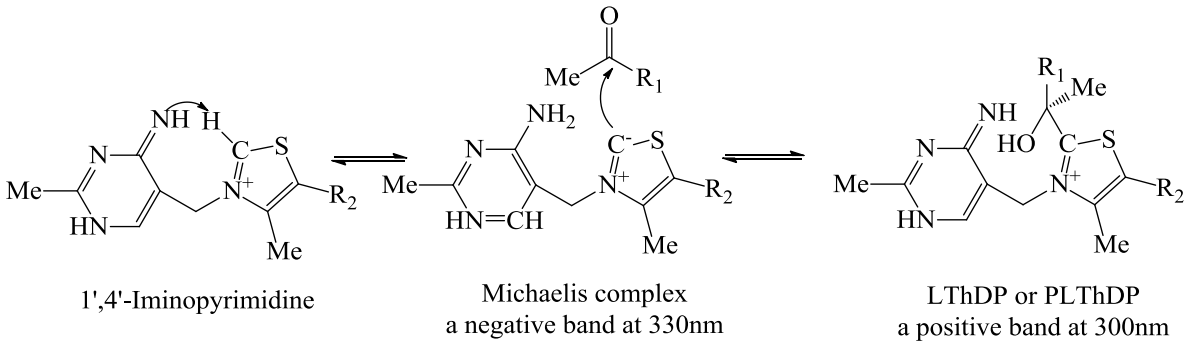


Figure 2.10 Michaelis Complex and 1',4'-Iminopyrimidine (IP)-ThDP Forms Detected by CD.

The calculated values of K_d for AcP^- were 320 μM (at 297 nm) and 310 μM (at 330 nm) (Figure 2.11, Table 2.6) as compared with $K_{M,\ 2\text{-}OG}$ of 90 μM (Table 2.5). Later, $SPME^-$, SP^{2-} and PP^-, analogs for 2-OG and 2-OV, respectively, were evaluated in the CD experiment (Table 2.6, Figures 2.12 and 2.13).

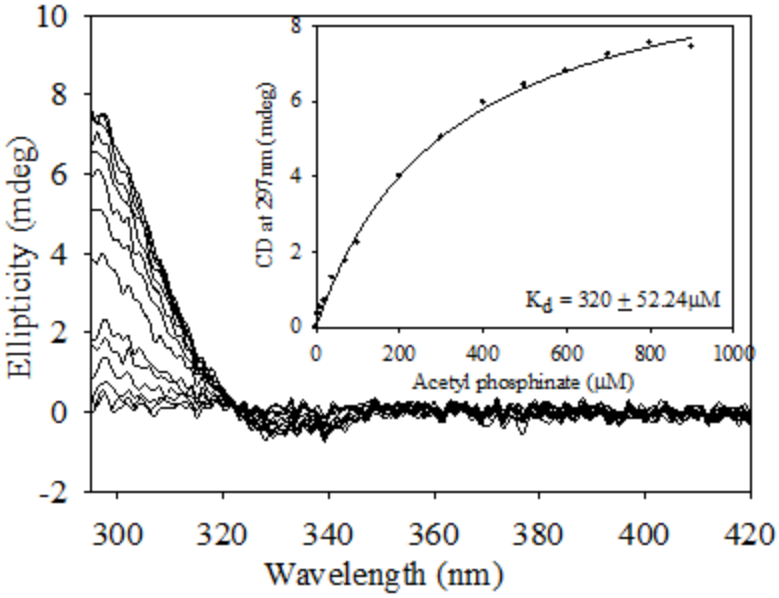


Figure 2.11 Circular Dichroism Titration of E1o by Acetyl Phosphinate. E1o (1.62 mg/mL, Concentration of Active Centers = 15.5 μM) was Titrated by AcP^- (5-800 μM) in 20 mM KH_2PO_4 (pH 7.0) Containing 2 mM $MgCl_2$ and 0.2 mM ThDP at 30 ^oC. Inset: Dependence of the Ellipticity at 297 nm on Concentration of AcP^-.

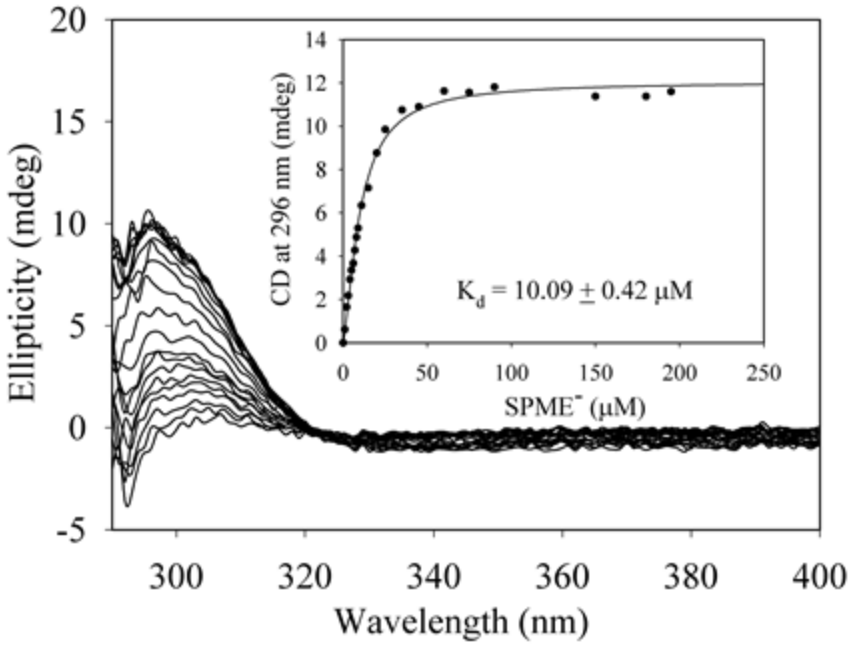


Figure 2.12 Circular Dichroism Titration of E1o with Succinyl Phosphonate Methyl Ester. E1o (2.5 mg/ mL, Concentration of Active Centers = 23.6 μM) in 20 mM KH_2PO_4 (pH 7.0) was Titrated by $SPME^-$ (1-200 μM) in 20 mM KH_2PO_4 (pH 7.0) Containing 2 mM $MgCl_2$ and 0.20 mM ThDP. Inset: Dependence of Ellipticity at 300 nm on the Concentration of $SPME^-$.

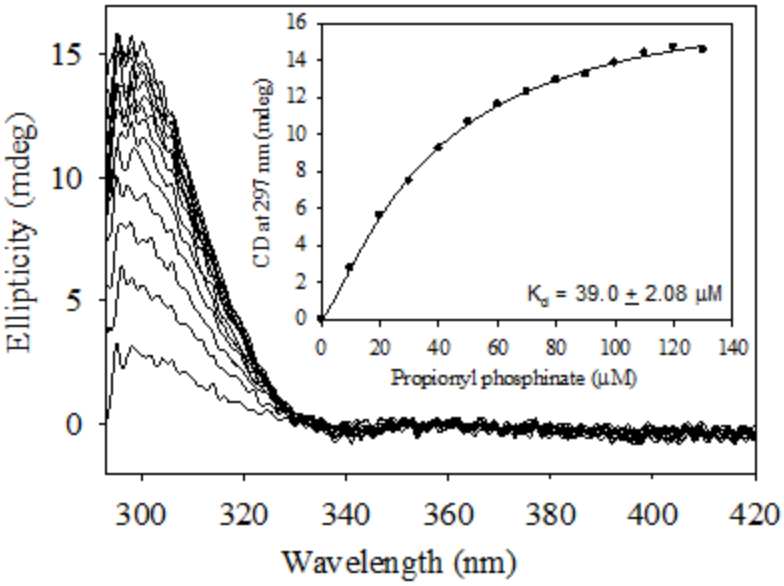


Figure 2.13 Circular dichroism titration of E1o with propionyl phosphinate. E1o (2.39 mg/mL, concentration of active centers = 22.6 μM) in 20 mM KH_2PO_4 (pH 7.0) containing 2 mM $MgCl_2$, 0.20 mM ThDP and 1.5 mM NaCl was titrated by PP^- (30-140 μM). Inset: Dependence of the ellipticity at 297 nm on concentration of PP^-.

Table 2.6 Circular Dichroism Determination of the Formation of Pre-decarboxylation Intermediate between E1o Variants and Substrate Analogs

E1o substitution	Substrate analogs	CD maximum (nm)	K_d (μM)
wt	AcP^-	297	320 ± 52.2
wt	MAP	No band detected	-
wt	PP^-	300	39.0 ± 2.08
wt	SP^{2-}	300	28.4 ± 1.12
wt	$SPME^-$	297	10.1 ± 0.42
His298Leu	$SPME^-$	297	Not saturated
His298Leu	PP^-	300	22.3 ± 0.98
His298Thr	$SPME^-$	297	6.07 ± 0.53
His298Thr	PP^-	297	7.00 ± 0.23
His298Asp	$SPME^-$	298	659 ± 11.5
His298Asp	PP^-	300	9.61 ± 0.20
His298Val	$SPME^-$	297	144 ± 11.6
His298Val	PP^-	297	5.17 ± 1.04
His260Glu/His298Asn	$SPME^-$	No band detected	-
His260Glu/His298Asn	PP^-	305	7.69 ± 2.50
His260Glu	$SPME^-$	No band detected	-
His260Glu	PP^-	No band detected	-

These are the first CD experiments to demonstrate that on addition of substrate analogs, E1o forms a tetrahedral ThDP-bound pre-decarboxylation intermediate analog, resembling those formed from substrates. The K_d values determined are in the μM range ($SPME^-$ phosphonate monoester gives the best K_d =10 μM, while the di-acid SP^{2-} is approximately three times weaker) demonstrating that some of the tested compounds could be powerful inhibitors of the OGDHc E1o component (Table 2.6). Similar CD experiments with the His298 E1o variants by phosphonate and phosphinate analogs of 2-oxoacids (Appendix) indicated that this substitution is not favorable for binding of $SPME^-$,

excepting His298Thr. For the His260Glu/His298Asn variant, no CD band was detected at 300 nm. On the other hand, His298 substitutions and the double His260Glu/His298Asn substitution were favorable for binding of PP^-, which is a substrate analog for 2-OV. The His298Asp variant has a K_d value of 9.6 μM as compared with 39 μM for E1o (Figure 2.14, Table 2.6). In general, the $K_{d,\ PP}^-$ were smaller (binding was stronger) than that for E1o, and $K_{d,\ PP}^-$ ranged from 5.17–22.3 μM (Appendix).

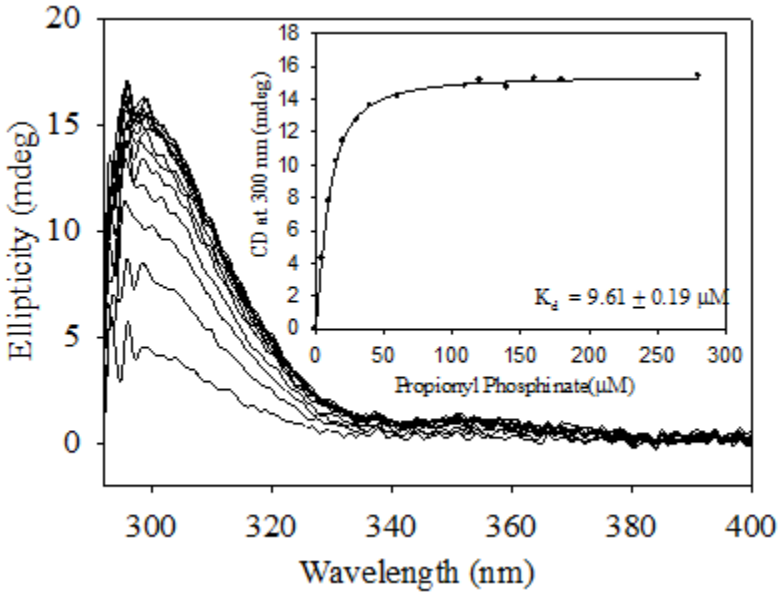


Figure 2.14 Circular dichroism titration of His298Asp with propionyl phosphinate. The His298Asp E1o (2.5 mg/mL, concentration of active centers=23.8 μM) in 20 mM KH_2PO_4 (pH 7.0) containing 2 mM $MgCl_2$ and 0.2 mM ThDP was titrated by PP^- (5-580 μM). Dependence of the ellipticity at 300 nm on the concentration of PP^-.

2.5 Discussion

The following could be concluded about the roles of His260 and His298 in E1o. The His260Glu, His298Thr, His298Val and His298Leu substitutions displayed activity with 2-OG. The His298Asp and His298Val substitutions led to active enzyme with 2-OV, displaying improvement in k_{cat}/K_M in comparison to the E1o. While finding a positively

charged or hydrophilic side chain in its place could be anticipated, the most active variant, His298Leu is unexpected, with a K_M comparable to that of E1o. Being only slightly larger than histidine, this substitution to leucine may only fulfill a volume constraint (the van der Waals volumes for leucine and histidine are 124 $Å^3$ and 118 $Å^3$, respectively). A study on the active center residues of the ThDP enzyme benzoylformate decarboxylase led to a similar conclusion on residue His281: it could be replaced by phenylalanine or leucine without significant activity loss [70]. Randomization at His260 was yielded only one E1o variant (His260Glu) with low activity toward 2-OG and much better activity towards 2-OV.

The active variants identified by E1-specific assay were shown to be functionally competent according to their ability to form pre-decarboxylation covalent intermediate analogs between the ThDP and phosphono- or phosphino- analogs of 2-OG and 2-OV as judged by CD. The values of K_d calculated from CD data are in the μM range as compared with reported values of $K_{M,\ 2\text{-oxoglutarate}}$ = 0.1 - 0.2 mM [67], and point to increasing binding potency with increasing chain length. For all His298 variants, PP^- was more firmly bound than $SPME^-$, suggesting conversion of function from 2-oxoglutarate dehydrogenase to 2-oxovalerate dehydrogenase, especially with the His298Asp and His298Val variants, which display relatively high activity with 2-OV. The single low activity His260Glu E1o variant did not display measurable CD signal with PP^- or $SPME^-$, consistent with kinetic analysis. The randomization experiment, kinetic study and CD detection of covalent intermediate analogs provide strong evidence that His260 is crucial and indispensable for 2-OG recognition.

Surprisingly, the E1o could decarboxylate 2-OV and pyruvate, in addition to 2-OG according to the DCPIP assay, an assay adequate to confirm decarboxylation of the substrates. To interrogate whether the second component would enable synthesis of acyl-coenzyme A derivatives from substrates accepted by the engineered E1o, hybrid complexes consisting of recombinant components of the *E. coli* OGDHc (o) and PDHc (p) were constructed (the E3 component is common to both). On reconstitution of E1o with E2o and E3 in OGDHc, the overall activity with 2-OG was 16.6 mol.min^{-1}.mg E1o^{-1} and correlated well with recently published data on E1o [6]. No NADH production was detected with pyruvate or 2-OV in the E1o-E2o-E3 and E1o-E2p-E3 hybrid complexes. Detection of succinyl-CoA formation by mass spectrometry, and of reductively acetylated and succinylated LD-E2p, and of activity of the reconstituted complex by NADH kinetic assay, allowed us to conclude that: (1) E1o-E2o-E3 and E1p-E2p-E3 produced the respective acyl-CoA products and NADH; (2) E1o-E2p-E3 could produce succinyl-CoA from 2-OG; (3) E1p-E2o-E3 could not produce acetyl-CoA from pyruvate; and (4) E1o could reductively acetylate, and reductively succinylate LD-E2p. Apparently, a different component is the 'gatekeeper' for specificity for acyl-CoA formation by these two important multienzyme complexes in Gram-negative bacteria, E1p for pyruvate, but E2o for 2-OG. The ability of E1o to reductively acetylate LD-E2p, and the ability of the hybrid E1o-E2p-E3 to produce succinyl-CoA provide strong confirmation of this statement.

The principal difference between this and earlier work is that here recombinant individual components were used, while earlier work used isolated complexes, PDHc and OGDHc, or their sub-complexes. Notably, Frey and associates demonstrated that 10% E1p co-purified with *E. coli* OGDHc, however, the overall activity was about 1% of that with

PDHc, already suggesting that the E2o also conferred substrate specificity in terms of rates [54]. deKok and coworkers used the OGDHc isolated from *Azotobacter vinelandii* and found modest overall activity with pyruvate, but no E1-specific activity was detected, in contrast to our findings [71]. 'Promiscuous' substrate utilization has been identified in several ThDP enzymes: 'engineering' by single amino acid substitutions has been shown to lead to changing both substrate and reaction specificity from a decarboxylase/dehydrogenase activity to carboligase-like activity in yeast pyruvate decarboxylase [72], E1p [50], benzoylformate decarboxylase [73, 74] among others; the specificity of acetohydroxyacid synthase toward pyruvate as donor has been attributed to hydrophobic residues [75].

These results rule out an acid-base or hydrogen-bonding role for the residue His298, but confirm a hydrogen-bonding role for His260. Hence, to create complexes capable of accepting alternate 2-oxoacids, it will also be necessary to engineer the E2o active center.

CHAPTER 3

THE E2 COMPONENT OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX FROM *ESCHERICHIA COLI* ALSO DICTATES SUBSTRATE SPECIFICITY, IN ADDITION TO THE E1 COMPONENT

3.1 Introduction

In Chapter 2, it was shown that the His298Asp E1o variant could accept the unnatural substrate 2-oxovalerate (2-OV) at a faster rate than the natural substrate 2-oxoglutarate (2-OG) (Figure 3.2).

A. **B.**

2-oxoglutarate (2-OG) 2-oxovalerate (2-OV)

Figure 3.1 Substrates for E1o. **A:** 2-Oxoglutarate (Natural Substrate). **B:** 2-Oxovalerate (Unnatural Substrate).

Next, His298Asp E1o was reconstituted with E2o and E3 and the multienzyme complex did not generate NADH in the overall reaction when using as 2-OV (Equation 3.1). Therefore, the overall complex failed to make the butyryl-CoA derivative.

$$\text{2-oxovalerate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{butyryl-CoA} + \text{CO}_2 + \text{NADH} \qquad (3.1)$$

The E2o component consists of an amino-terminal lipoyl domain (E2oLD, 12 kDa) [17], followed by a peripheral E1o and E3 subunit binding domain (4 kDa) and a catalytic domain (E2oCD, 28 kDa) (Figure 3.1).

Figure 3.2 Domains in E2o of OGDHc from *E. coli*.

There are two possibilities for the failure to form NADH from 2-OV. Substrate specificity is determined at on two levels: reductive acylation is not occurring in the E2oLD or acyl transfer is not taking place from E2oCD to CoA in E2oLD (Figure 3.3). It is also possible that neither E2oLD nor E2oCD is capable of accepting the unnatural substrate. The *E. coli* E2oLD crystal and NMR structures have been determined [7, 8] (Figure 3.3). In addition, the structure of E2oCD has been solved.

A. **B.**

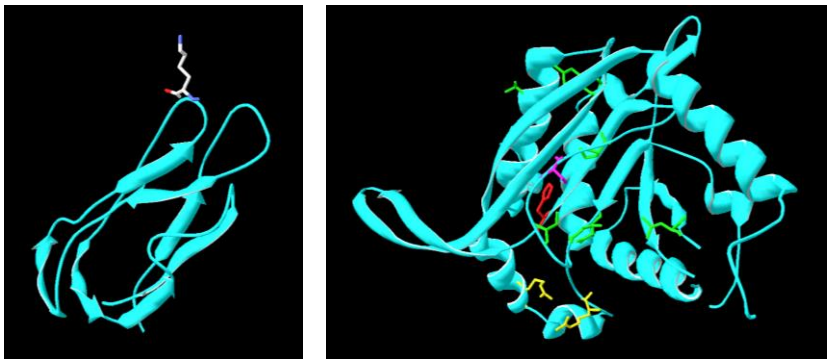


Figure 3.3 A: Ribbon Diagram of the Lipoyl Domain of E2o (E2oLD). Lys43 is Shown as Sticks. **B:** Ribbon Diagram of E2o Catalytic Domain (E2oCD). The Carboxyl-binding Residue (Ser333) is Shown in Magenta. The Carboxylate-binding Group (His348) is Shown in Red.

Initially, the E1o component that contains the ThDP-bound post-decarboxylation intermediate interacts with E2oLD, resulting in reductive acylation of the E2o-bound lipoic acid. It is known that the lipoyl domains from the pyruvate dehydrogenase complex (PDHc) and OGDHc complexes only function as efficient substrates for their respective E1p and E1o components [76-78]. As a result, lipoyl domains may be critical to the specificity of those multienzyme complexes.

The ability of E2oLD to undergo reductive acylation was first examined. If E2oLD does not accept the substrate from E1o then E2oLD necessitates engineering. If E2oLD does accept the substrate then E2oCD requires optimization. Experiments were designed to test these possibilities. First, E1o will be incubated with E2oLD. It was previously shown that wild-type E1o accepts 2-OV (Chapter 2). Then, 2-OV will be added and electrospray ionization mass spectrometry (ESI-MS) will be performed to detect whether reductive acylation has occurred. 2-OG will be used as a positive control. Second, the E1o will be incubated with E2pLD from PDHc from *E. coli*. It should be noted that E2pLD has three lipoyl domains. Then, 2-OG will be added and ESI-MS will be performed to detect whether reductive acylation has occurred. It was previously demonstrated that E1o-E2p-E3 was active with 2-OG in the overall reaction. When 2-OV was substituted for 2-OG, the reconstituted complex was inactive in the overall reaction. In addition, similar analysis was carried out with the E1o H298D variant, which showed higher activity toward the unnatural substrate 2-OV.

3.2 Materials

2-Oxoglutarate and 2-oxovalerate, formic acid and methanol were from Sigma-Aldrich. Restriction enzymes were purchased from Invitrogen. Lipoyl protein ligase, ATP and DL-α-lipoic acid were from USB. Primers were obtained from Fisher. *E. coli* strain JW0715 containing the plasmid pCA24N encoding the OGDHc-E1 (E1o) component [ASKA clone (-)] was obtained from National Bio Resource Project (NIG, Japan). Amicon® Ultra-4 Centrifugal Filter Units are from EMD Millipore. Vivaspin 500 units are from GE Healthcare Biosciences, Piscataway, NJ.

3.3 Methods

3.3.1 Protein Expression and Purification

The expression and purification of E1o, E1o H298D, E2o, and E3 were described in Chapter 2.

E. coli cells from a frozen stock, which harbor the plasmid containing E2oLD, were streaked on LB agar plates containing chloramphenicol (30 μg/mL) and incubated at 37 ℃ overnight. A single colony was used to inoculate 20 mL LB containing chloramphenicol (30 μg/mL). The overnight culture was used to inoculate 1 L of LB medium containing chloramphenicol (30 μg/mL) and lipoic acid (0.3 mM). The culture was induced with IPTG (1.0 mM) when the OD_{600} reached 0.6 and incubated at 37 ^{o}C for 5 h with shaking at 250 RPM overnight. The cells were centrifuged at 4400 *g* at 4 ℃, and the pellets were stored at -20 ℃.

All subsequent steps were carried out at 4 ℃. The cells were resuspended in KH_2PO_4 (20 mM, pH 7.2) containing NaCl (0.5 M), EDTA (0.50 mM), DTT (1.0 mM),

benzamidine hydrochloride (1.0 mM), PMSF (1.0 mM), lysozyme (0.6 mg/mL) and incubated on ice for 20 min. The cells were sonicated for 4 min (10 s pulsar "on" and 10 s pulsar "off") using the Sonic Dismembrator Model 550 (Fisher Scientific). The lysate was centrifuged at 30,000 *g* at 4 ℃ for 30 min. The supernatant was applied to a Ni Sepharose 6 Fast Flow Column (GE Healthcare) that was equilibrated with KH_2PO_4 (20 mM, pH 7.2), which contained NaCl (0.5 M), EDTA (0.50 mM), DTT (1.0 mM), benzamidine hydrochloride (1 mM). The enzyme was eluted with KH_2PO_4 (20 mM, pH 7.2) containing NaCl (0.5 M), imidazole (150 mM), and EDTA (0.5 mM). Fractions with enzyme were combined, dialyzed against KH_2PO_4 (20 mM, pH 7.2) containing NaCl (0.5 M), EDTA (0.5 mM) and benzamidine hydrochloride (1.0 mM). Next, the enzyme was concentrated by ultrafiltration with a cutoff of 30 kDa. The purity was confirmed by SDS-PAGE.

E2pLD was purified as described previously [62] except that no DL-α-lipoic acid was added to cell culture.

3.3.2 Lipoylation of E2pLD

As shown by ESI-FTMS, the purified E2oLD was fully lipoylated, while E2pLD was completely unlipoylated. To achieve full lipoylation of E2pLD, the lipoylation reaction for E2pLD was performed *in vitro* with *E. coli* lipoyl protein ligase [78]. E2pLD (50 μM) was incubated in ammonium bicarbonate (20 mM, pH 7.0) containing ATP (1.2 mM), $MgCl_2$ (1.2 mM), DL-α-lipoic acid (0.6 mM), lipoyl protein ligase (0.8 μM,) for 1 h at room temperature.

3.3.3 Reaction of the Enzyme with Substrates

The purified proteins (E2oLD, wild-type E1o, and E1o H298D) in KH_2PO_4 buffer (20 mM, pH 7.2) and lipoylated E2pLD were dialyzed against ammonium bicarbonate (20 mM, pH 7.0) buffer by Vivaspin 500 units (3K MWCO for E2oLD and E2pLD; 10K MWCO for wild-type E1o and E1o H298D). Wild-type E1o or E1o H298D (1 or 30 μM) and E2oLD or E2pLD (30 μM) were incubated in ammonium bicarbonate (20 mM, pH 7.0) containing $MgCl_2$ (0.1 mM), thiamin diphosphate (ThDP, 0.05 mM) and substrate (2-OG or 2-OV, 5 mM) for 10 min at room temperature.

3.3.4 Measurement of the Acylation of E2oLD and E2pLD with ESI-FTMS

Mass spectrometric analysis was carried out on a Bruker Daltonics Apex Ultra 7.0 FT-MS Mass Spectrometer. All protein samples were diluted into 1 μM with a buffer consisting of a 50: 50: 0.1 (v: v: v) mixture of methanol, water and formic acid. The samples were introduced into the mass spectrometer through a KD Scientific KDS-100 syringe pump at a flow rate of 120 μL/h. Mass spectra were acquired with electrospray in the positive ionization mode. To achieve high accuracy in the mass measurement, the mass spectrometer was calibrated using Agilent ES Tuning Mix over a mass to charge ratio of 300 to 3,000. The acquired mass spectra were analyzed and deconvoluted with Bruker Daltonics DataAnalysis 4.0.

3.4 Results and Discussion

3.4.1 ESI-MS Analysis of E2oLD in the Reaction with E1o and 2-OV

Here, the most abundant isotopic mass is used for mass comparison. The theoretical mass was calculated by Bruker Daltonics IsotopePattern 1.3, and the experimental mass was read from Bruker Daltonics DataAnalysis 4.0.

As a control, it was shown by ESI-MS that E1o, E2oLD and 2-OG forms succinylated E2oLD (Figure 3.4). The theoretical mass difference between succinylated E2oLD (11523.82 Da) and E2oLD (11422.82 Da) is 101.00 Da. The experimental value was 100.01 Da. Next, E1o, E2oLD, and 2-OV were incubated together and the reaction mixture was analyzed by ESI-MS. E2oLD was reductively butyrylated by E1o (Figure 3.5). The theoretical mass difference between butyrylated E2oLD (11493.85 Da) and E2oLD (11422.85 Da) was 71.85 Da. The experimental value is 71.85 Da. However, E1o-E2o-E3 could not produce NADH in the overall reaction with 2-OV. Hence, the reaction is stopped in E2oCD, i.e., the catalytic center responsible for forming acyl-CoA from acyl-dihydrolipoyl-E2o would not accept the butyryl group. (Figure 3.5)

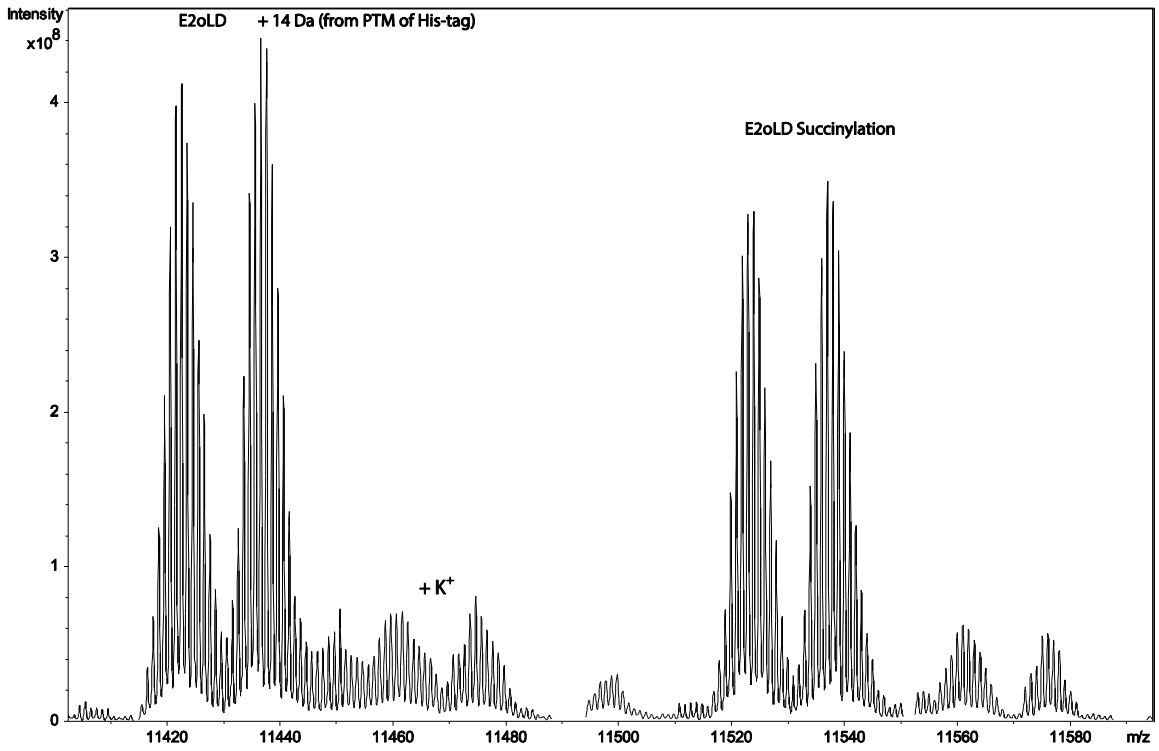


Figure 3.4 Mass Spectrum of Succinylated E2oLD Produced by E1o and 2-OG.

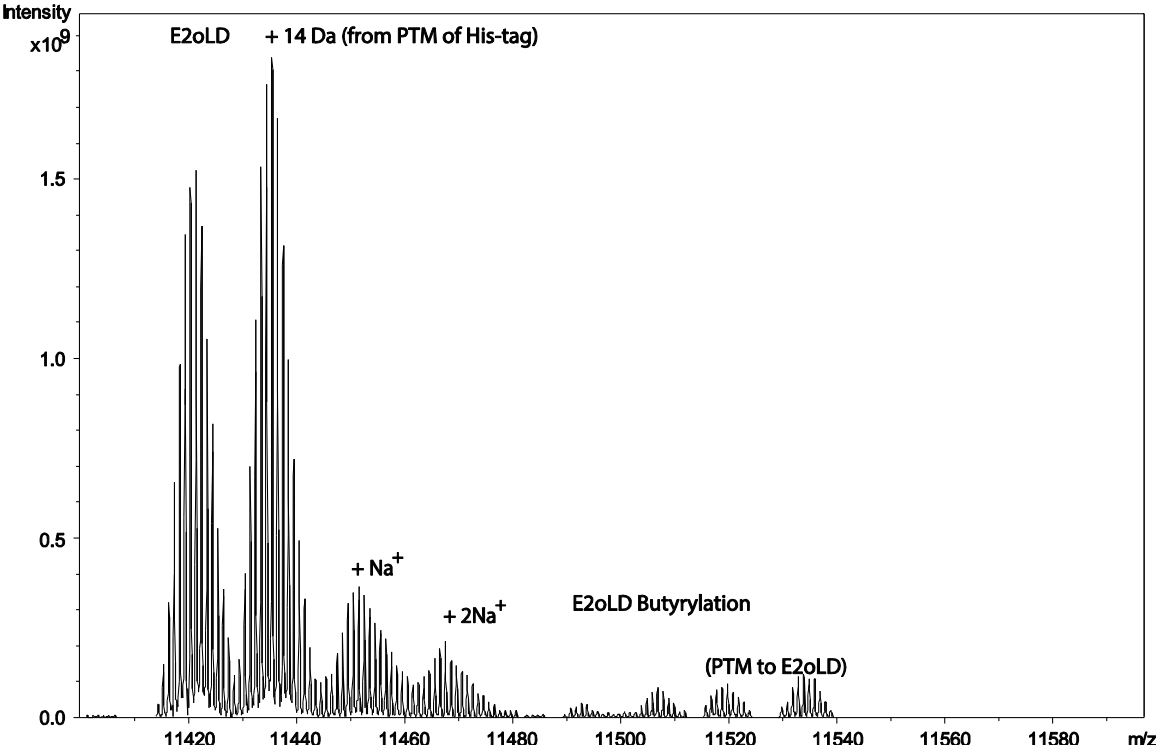


Figure 3.5 Mass Spectrum of Butyrylated E2oLD Produced by E1o and 2-OV.

3.4.2 ESI-MS Analysis of E2oLD in the Reaction with H298 E1o and 2-OV

It was previously shown that His298Asp E1o reconstituted with E2o-E3 produces NADH in the overall reaction with 2-OG. As control, His298Asp E1o, E2oLD, and 2-OG produced succinylated E2oLD (Figure 3.6). The experimental value for the difference of succinylated E2oLD (11422.85 Da) and E2oLD (11522.86 Da) was 100.01 Da, is in agreement with the theoretical value. Next, His298Asp E1o, E2oLD, and 2-OV were incubated together and the reaction mixture was analyzed by ESI-MS. E2oLD was reductively butyrylated by E1o (Figure 3.7) and the experimental value 71.05 Da, which is in agreement with the theoretical value. However, His298Asp E1o-E2o-E3 could not produce NADH in the overall reaction. Hence, the reaction is stopped at E2oCD.

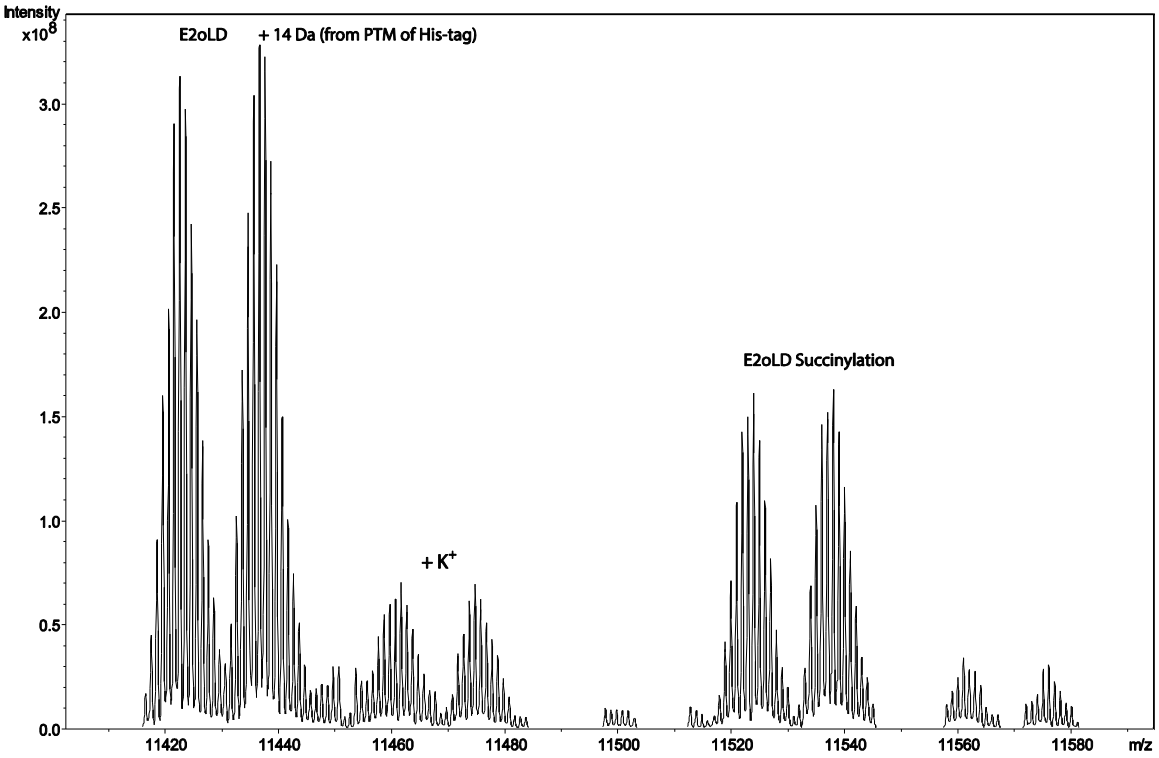


Figure 3.6 Mass Spectrum of Succinylated E2oLD Produced by His298Asp E1o and 2-OG.

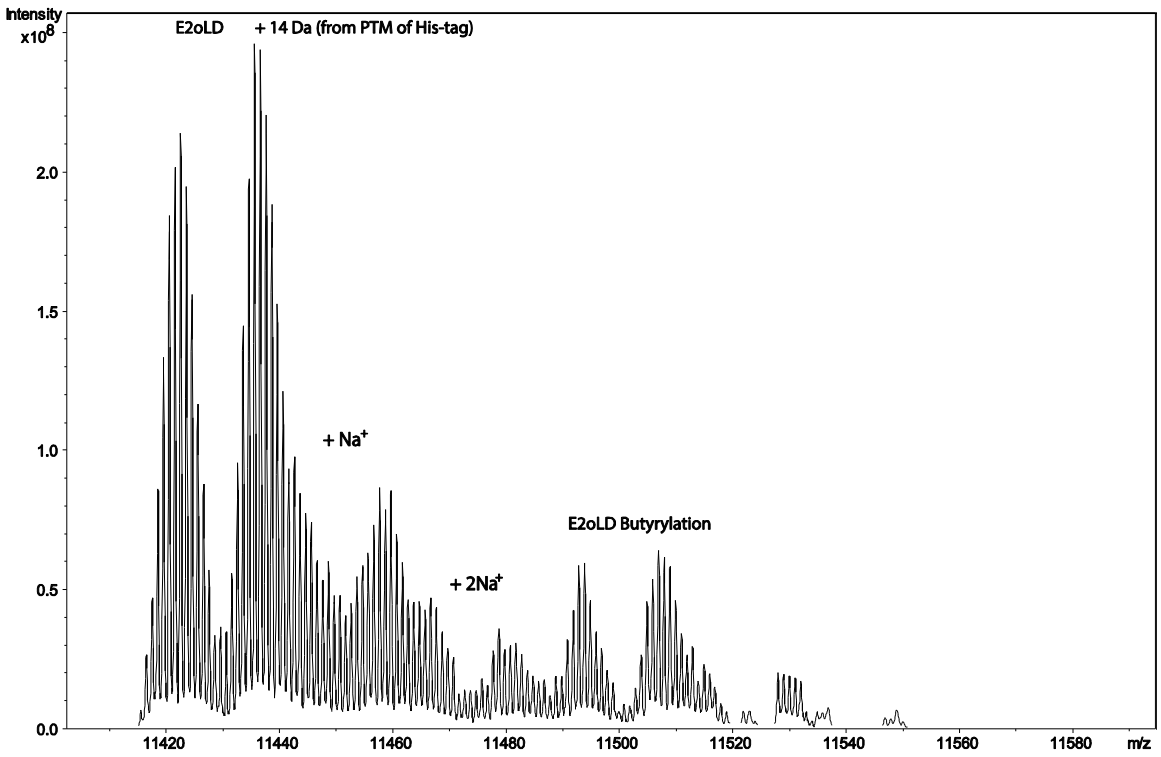


Figure 3.7 Mass Spectrum of Butyrylated E2oLD Produced by His298Asp E1o and 2-OV.

3.4.3 ESI-MS Analysis of E2pLD in the Reaction with His298Asp E1o and 2-OV

It was previously shown E1o-E2p-E3 and His298Asp E1o-E2p-E3 were able to produce NADH in the overall reaction with 2-OG, but the reactions occurred at a slow rate. On the other hand, E1o-E2p-E3 and His298Asp E1o-E2p-E3 did not generate NADH in the overall reaction with 2-OV or it generate too little to be detected.

E1o, E2pLD, and 2-OG were incubated and the reaction mixture was analyzed by ESI-FTMS. E2pLD can be reductively succinylated by wild-type E1o (Figure 3.8) or by His298Asp E1o (Figure 3.9).

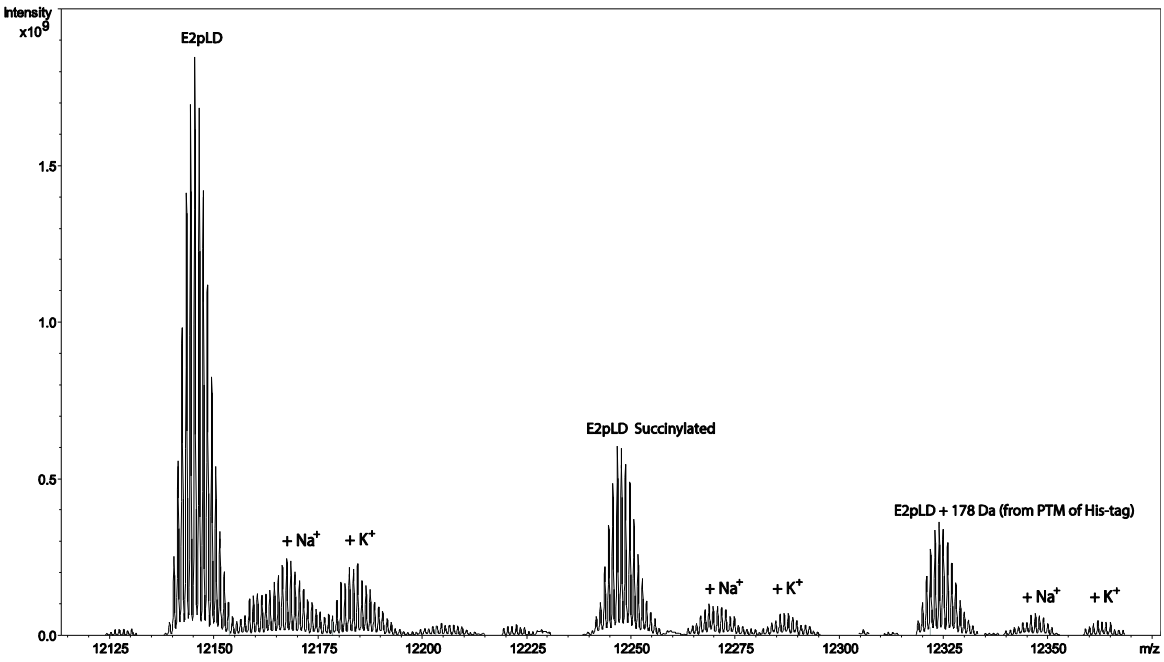


Figure 3.8 Mass Spectrum of Succinylated E2pLD Produced by E1o and 2-OG.

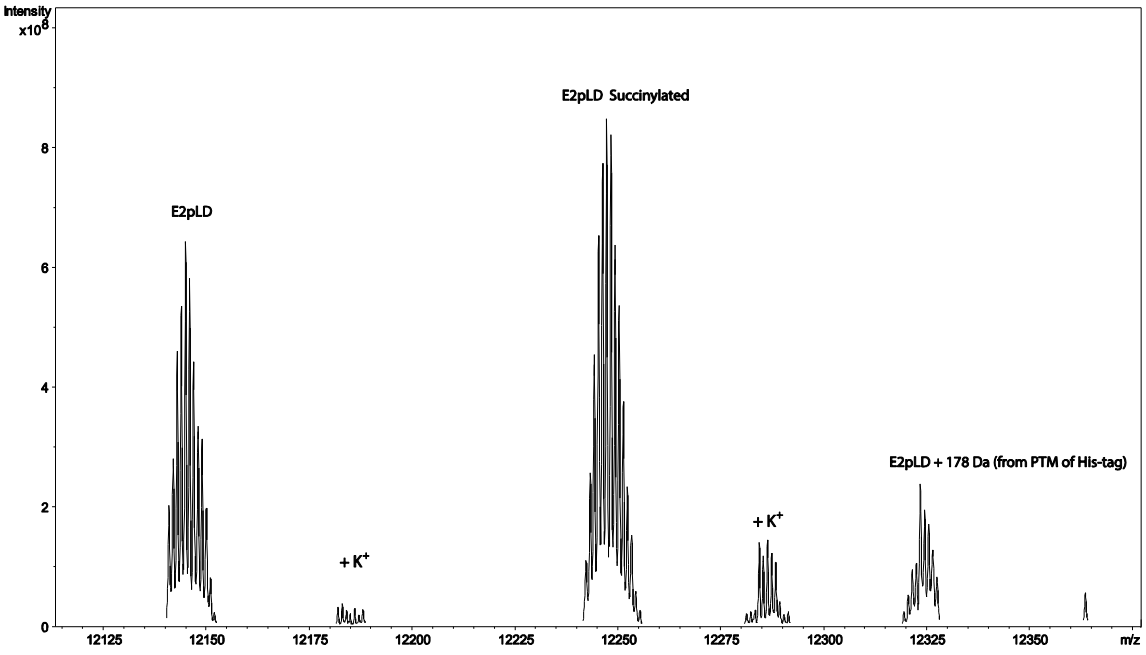


Figure 3.9 Mass Spectrum of Succinylated E2pLD Produced by His298Asp E1o and 2-OG.

E1o, E2pLD, and 2-OV were incubated and the reaction mixture was analyzed by ESI-FTMS. No reductive butyrylation on E2pLD was observed with wild-type E1o (Figure 3.10) or with His298Asp E1o (Figure 3.11). These results further support the conclusions in Chapter 2. E1o-E2p-E3 forms NADH in the overall reaction with 2-OG, but the complex does not form NADH with 2-OV. Hence, E2p requires engineering to accept unnatural substrates. In addition, it was shown that E1p has strict substrate specificity for the natural substrate. This demonstrates that the E1p and E2p components are not a good starting point for synthesizing derivatives of CoA. Whereas, E1o, His298Asp, and E2oLD have a broad specificity and only the E2oCD domain needs optimization.

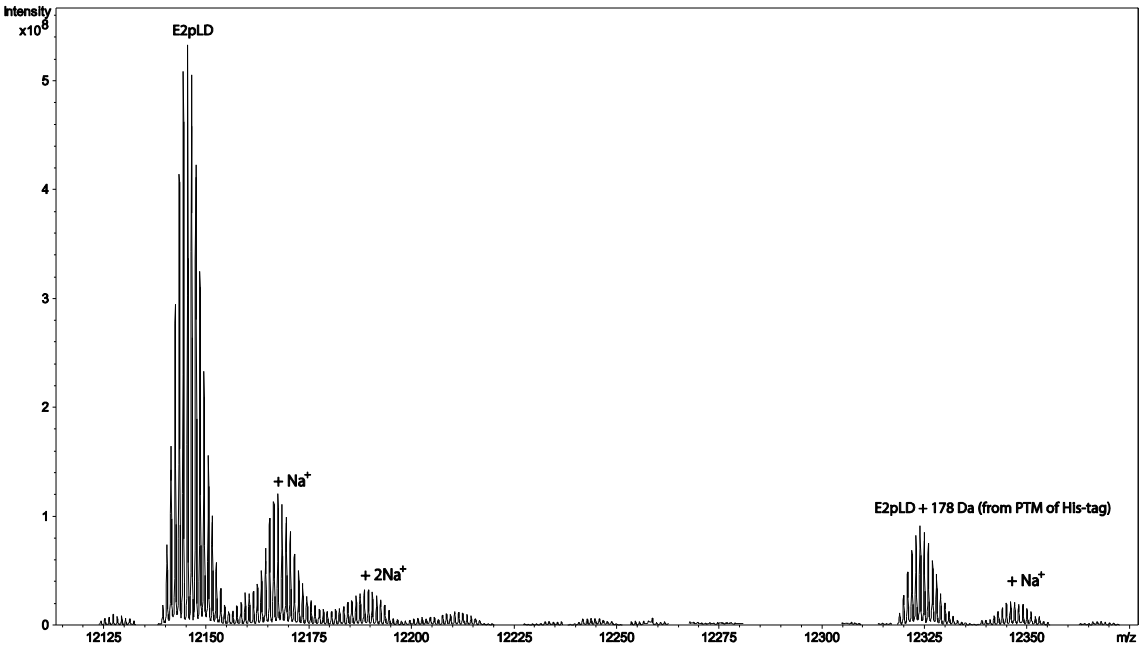


Figure 3.10 Mass Spectrum of the Product of a Reaction with E1o, E2pLD and 2-OV.

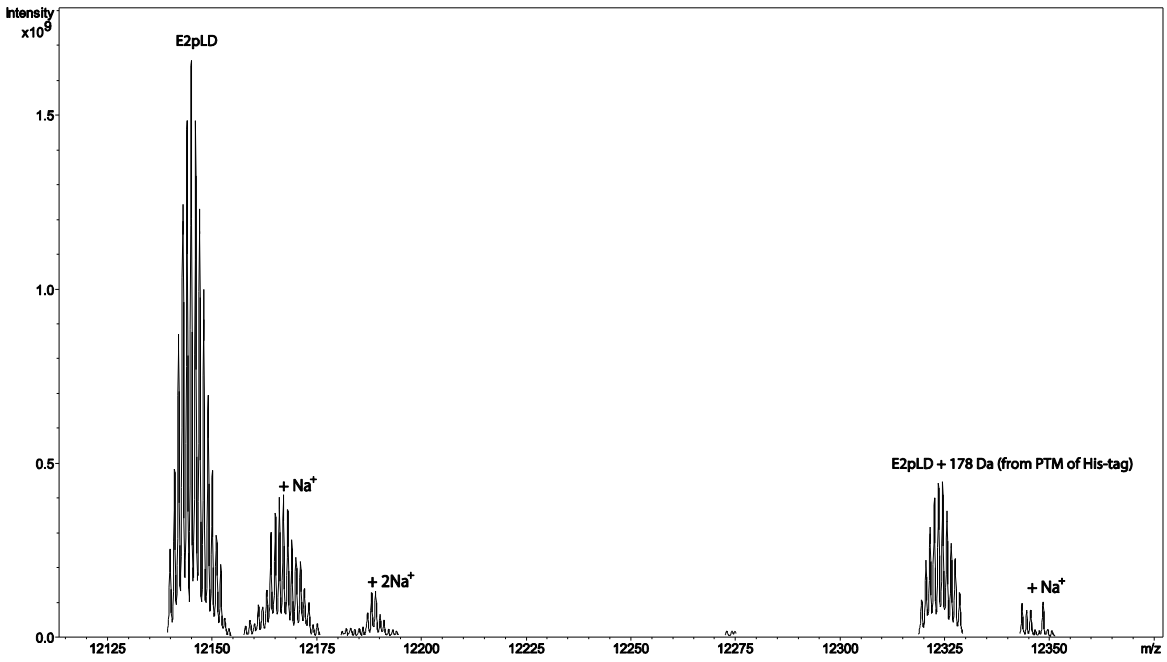


Figure 3.11 Mass Spectrum of the Product Resulting from a Reaction of His298Asp E1o, E2pLD and 2-OV.

3.5 Conclusions

In conclusion, E1o and His298Asp E1o can be reductively succinylate and butyrylate E2oLD with 2-OG and 2-OV, respectively. These results were confirmed with ESI-FTMS. However, NADH production was not detected for 2-OV in the overall reaction with either E1o-E2o-E3 or His298Asp E1o-E2o-E3. Therefore, butyryl transfer from the lipoyl domain to the catalytic domain is hindered. The E1p and E2p components from PDHc both require engineering to be used in the synthesis of unnatural CoA derivatives. However, only the E2oCD domain from OGDHc requires optimization. Therefore, OGDHc or variants of OGDHc are a suitable starting point to form unnatural CoA derivatives.

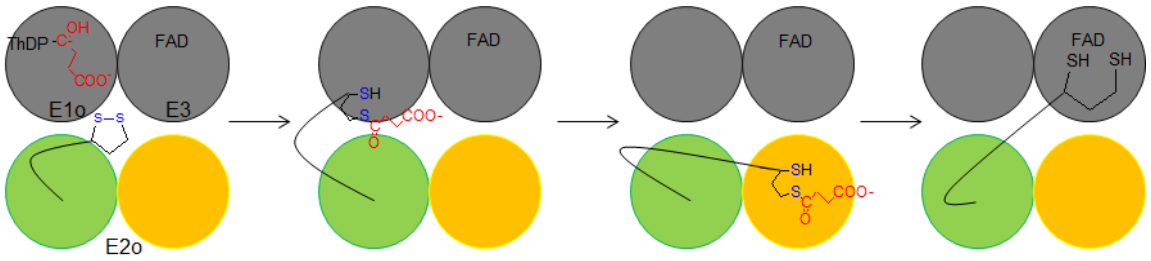
CHAPTER 4

INTERCHAIN SUCCINYL TRANSFER IN THE E2 COMPONENT OF THE *ESCHERICHIA COLI* 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

4.1 Introduction

For the 2-oxoglutarate dehydrogenase complex (OGDHc) from *Escherichia coli*, a stoichiometry of 6 E1o dimers: 8 E2o trimers: 6 E3 dimers was proposed, [7, 15, 79, 80]. All the subunits of E2o have a single lipoyl domain (LD) and catalytic domain (CD). The LD is succinylated by E1o and then the succinyl group is transferred to the CD. In the CD, the succinyl group is transferred to CoA producing succinyl-CoA. LD mobility is afforded by a flexible Ala-Pro rich linker between the LD and CD [17]. Succinyl transfer to the CD domain may occur through an intrachain (Figure 4.1 A) or interchain (Figure 4.1 B) pathway. In this chapter, the possibility of interchain transfer will be determined.

A.



B.

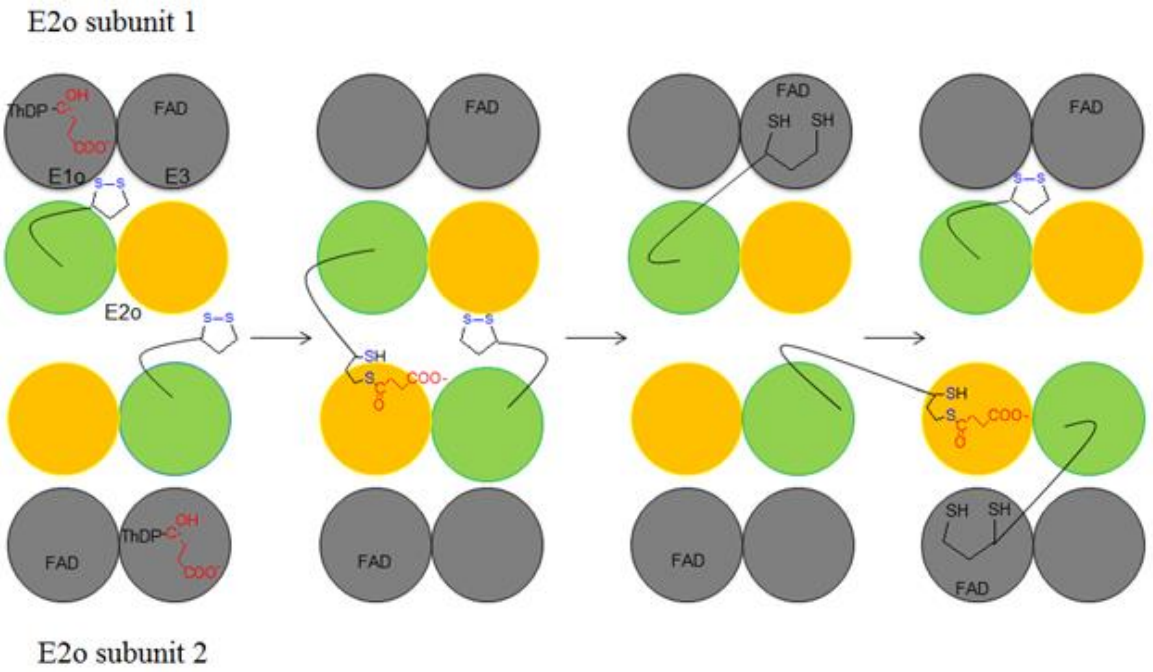


Figure 4.1 Succinyl Group Transfer in *E. coli* E2o Component. **A:** Intrachain Succinyl Group Transfer. **B:** Interchain Succinyl Group Transfer (Gray: Sub-complex of *E. coli* E1o and E3 Component. Green: LD of *E. coli* E2o Component. Orange: CD of *E. coli* E2o Component).

Two E2o variants were constructed by site directed mutagenesis.

1. The E2o domain alone (E2oLD). This construct does not have the CD domain (Figure 4.2)

2. E2o with a lysine to alanine substitution at position 43 (E2oK43A). This variant is incompetent towards posttranslational ligation of lipoic acid. Therefore, it cannot be

reductively succinylated by E1o.

Figure 4.2 The Lipoyl Domain of the E2o Component of *E. coli* OGDHc. Lys43 Side Chain is shown as Sticks.

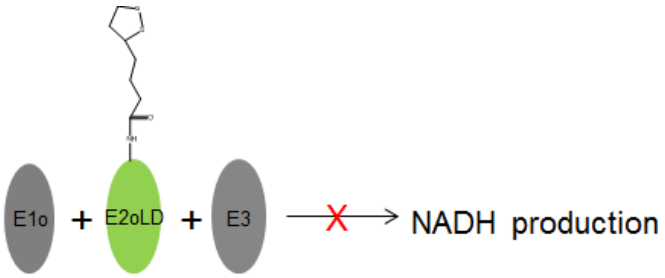
A crossover experiment was designed to test this hypothesis. The overall reaction of OGDHc is the following (Equation 4.1).

$$\text{2-oxoglutarate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{succinyl CoA} + \text{CO}_2 + \text{NADH} \qquad (4.1)$$

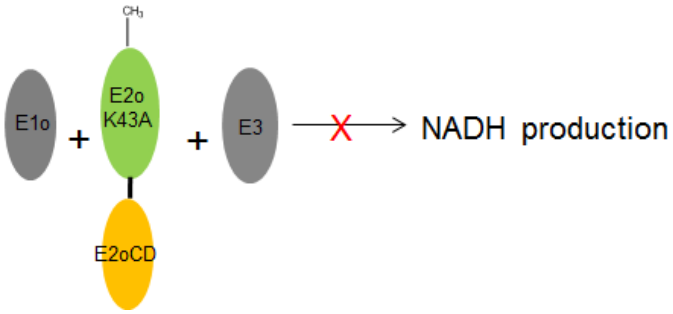
First, E1o, E2oLD and E3 will be reconstituted and no NADH should be produced in the overall reaction of OGDHc (Figure 4.3 A). Second, E1o, E2oK43A and E3 will be reconstituted and it is also expected not to produce NADH in the overall reaction (4.3 B). Finally, E2oLD will be added to the reconstituted complex that contains E1o, E2oK43A

and E3. As a result, E2oLD should rescue the activity, and NADH should be produced.

A.



B.



C.

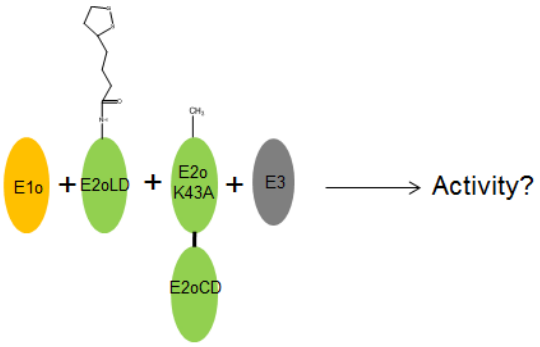


Figure 4.3 Crossover Experiment of Interchain Succinyl Transfer of E2o. Gray: E1o and E3 Components of OGDHc. Green: Reconstituted E2o Complexes.

4.2 Materials

Thiamin diphosphate (ThDP), dithiothreitol, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from U.S. Biochemical Corp. NAD^+, coenzyme A (CoA), and succinyl coenzyme A (succinyl CoA), phenylmethanesulfonyl fluoride (PMSF) were from Sigma-Aldrich. Restriction enzymes were purchased from Invitrogen. Primers were procured from Fisher.

DNA sequencing was done at the Molecular Resource Facility of the New Jersey Medical School (Newark, NJ).

4.3 Methods

4.3.1 Site Directed Mutagenesis

Site-directed saturation mutagenesis libraries were constructed using a modified QuikChange procedure for lipoyl domain of E2oLD and E2oK43A variants. A typical 50 μL reaction contained 10x PfuUltra buffer, dNTP (0.2 mM) and PfuUltra DNA polymerase (1 unit), pCA24N encoding the E2o subunit, and primers are shown in Table 4.1.

Table 4.1 List of Primers for Creating Variants of E2o

No	Site	5' Primers 3'
1	E2oLD_F	GGTAAAGAAACCAGCGCCTAATCTGAAGAGAAAGCGTCC
2	E2oLD_R	GGACGCTTTCTCTTCAGATTAGGCGCTGGTTTCTTTACC
3	E2oK43A_F	GAAATCGAAACTGACGCGGTGGTACTGGAAGTACCG
4	E2oK43A_R	CGGTACTTCCAGTACCACCGCGTCAGTTTCGATTTC

The PCR product was transformed into BL21 *E. coli* competent cells. The plasmid was isolated and the gene was confirmed by DNA sequencing. Frozen stock of cells were stored at -80 ^{o}C.

4.3.2 Protein Expression and Purification

The protein expression and purification of E1o, E2o, and E3 were described in Chapter 2. The E2oK43A variant was purified using same method as wild type E2o. The purification of E2o LD was described in Chapter 3. The E2oK43A variant was overexpressed and purified using the procedure developed for wild type E2o. Purified proteins were stored at -20 ℃.

4.3.3 Measurement of the OGDHc Activity

Overall activity of the OGDHc consisting of E2o and its variants was measured after reconstitution of the complex with independently expressed E1o and E3 components. A Varian DMS 300 spectrophotometer was used to monitor the 2-oxoglutrate dependent reduction of NAD^+ at 340 nm. First, E2oK43A was titrated with increasing concentrations of E2oLD in order to determine at optimal mass ratio of E2oLD:E2oK43A (0.25:1 to 2.5:1). Solutions that contain E2oLD and E2oK43A were preincubated at room temperate for 20 min in KH_2PO_4 (20 mM, pH 7.2) containing NaCl (0.5 M). Then, E1o and E3 were added and incubated for 20 min at room temperature in the same buffer. The assay medium contained: 0.1 M Tris-HCl (pH 8.0), $MgCl_2$ (1.0 mM), ThDP (0.2 mM), NAD^+ (2.5 mM) and CoA (0.13 mM). The reaction was initiated by the addition of 2-OG (2.0 mM). The initial steady state velocities were determined from the progress curves recorded at 340 nm and 30 ^{o}C. One unit of activity is defined as the amount of NADH produced (μmol/min/mg of complex). Overall activity for wild type OGDHc was described in Chapter 2. The

overall activity of reconstituted complex containing E1o/E2oK43A/E3 E1o/E2oLD/E3 was identical to that of wild type OGDHc.

4.4 Results and Discussion

4.4.1 Substitutions of Lys43Ala E2o and E2oLD Affected the Overall OGDHc Activities

The overall activity of reconstituted complex, which contains E1o, E2oLD, and E3 did not produce detectable amounts of NADH. It is expected that when E1o, E2oK43A, and E3 are reconstituted together, the complex should not show NADH production. However, NADH was generated (1.7%) at a low rate (Table 4.2), and this value was used as the baseline.

4.4.2 Complementation of E2oLD with E2oK43A

E2oK43A was titrated with increasing concentrations of E2oLD, and then E1o and E3 were added to form a reconstituted complex (Figure, 4.4, Table 4.2). NADH was formed in the overall reaction above the baseline. Maximum activity of 4.9 % above the background was found with a ratio 0.5 E2oLD: 1.0 E2oK43A.

Table 4.2 Overall OGDHc Activity of E2oLD/Lys43Ala with Various Mass Ratios

Enzyme	**Activity (units/mg complex)**
E1o : E2o : E3	2.06
E1o : E2oLD: E3	No Activity
E1o : E2oK43A: E3	0.035 (1.7%)
E1o : (E2oLD : E2oK43A = 0.25:1): E3	0.046 (2.2%)
E1o : (E2oLD : E2oK43A = 0.5:1): E3	0.101 (4.9%)
E1o : (E2oLD : E2oK43A=1:1) : E3	0.093 (4.5%)
E1o : (E2oLD : E2oK43A=2:1) : E3	0.062 (3.0%)
E1o : (E2oLD : E2oK43A=2.5:1) : E3	0.074 (3.6%)

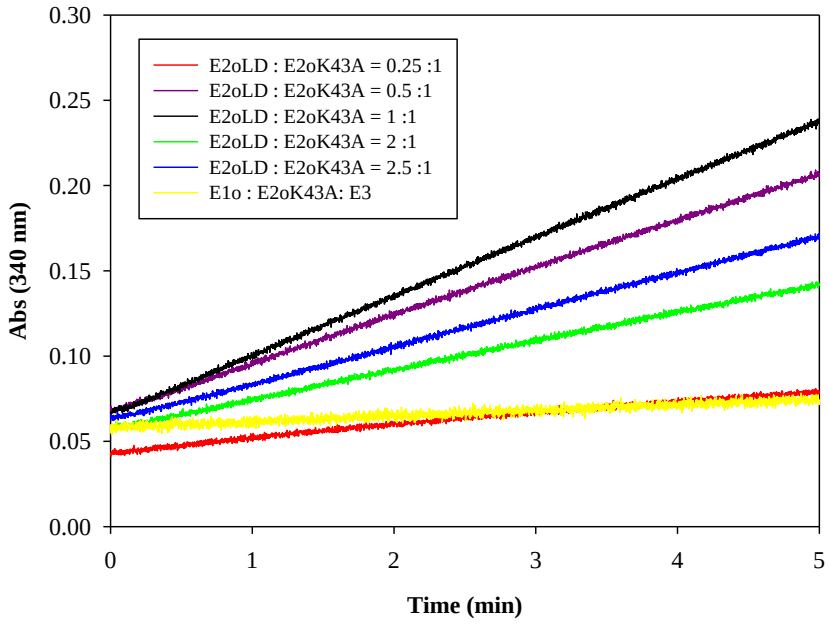


Figure 4.4 Activity Assay for E2o Variants Reconstituted with E1o and E3. Mixtures of E2oK43A and E2oLD in Various Mass Ratios were Incubated and E1o and E3 were Added Enabling Use of the NADH Production Assay for Overall Activity of the Complex.

These results clearly show that E2oLD was capable of rescuing the crippled E2oK43A variant. Therefore, an interchain mechanism is likely between two different E2o subunits.

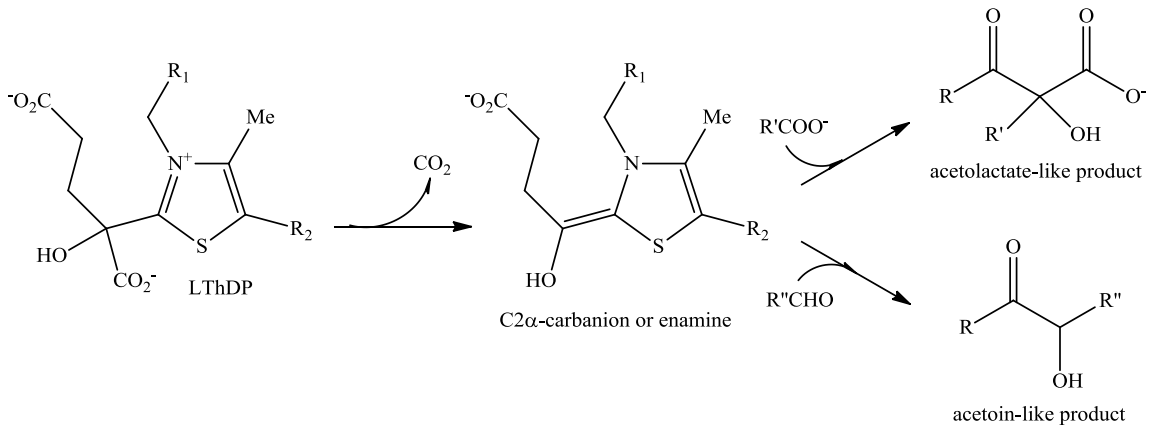
CHAPTER 5

CARBOLIGASE REACTION OF THE E1 COMPONENT OF THE 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

5.1 Introduction

Thiamin diphosphate (ThDP)-dependent enzymes have the potential to be used for chemical synthesis. These enzymes share a common feature in that they catalyze carboligase reactions [81, 82]. Carboligation is the native activity for several enzymes, but it is a side reaction for nearly all ThDP-dependent 2-oxo acid dacarboxylases. The Jordan research group at Rutgers-Newark have previously constructed several active site variants in yeast pyruvate decarboxylase (YPDC) from *Saccharomyces cerevisiae* and in the E1 subunit of *Escherichia coli* pyruvate dehydrogenase complex (E1p), and they are capable of carrying out such reactions [49]. The E477Q variant of YPDC was an effective acetoin synthase, while the D28A or D28N variants catalyze acetolactate formation [72]. The E636Q and E636A E1p active site variants were also created and they also became acetolactate synthases [50]. Remarkably, although YPDC and E1p both catalyze decarboxylation of pyruvate in the first step, the opposite enantiomers of acetoin were produced.

The E1 subunit (E1o) of *E. coli* 2-oxoglutarate dehydrogenase complex (OGDHc) also catalyzes carboligation reactions. The central ThDP-bound enamine intermediate reacts with the electrophilic substrate, which results in the formation of acetoin-like or acetolactate-like ligated products (Scheme 3).



Scheme 3 Carboligase Reaction of ThDP-dependent Enzymes from the Central ThDP-bound Enamine Intermediate.

The E1o component of *E. coli* OGDHc was reacted with substrates that vary in the size and functional groups to determine the versatility for carboligase reactions (Figure 5.1). The products were confirmed by circular dichroism (CD), nuclear magnetic resonance (NMR) and chiral gas chromatography (GC).

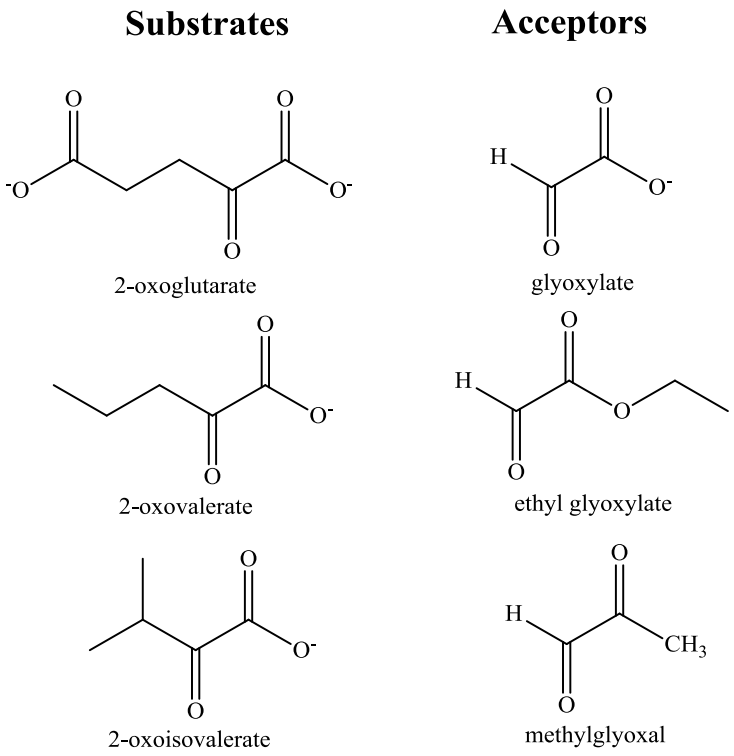


Figure 5.1 Substrates and Acceptors for Carboligase Reaction by E1o.

5.2 Materials

2-Oxoglutarate, 2-oxovalerate, 2-oxoisovalerate, glyoxylate, ethyl glyoxylate, and methylglyoxal were from Sigma-Aldrich. Restriction enzymes were purchased from Invitrogen. Primers were procured from Fisher. *E. coli* strain JW0715 containing the plasmid pCA24N encoding the OGDHc-E1 (E1o) component [ASKA clone (-)] was obtained from National Bio Resource Project (NIG, Japan). Amicon® Ultra-4 Centrifugal Filter Units are purchased from EMD Millipore.

5.3 Methods

5.3.1 CD Analysis

CD experiments were carried out on a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK). First, E1o (concentration of active centers = 19 μM) in KH_2PO_4 (20 mM, pH 7.0) containing $MgCl_2$ (2 mM), ThDP (0.2 mM) was incubated overnight with 2-OG (2 mM). Next, the acceptors glyoxylate (1 mM), ethyl glyoxylate (1 mM), or methylglyoxal (1 mM)) were added and CD spectrum was recorded (reaction 1). Similar reactions were performed using 2-OV (10 mM, reaction 2) or 2-OiV (10 mM, reaction 3) as the substrates with the acceptors. The protein was seperated from the carboligase product using an Amicon® Ultra-4 Centrifugal Filter Units. The filtrate was collected and the CD spectra were recorded between 260 nm and 400 nm.

5.3.2 NMR Analysis

After CD experiment, the carboligase product was extracted with chloroform. Then, sodium sulfate was added to remove dry the organic layer. Spectra were recorded on a Varian 500 MHz INOVA spectrometer at 25 ^{o}C. The spectra were record by Hetalben Patel at Rutgers.

5.3.3 Chiral GC Analysis

Products were extracted from the reaction mixture with choloroform and analyzed by GC. GC analysis was carried out on a Varian CP-3800 gas chromatograph equipped with a Chiraldex B-DM chiral column (Astec, Advanced Separation Technologies, Inc.) and a flame ionization detector at a flow rate of 1.5 ml/min. The enantiomers were assigned according to their relative retention time. It is known that the (*S*)-enantiomer interacts

more favorably with the matrix than the (*R*)-enantiomer. Hence, the (*S*)-enantiomer has a longer retention time.

5.4 Results and Discussion

5.4.1 Acetoin-like Product Formation by E1o with 2-OG and Glyoxylate

No CD band was produced on reacting E1o and 2-OG showing the E1o could not form a carboligase product with 2-OG alone. The active site may not be large enough to accommodate a free 2-OG as an acceptor or a racemic mixture was produced. Hence, glyoxylate, which is a smaller acceptor, was next used.

The reaction of E1o with 2-OG and glyoxylate (Figure 5.2) produced a negative CD band at 278 nm and positive band at 312 nm (Figure 5.3). The negative band indicates (*R*)-acetoin-like product formation (Figure 5.3). The product was confirmed by chiral GC (Figure 5.4) and ^{1}H NMR (Figure 5.5).

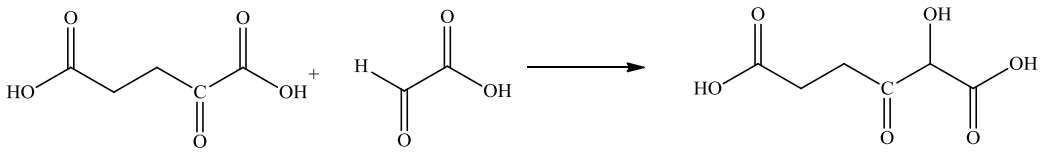


Figure 5.2 The Acetoin-like Product Produced from 2-OG and Glyoxylate.

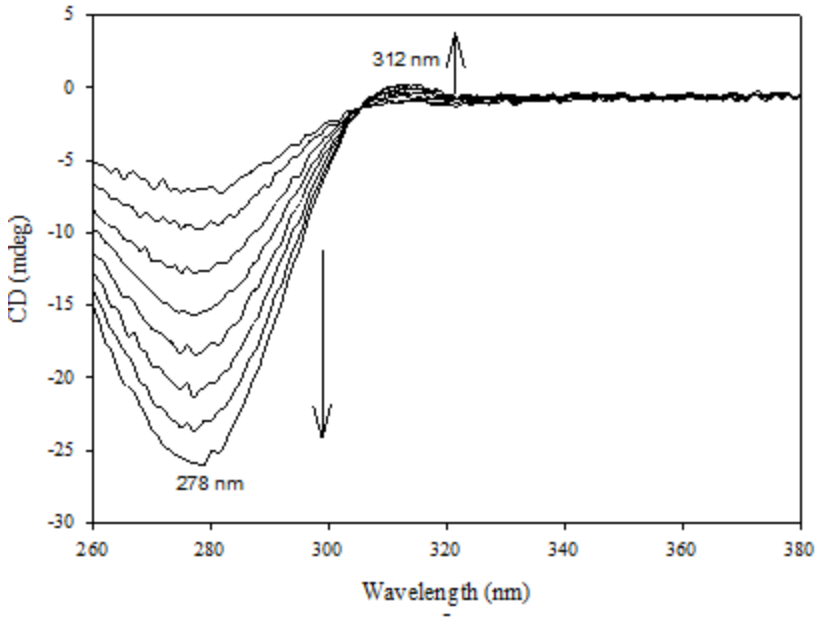


Figure 5.3 CD Spectrum of the Compound Produced by E1o (19 μM Active Center Concentration) in the Presence of 2-OG (2 mM), Glyoxylate (1 mM), $MgCl_2$ (2 mM), ThDP (0.2 mM).

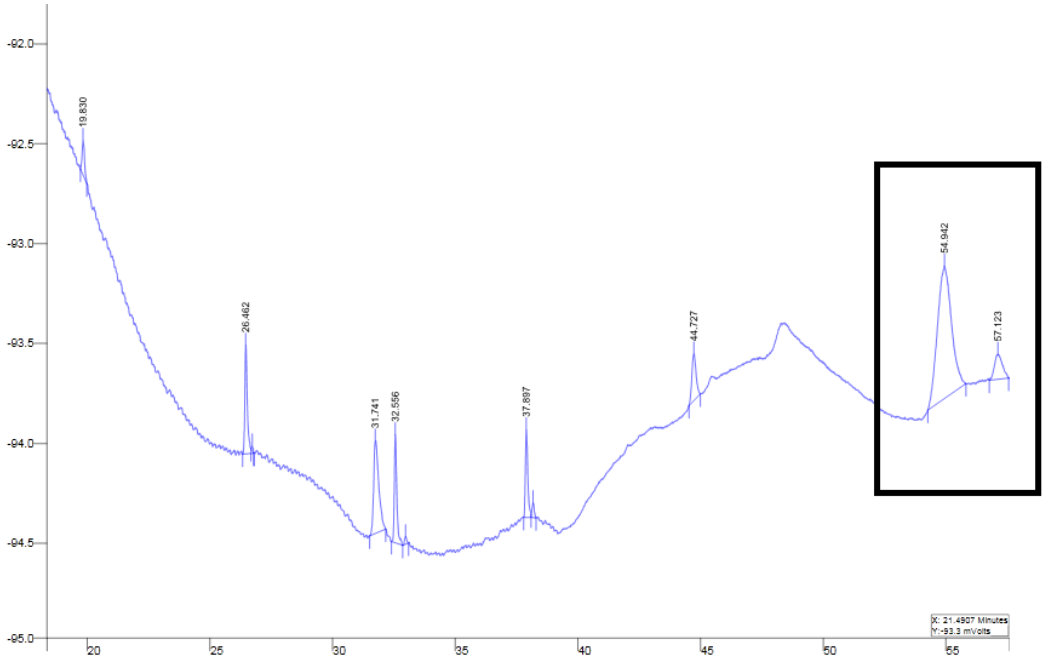


Figure 5.4 Chiral GC of the Product from E1o with 2-OG and Glyoxylate.

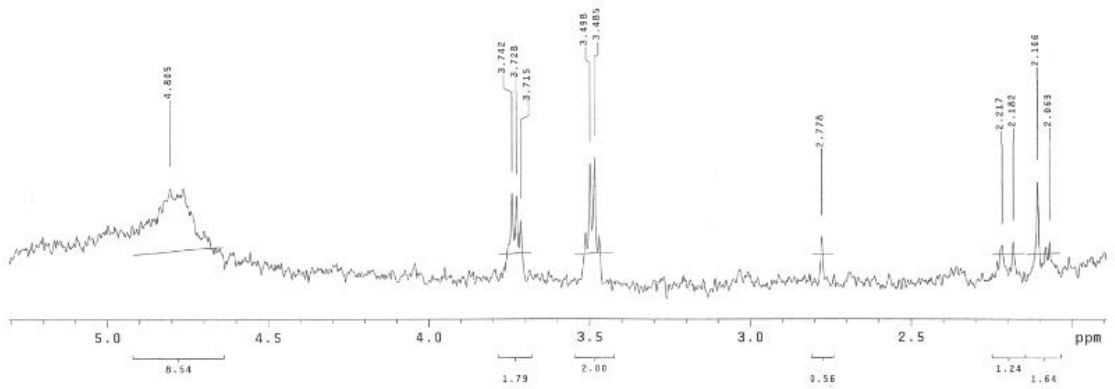


Figure 5.5 ^{1}H NMR Spectrum of the Product Produced by E1o with 2-OG and Glyoxylate.

5.4.2 Acetoin-like Product Formation by E1o with 2-OV and Glyoxylate

No CD band was produced when E1o was reacted with 2-OV, showing that the enzyme could not form a carboligase product from 2-OV alone. The active site may not be large enough to accommodate a free 2-OV as an acceptor or a racemic mixture was produced. Again, the acceptor glyoxylate was next tried.

The reaction of E1o with 2-OV and glyoxylate (Figure 5.6) produced a positive CD band at 278 nm (Figure 5.7). The positive band indicates (*S*)-acetoin-like product, which was confirmed by chiral GC (Figure 5.8) and ^{1}H NMR (Figure 5.9).

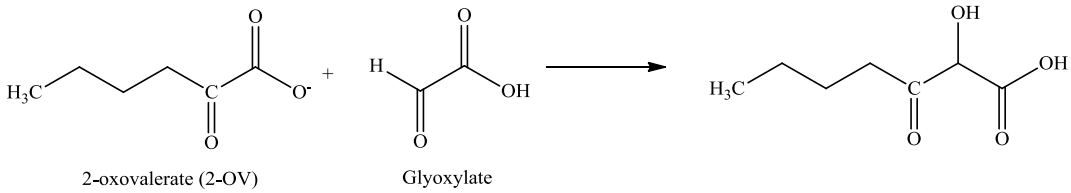


Figure 5.6 The Acetoin-like Product Produced by E1o from 2-OV and Glyoxylate.

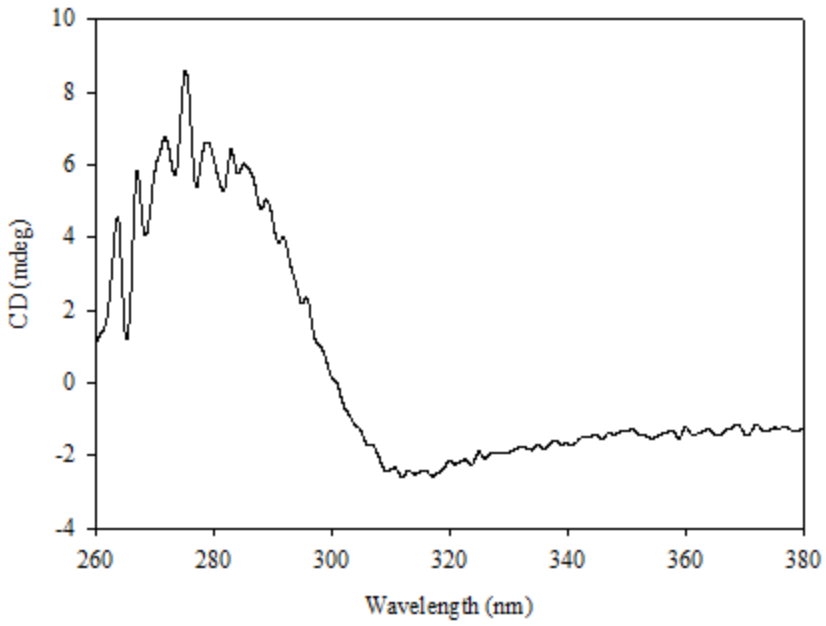


Figure 5.7 CD Spectrum of the Compound Produced by E1o (19 μM Active Center Concentration) in the Presence of 2-OV (5 mM), Glyoxylate (10 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

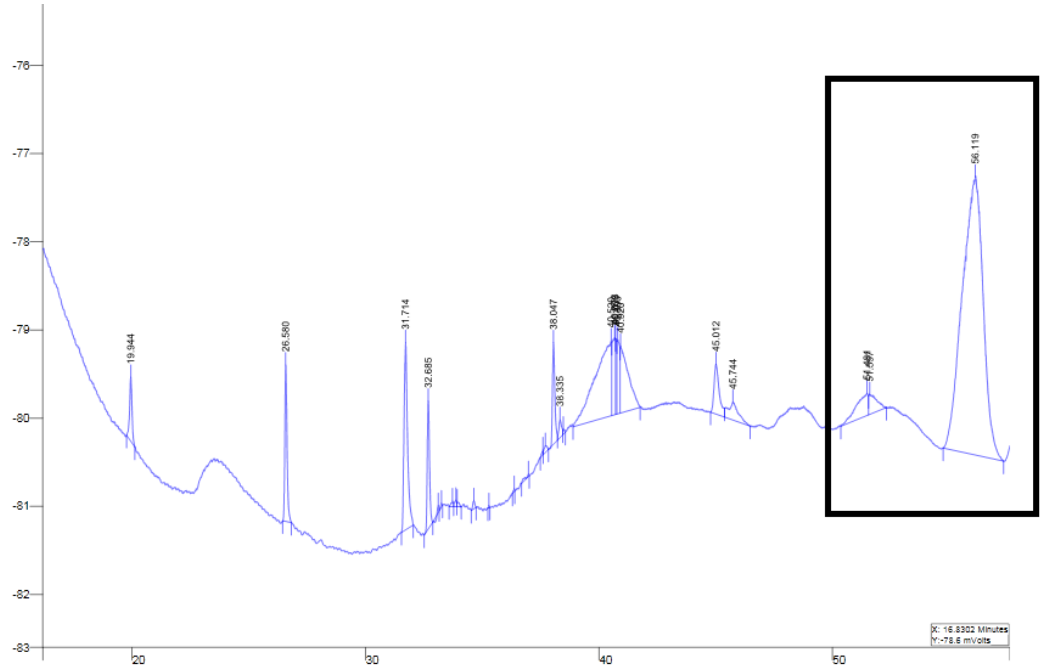


Figure 5.8 Chiral GC of the Product Produced by E1o with 2-OV and Glyoxylate.

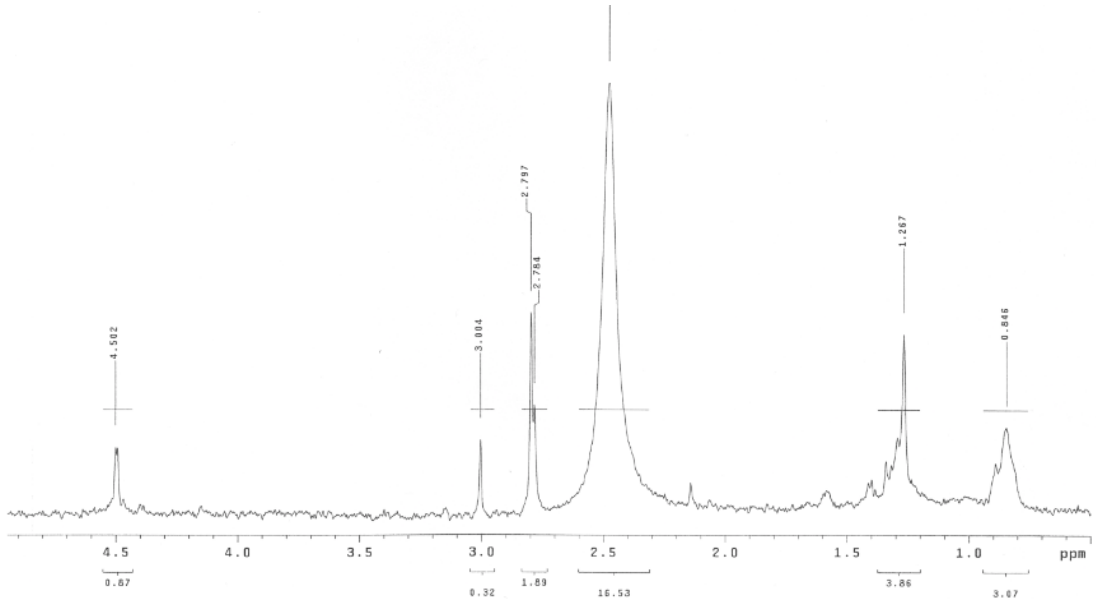


Figure 5.9 ^{1}H NMR Spectrum of the Product Produced by E1o with 2-OV and Glyoxylate.

5.4.3 Acetoin-like Product Formation by E1o with 2-OiV and Glyoxylate

No CD band was produced when E1o was reacted with 2-OiV, showing that the enzyme could not form a carboligase product from 2-OiV itself. The E1o active site may not be large enough to accommodate a free 2-OiV as acceptor or a racemic mixture was produced. Again, the acceptor glyoxylate was next tried.

The reaction of E1o with 2-OiV and glyoxylate (Figure 5.10) produced a positive CD band at 280 nm (Figure 5.11). The positive band indicates an (*S*)-acetoin-like product, which was confirmed by chiral GC (Figure 5.12) and ^{1}H NMR (data not shown).

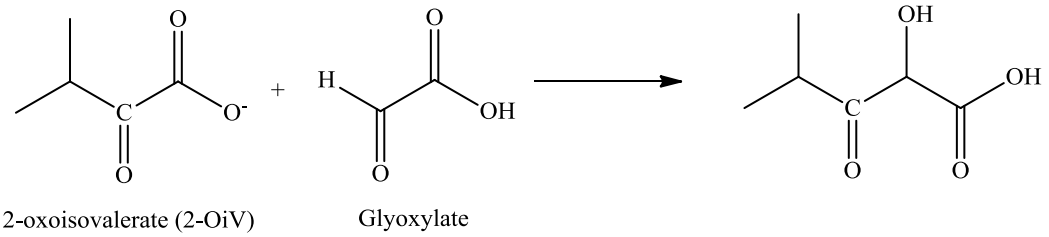


Figure 5.10 The Acetoin-like Product Produced from 2-OiV and Glyoxylate.

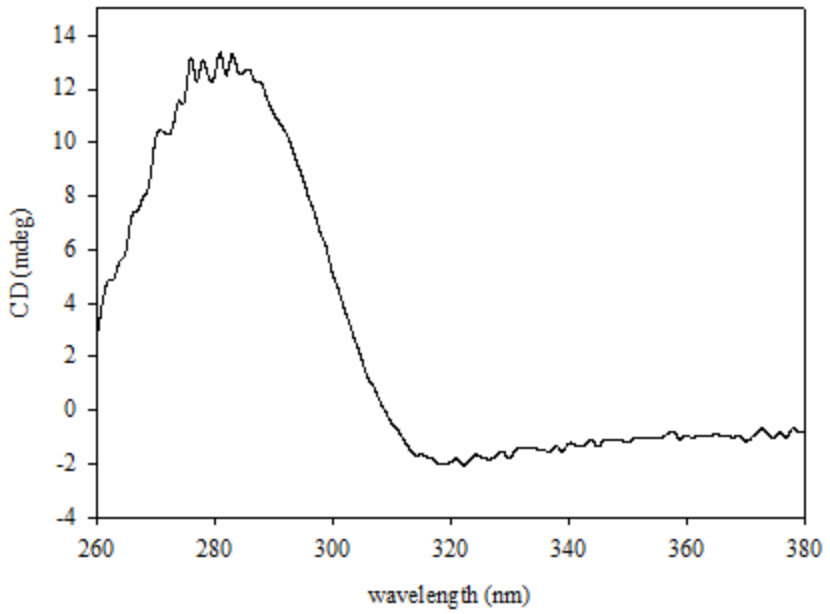


Figure 5.11 CD Spectrum of the Compound Produced by E1o (19 μM Active Center Concentration) in the Presence of 2-OiV (5 mM), Glyoxylate (10 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

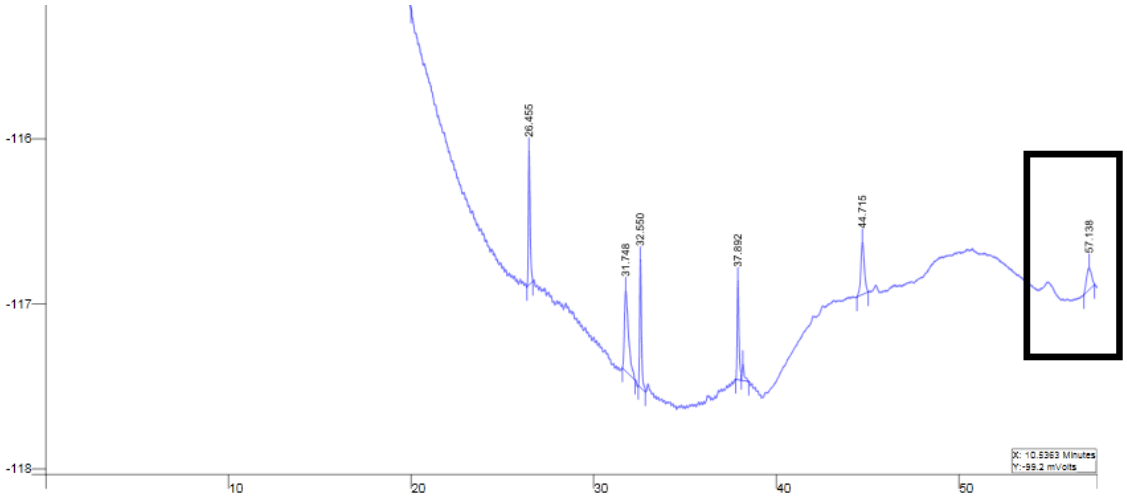


Figure 5.12 Chiral GC of the Product Produced by E1o with 2-OiV and Glyoxylate.

5.4.4 Acetoin-like Product Formation by E1o with 2-OG and Ethyl Glyoxylate

The reaction of E1o with 2-OG and ethyl glyoxylate (Figure 5.13) produced a negative CD band at 280 nm (Figure 5.14). The negative band indicates an (*R*)-acetoin-like product, which was confirmed by chiral GC (Figure 5.15) and ^{1}H NMR (Figure 5.16).

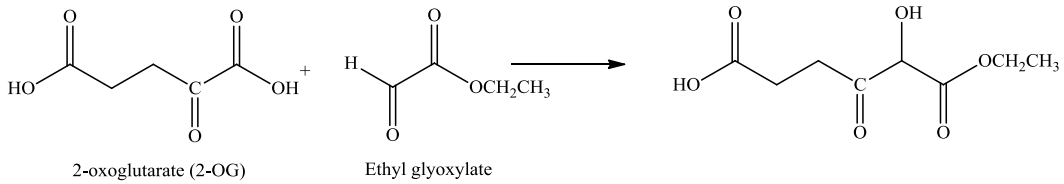


Figure 5.13 The Acetoin-like Product Produced by E1o from 2-OG and Ethyl Glyoxylate.

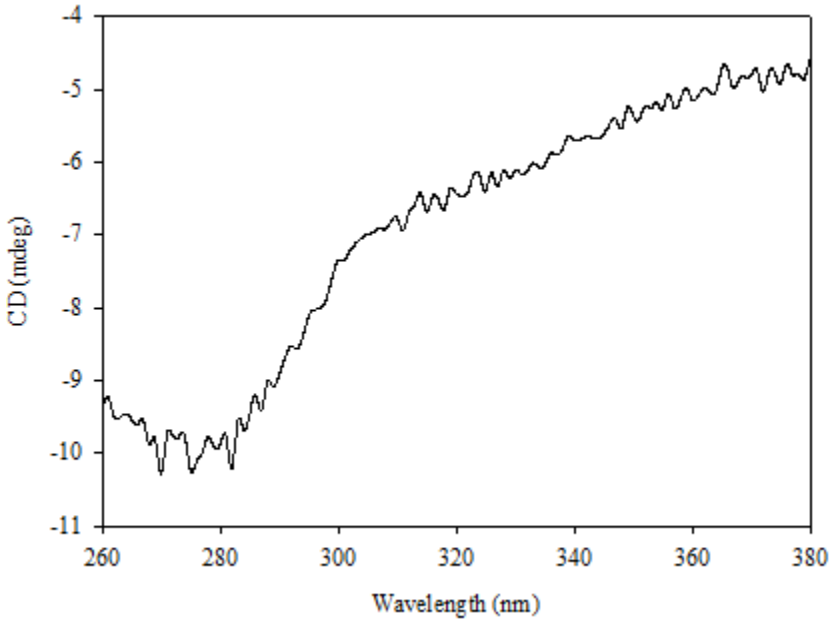


Figure 5.14 CD Spectrum of the Compound Produced by E1o (19 μM Active Center concentration) in the Presence of 2-OG (2 mM), Ethyl Glyoxylate (1 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

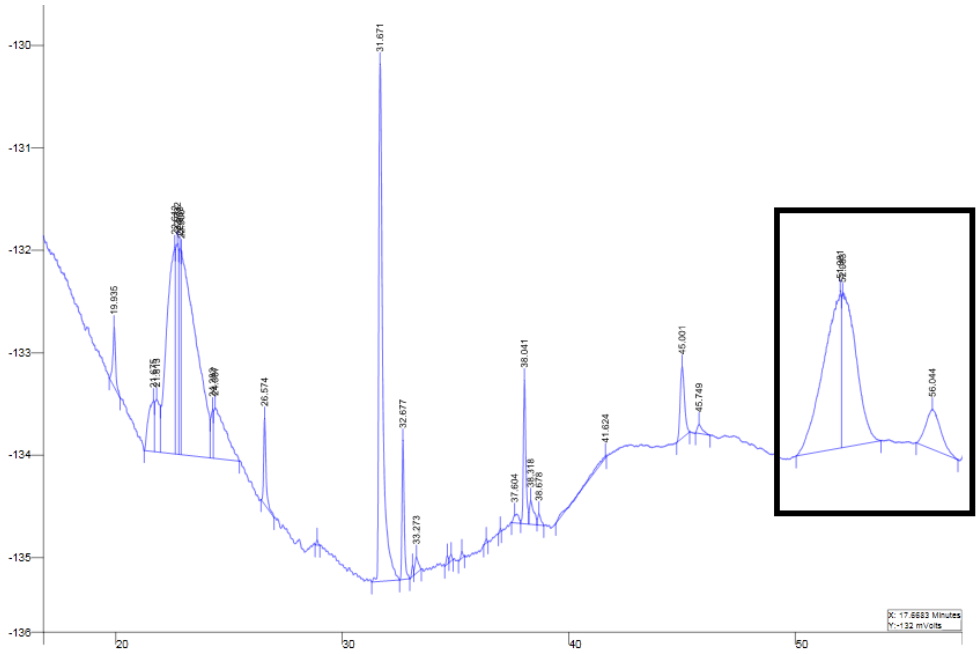


Figure 5.15 Chiral GC of the Reaction Product Formed by E1o with 2-OG and Ethyl Glyoxylate.

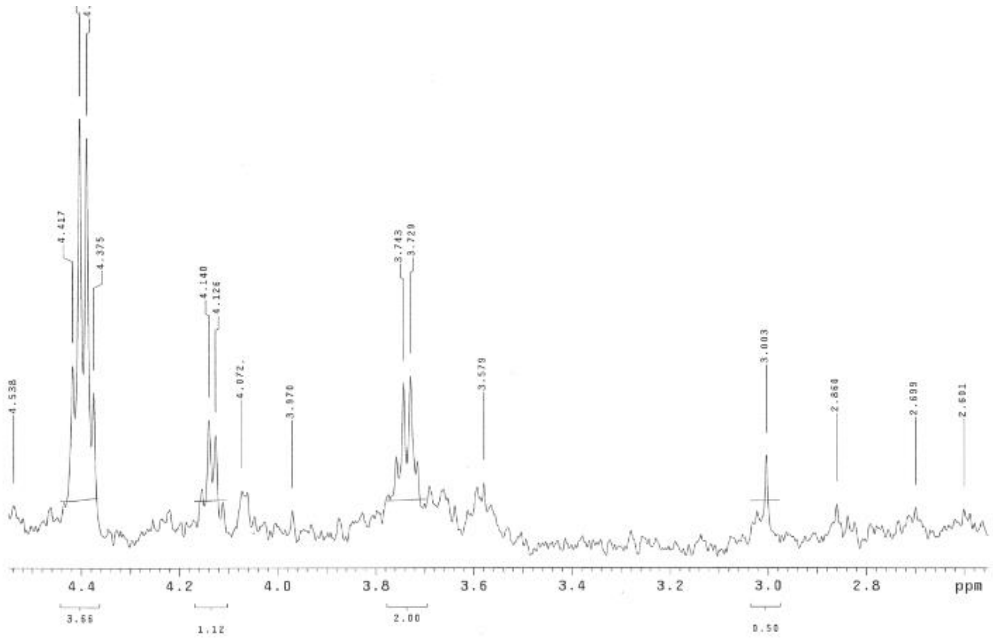


Figure 5.16 ^{1}H NMR Spectrum of the Product Formed by E1o with 2-OG and Ethyl Glyoxylate.

5.4.5 Acetoin-like Product Formation by E1o with 2-OV and Ethyl Glyoxylate

The reaction of E1o with 2-OV and ethyl glyoxylate (Figure 5.17) produced a weak positive band CD band at 280 nm (Figure 5.18). The positive band indicates (*S*)-acetoin-like product, which was confirmed by chiral GC (Figure 5.19) and ^{1}H NMR (Figure 5.20).

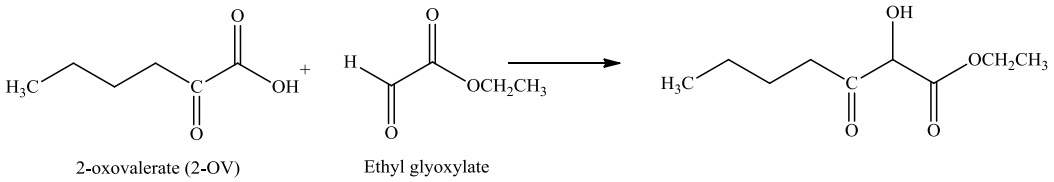


Figure 5.17 The Acetoin-like Product Produced Formed by E1o 2-OV and Ethyl Glyoxylate.

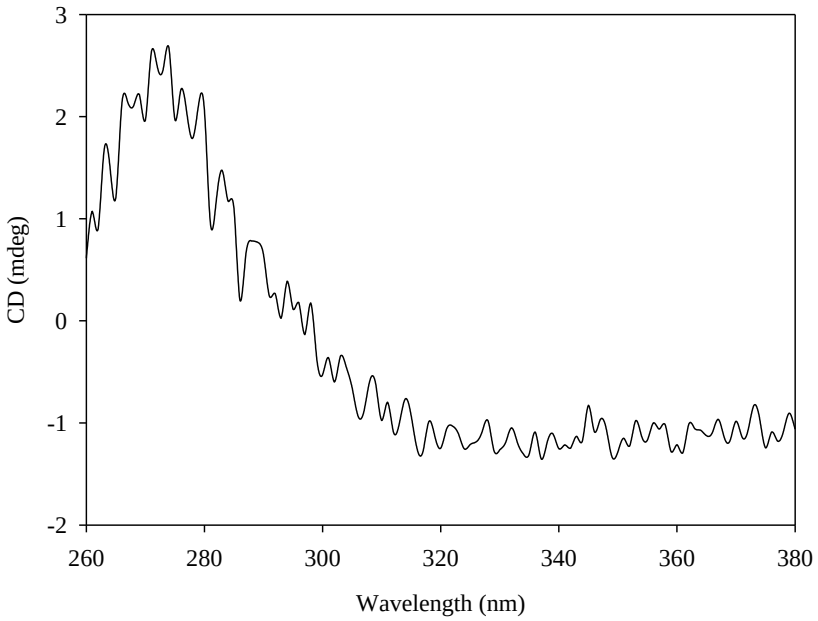


Figure 5.18 CD Spectrum of the Compound Produced by E1o (19 μM Active Center Concentration) in the Presence of 2-OV (5 mM), Ethyl Glyoxylate (1 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

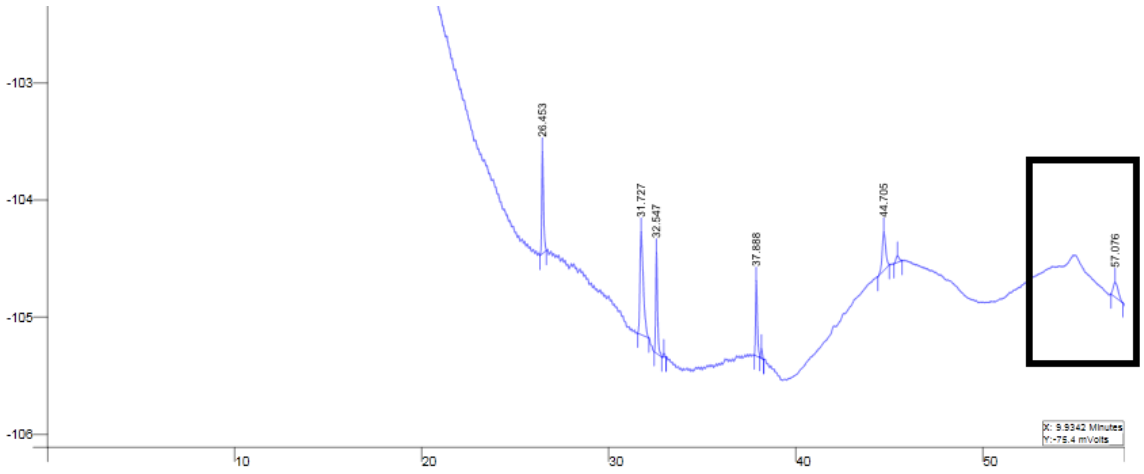


Figure 5.19 Chiral GC of the Product Formed by E1o with 2-OV and Ethyl Glyoxylate.

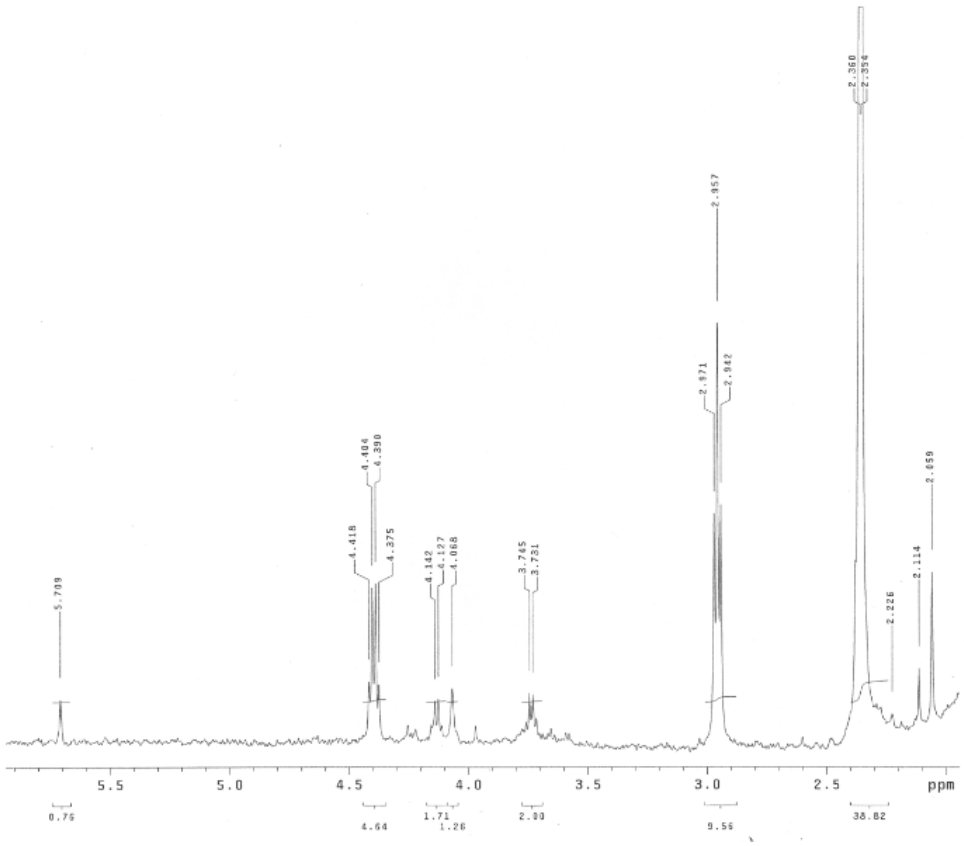


Figure 5.20 ^{1}H-NMR Spectrum of the Compound Produced by E1o (19 μM Active Center Concentration) in the Presence of 2-OV (2 mM), Ethyl Glyoxylate (1 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

5.4.6 Acetoin-like Product Formation by E1o with 2-OiV and Ethyl Glyoxylate

The reaction of E1o with 2-OiV and ethyl glyoxylate (Figure 5.21) produced a positive CD band at 283 nm (Figure 5.22). The positive band indicates (*S*)-acetoin-like product, which was confirmed by chiral GC (Figure 5.23) and ^{1}H NMR (Figure 5.24).

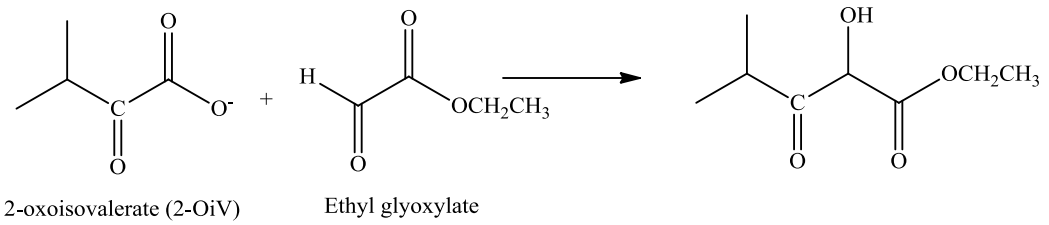


Figure 5.21 The Acetoin-like Product Produced by E1o from 2-OiV and Ethyl Glyoxylate.

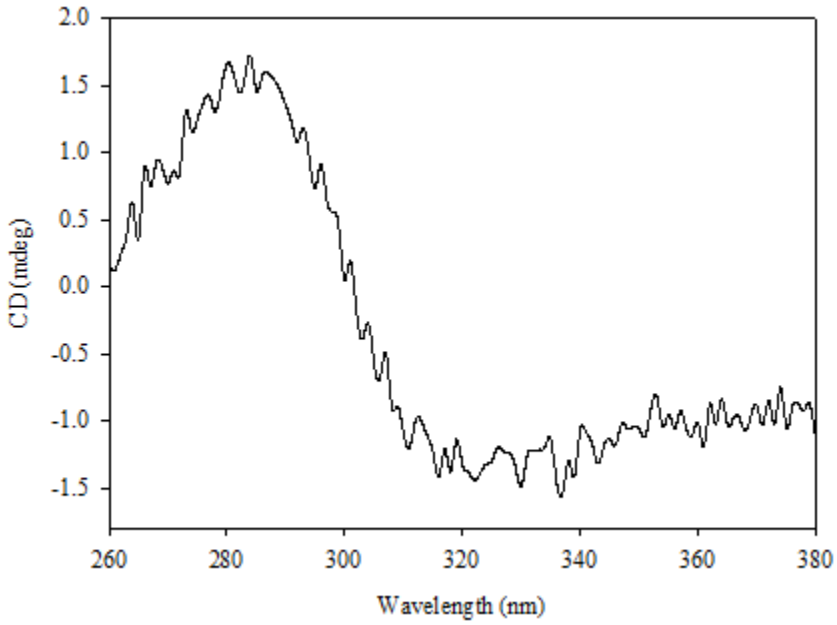


Figure 5.22 CD Spectrum of the Product Formed by E1o (19 μM Active Center Concentration) in the Presence of 2-OiV (5 mM), Ethyl Glyoxylate (10 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

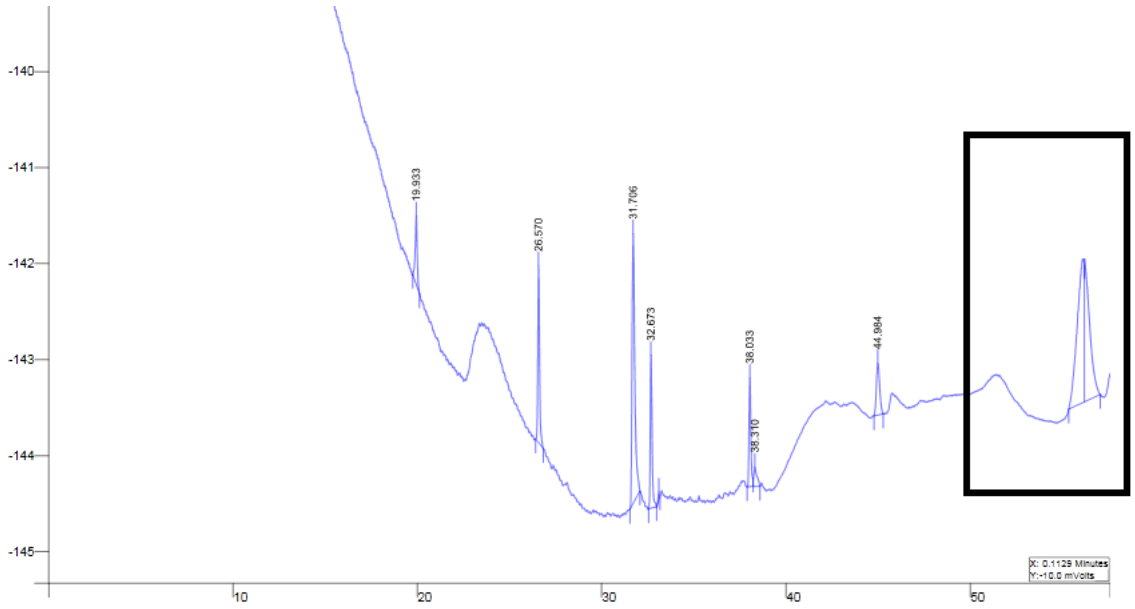


Figure 5.23 Chiral GC of the Product Formed by E1o with 2-OiV and Ethyl Glyoxylate.

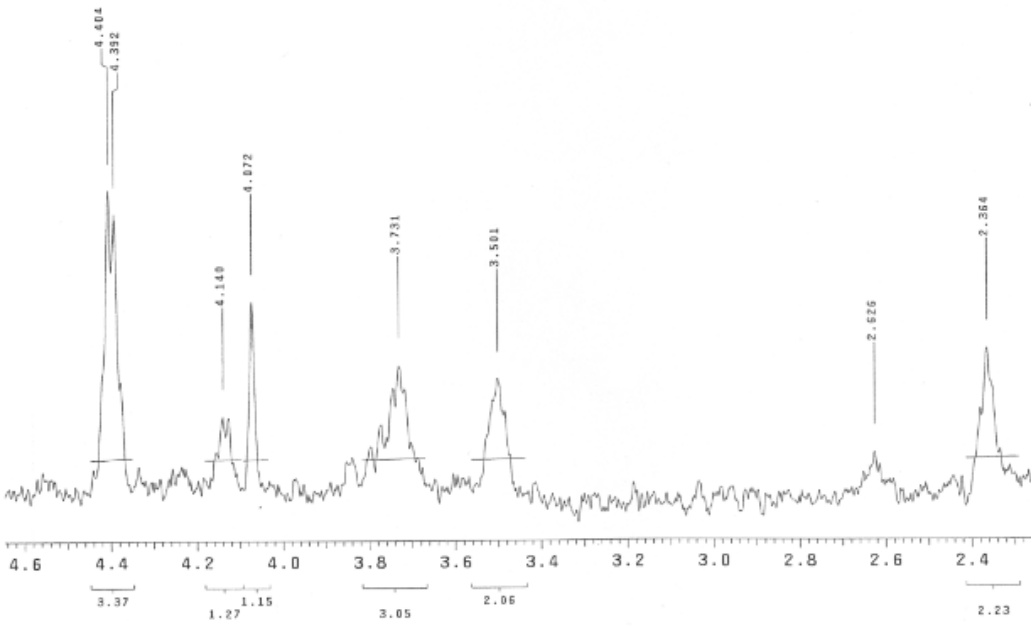


Figure 5.24 ^{1}H NMR Spectrum of the Product Formed by E1o (19 μM Active Center Concentration) in the Presence of 2-OiV (5 mM), Ethyl Glyoxylate (10 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

5.4.7 Acetoin-like Product Formation by E1o with 2-OG and Methylglyoxal

The reaction of E1o with 2-OG and methylglyoxal (Figure 5.25) produced a weak positive CD band at 280 nm (Figure 5.26). The positive band suggests (*S*)-acetoin-like product, which was confirmed by chiral GC (Figure 5.27) and ^{1}H NMR (Figure 5.28).

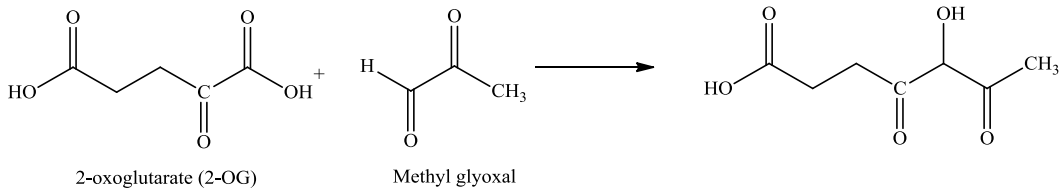


Figure 5.25 The Acetoin-like Product Produced from 2-OG and Methylglyoxal.

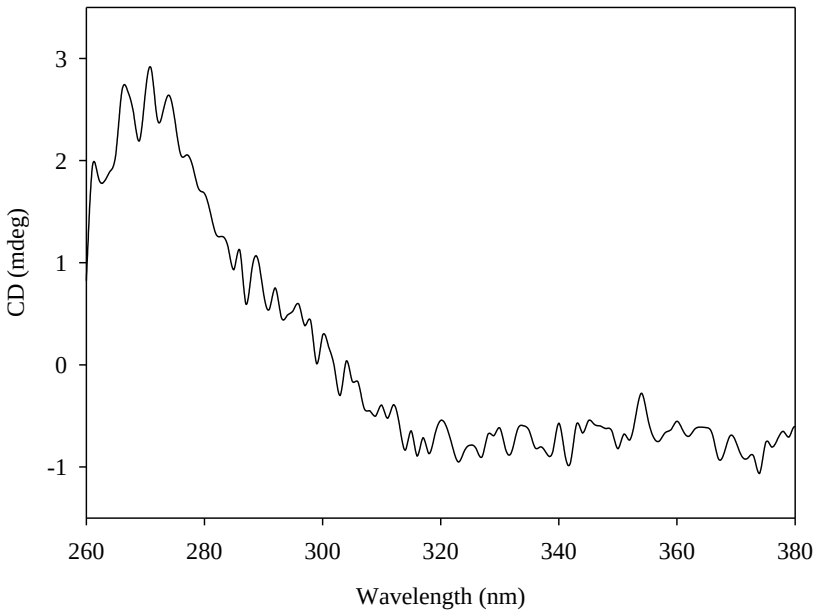


Figure 5.26 CD Spectrum of the Product Formed by E1o (19 μM Active Center Concentration) in the Presence of 2-OG (2 mM), Methylglyoxal (1 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

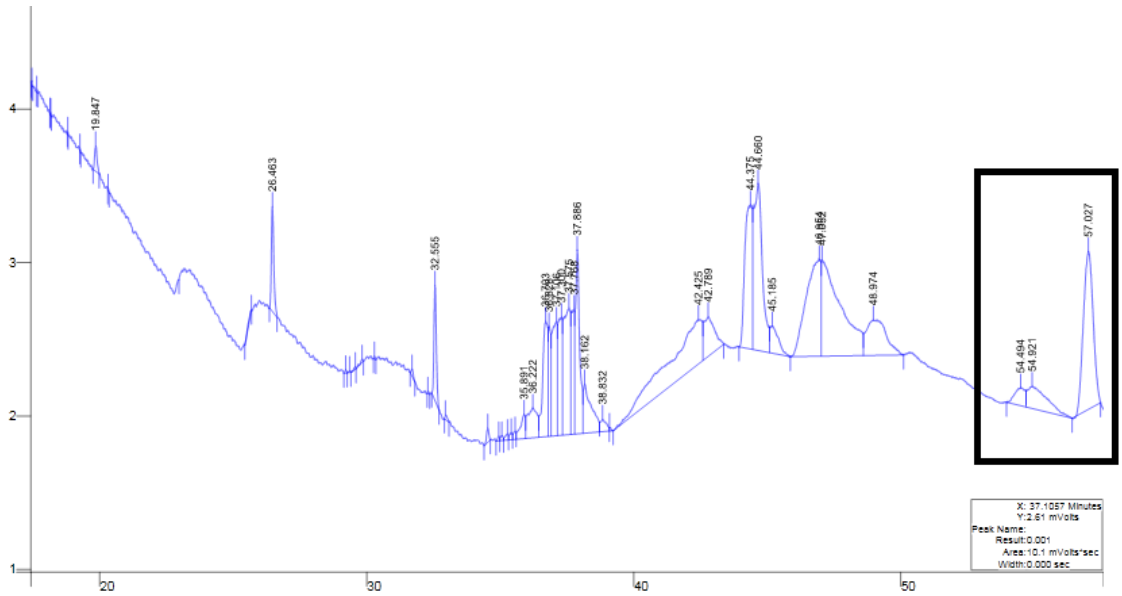


Figure 5.27 Chiral GC of the Product Formed by E1o with 2-OG and Methylglyoxal.

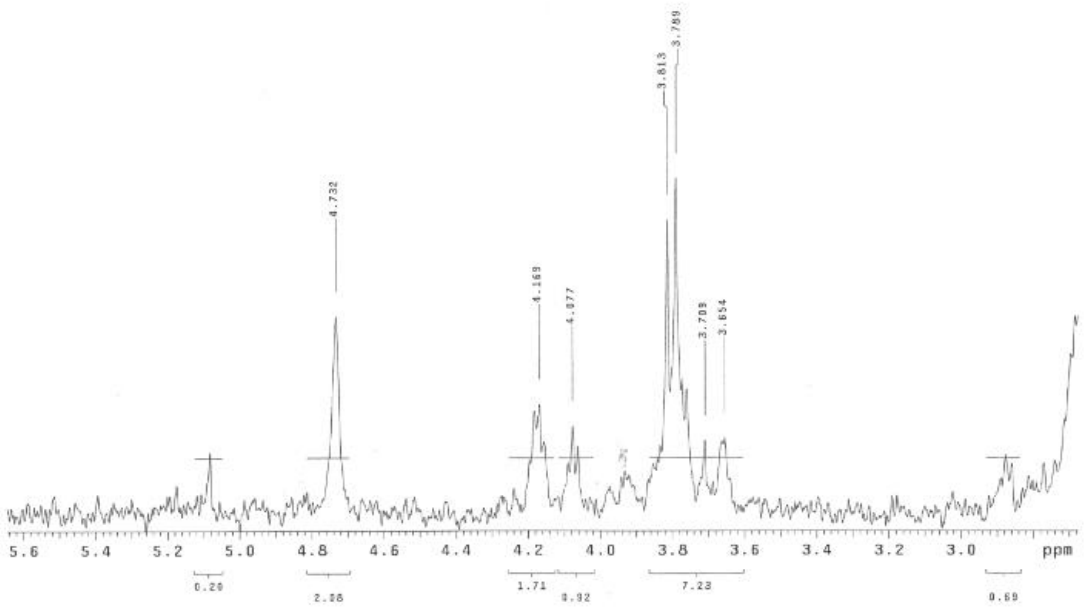


Figure 5.28 ^{1}H NMR Spectrum of the Product Formed by E1o (19 μM Active Center Concentration) in the Presence of 2-OG (2 mM), Methylglyoxal (1 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

5.4.8 Acetoin-like Product Formation by E1o with 2-OV and Methylglyoxal

The reaction of E1o with 2-OV and methylglyoxal (Figure 5.29) produced a weak negative CD band at 280 nm (Figure 5.30). The negative band indicates a (*R*)-acetoin-like product, which was confirmed by chiral GC (Figure 5.31) and ^{1}H NMR (Figure 5.32).

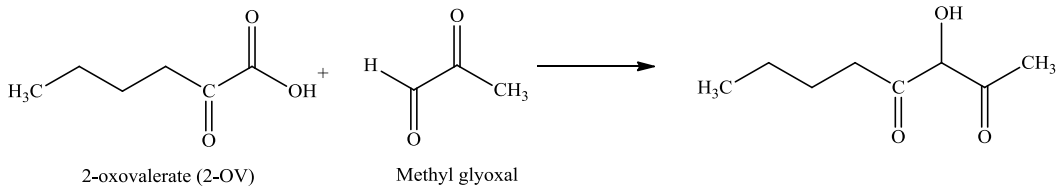


Figure 5.29 The Acetoin-like Product Produced from 2-OV and Methylglyoxal.

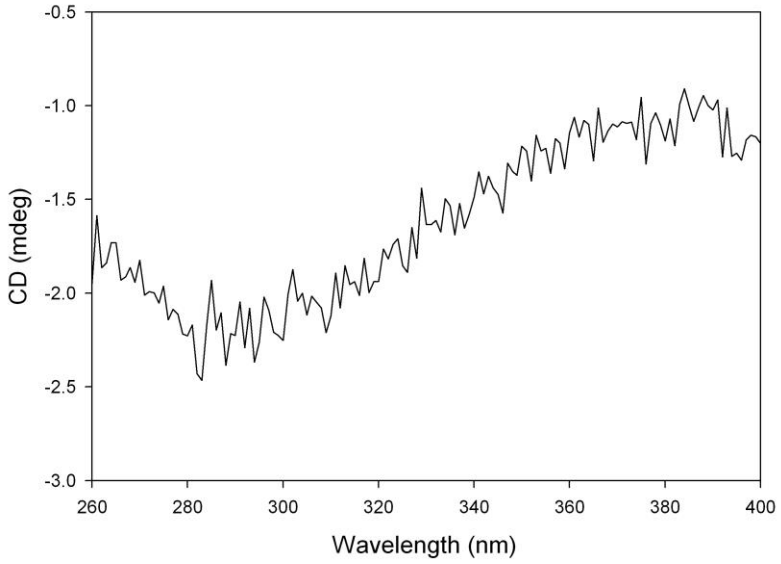


Figure 5.30 CD Spectrum of the Product Formed by E1o (19 μM Active Center Concentration) in the Presence of 2-OV (5 mM), Methylglyoxal (10 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

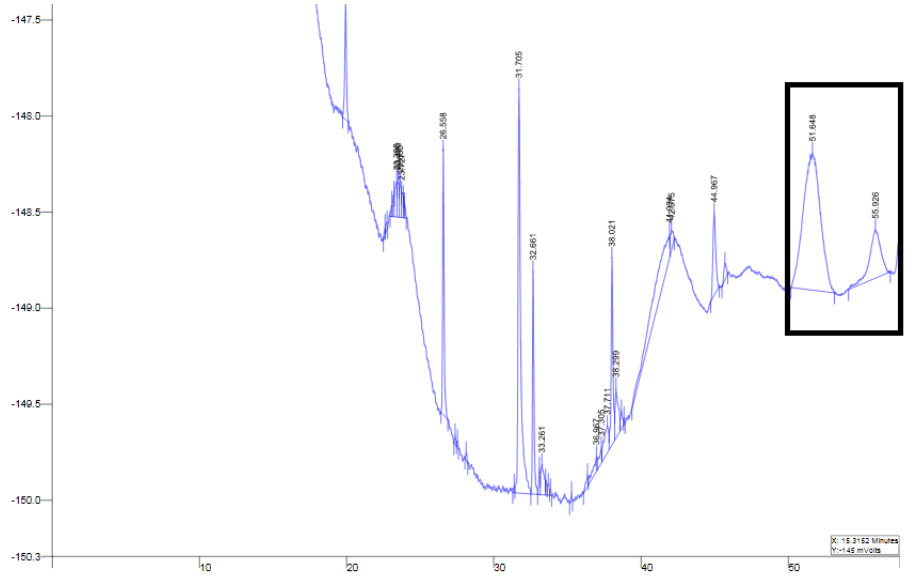


Figure 5.31 Chiral GC of the Product Formed by E1o with 2-OV and Methylglyoxal.

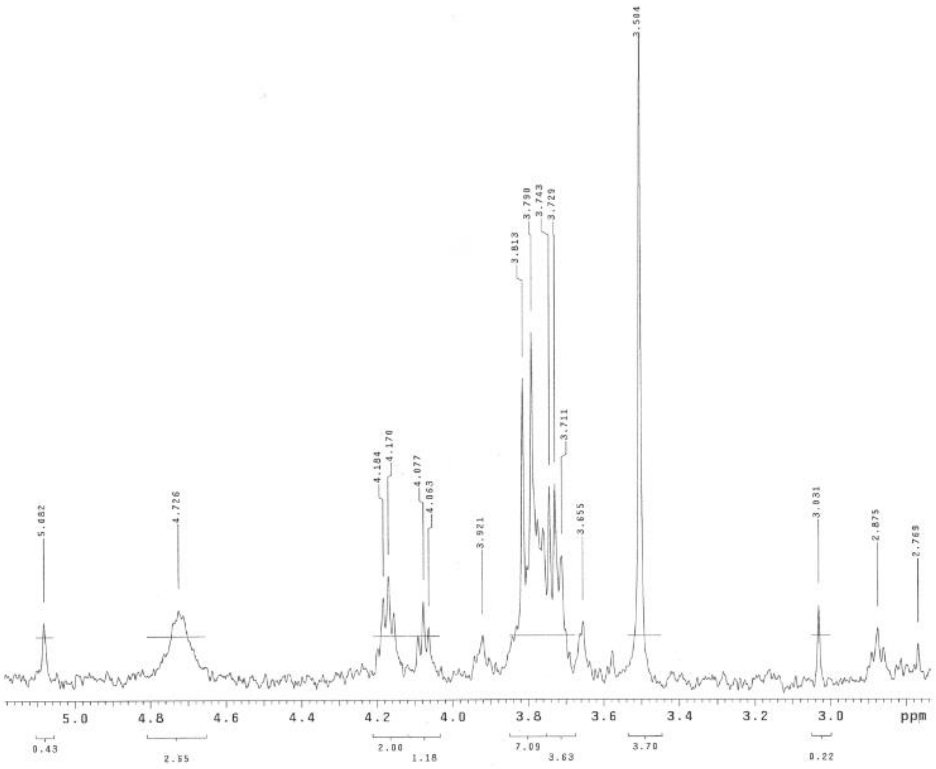


Figure 5.32 ^{1}H NMR Spectrum of the Product Formed by E1o (19 μM Active Center Concentration) in the Presence of 2-OV (5 mM), Methylglyoxal (10 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

5.4.9 Acetoin-like Product Formation by E1o with 2-OiV and Methylglyoxal

The reaction of E1o with 2-OiV and methylglyoxal (Figure 5.33) produced a weak positive band CD band at 280 nm (Figure 5.34). The positive band indicates (*S*)-acetoin-like product, which was confirmed by chiral GC (Figure 5.35) and ^{1}H NMR (Figure 5.36).

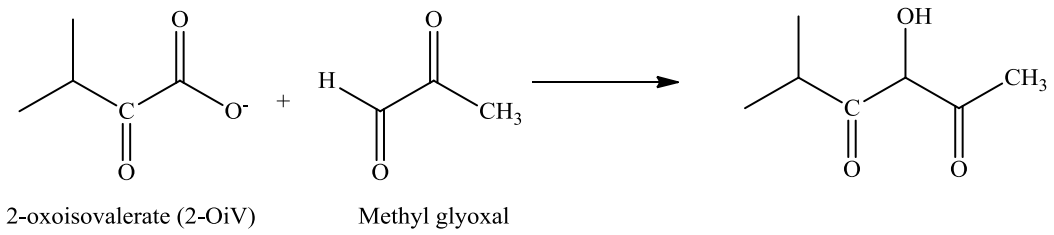


Figure 5.33 The Acetoin-like Product Produced by E1o from 2-OiV and Methylglyoxal.

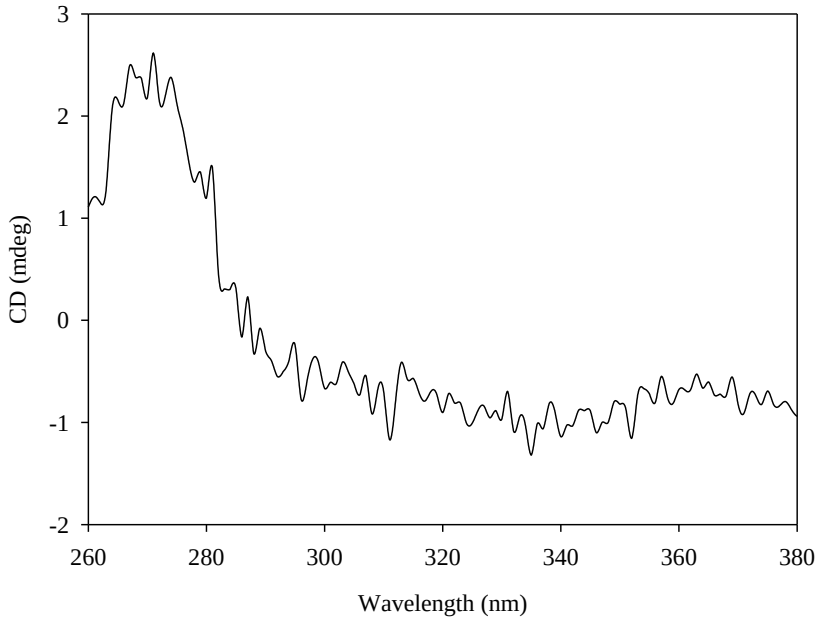


Figure 5.34 CD Spectrum of the Product Formed by E1o (19 μM Active Center Concentration) in the Presence of 2-OiV (5 mM), Methylglyoxal (10 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

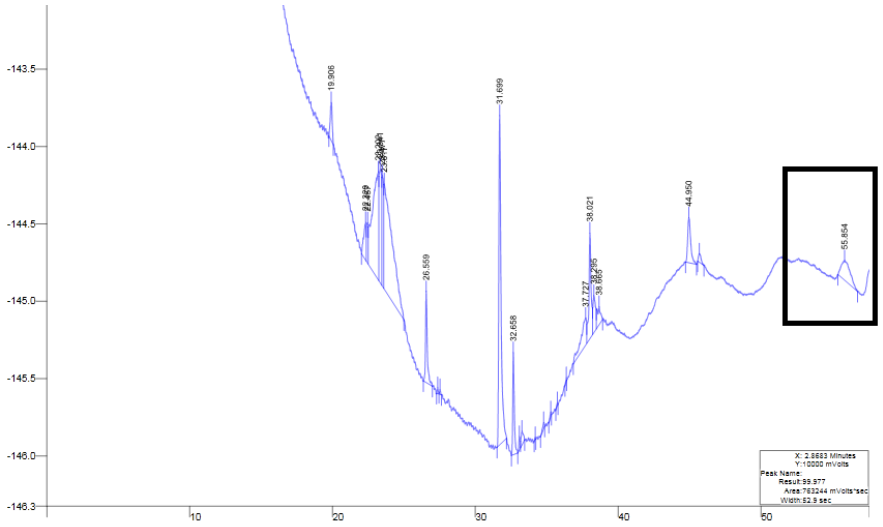


Figure 5.35 Chiral GC of the Product Formed by E1o with 2-OiV and Methylglyoxal.

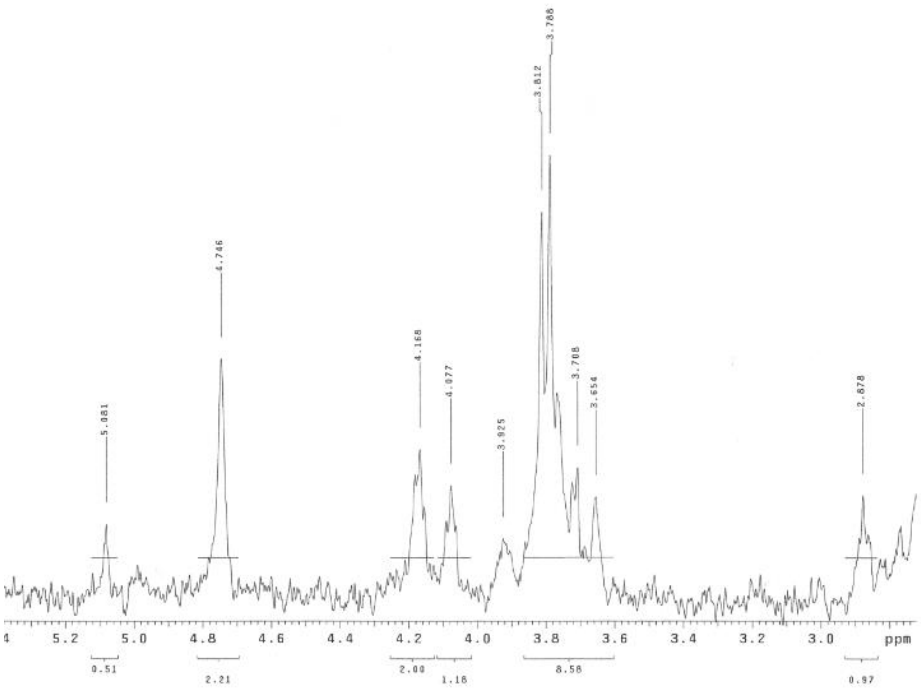


Figure 5.36 ^{1}H NMR Spectrum of the Product Formed by E1o (19 μM Active Center Concentration) in the Presence of 2-OiV (2 mM), Methylglyoxal (1 mM), $MgCl_2$ (2 mM), and ThDP (0.2 mM).

5.4.10 Chiral GC Analysis

E1o catalyzes the formation of acetoin-like products for all the reactions in high enantiomeric excess, as determined with chiral GC (Table 5.1). In addition, E1o demonstrates the versatility to yield products with different enantiomeric excess. This enantiomer depends on the substrate and the accepter being used. For example, E1o yields the (*R*)-enantiomer with 2-OG as the substrate and glyoxylate acceptor. On the other hand, (*S*)-enantiomer is produced with 2-OV as the substrate and glyoxylate as acceptor.

Table 5.1 Enantiomeric Excess of Acetoin-like Product Formed by OGDHc-E1o by Chiral GC[a]

Substrate	**Acceptor**	**Enantiomeric excess[b]**
2-oxoglutarate	glyoxylate	90.4% (*R*)
	ethyl glyoxylate	80.5% (*R*)
	methyl glyoxal	82.9% (S)
2-oxovalerate	glyoxylate	96.4% (*S*)
	ethyl glyoxylate	(*S*) [c]
	methyl glyoxal	81.2% (R)
2-oxoisovalerate	glyoxylate	(*S*)[c]
	ethyl glyoxylate	(*S*) [c]
	methyl glyoxal	(S) [c]

[a] See reaction conditions in Material and Methods.
[b] Yield is ~ 100% for all the acetoin products.
[c] Due to weak formation of *R* in GC could not calculate the *ee*% but clearly shows that *S* is in excess.

5.5 Conclusions

The ThDP-dependent enzymes are capable of catalyzing a broad range of carboligase reactions [73]. The variety of activities with respect to substrate range, catalytic activity, and enantioselectivity is a result of the protein environment surrounding ThDP. E1o of OGDHc from *E. coli* was catalyzes carboligase production as a side reaction (Scheme 3). The E1o is able to use a variety of substrates, which differ in size and polarity. While the natural substrate 2-OG has a negatively charged carboxylate group, both, 2-OV and 2-OiV have a hydrophobic tail. Furthermore, 2-OV has a straight chain, while 2-OiV is branched (Figure 5.1). Next, E1o forms carboligase products with a variety of acceptors, which also contain a range of size and polarity (Figure 5.1). Finally, the products for the carboligase reactions are produced with significant *ee* values. The trend for substrates 2-OV and 2-OiV reacting with the acceptors glyoxylate and ethyl glyoxylate is to produce the (*S*) enantiomer; whereas, 2-OG forms the (*R*) enantiomer. This may be due to 2-OV and 2-OiV both having hydrophobic tails, while 2-OG is hydrophilic. In contrast, there is no clear trend for the acceptor methylglyoxal with the three substrates.

The Westerfeld group previously demonstrated formation of carboligase products using the substrate 2-OV and the acceptors acetaldehyde and glyoxylate and enzymes from rat and beef heart particulate fractions [83]. However, no *ee* was reported. In this chapter, recombinant E1o from *E. coli* was also shown to form carboligase reactions. NMR confirmed product formation, and chiral GC and CD established the *ee*. In addition, it was shown that E1o is capable of forming esters. This is important because when the carboligase reactions produce β-ketoacids, these products are unstable and prone to decarboxylation. E1o of OGDHc can catalyze carboligase reactions from a variety of

substrate and aldehyde acceptors. These results indicate that E1o is a good starting point for protein engineering and optimization to synthesize stable chiral intermediates for fine chemical synthesis.

APPENDIX

CD TITRATION

Figures A.1 to A.8 show CD titration and dependence of ellipticity of E1o variants with succinyl phosphonate methyl ester and propionyl phosphinate.

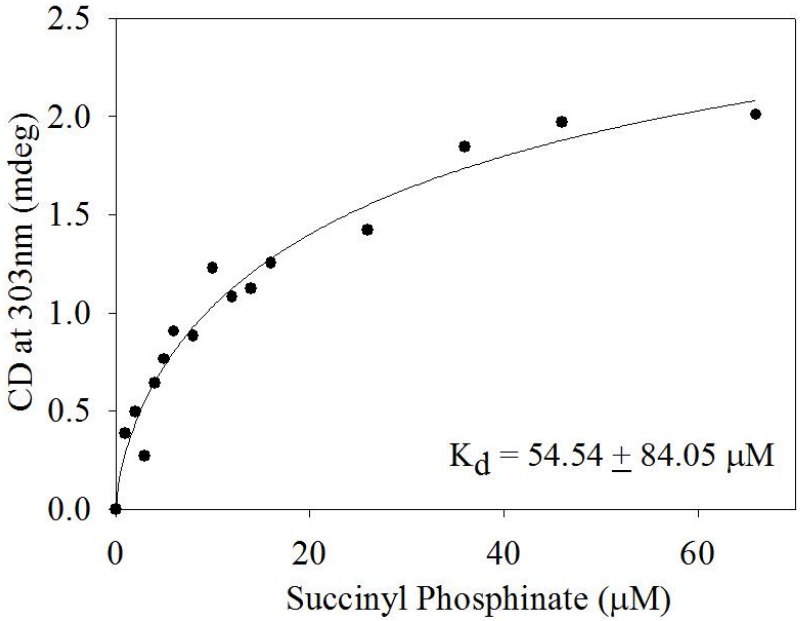


Figure A.1 Dependence of Ellipticity of His298Leu with Succinyl Phosphonate Methyl Ester at 303nm on the Concentration of Succinyl Phosphonate Methyl Ester.

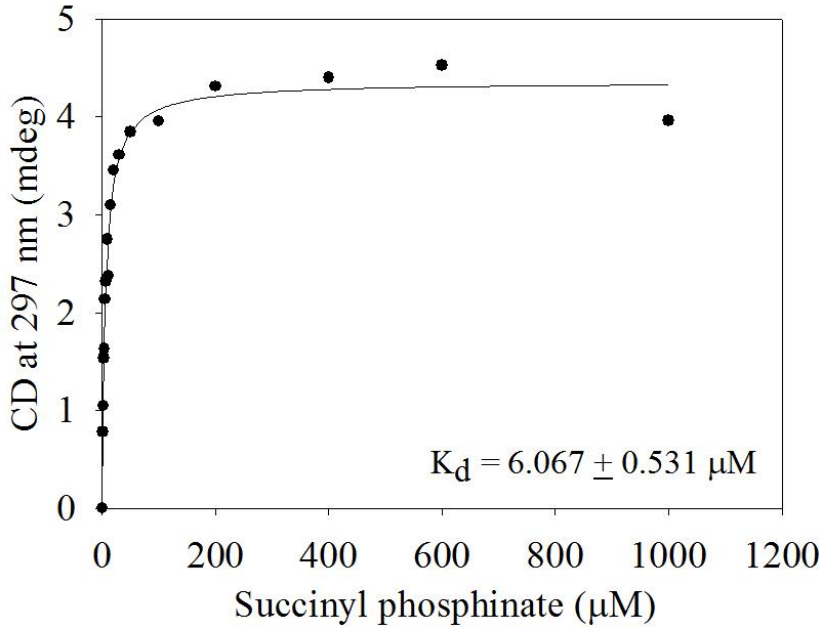


Figure A.2 Dependence of Ellipticity of His298Thr with Succinyl Phosphonate Methyl Ester at 297nm on the Concentration of Succinyl Phosphonate Methyl Ester.

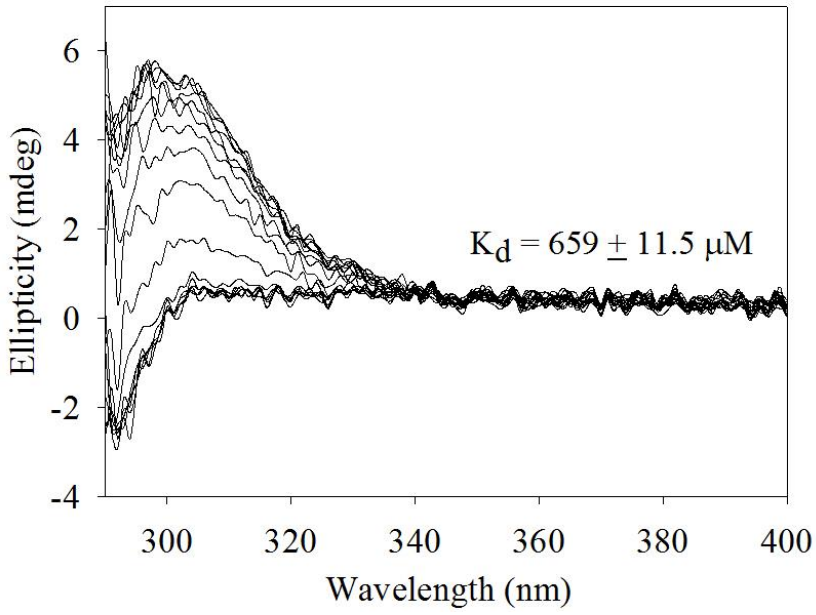


Figure A.3 CD titration of His298Asp with Succinyl Phosphonate Methyl Ester.

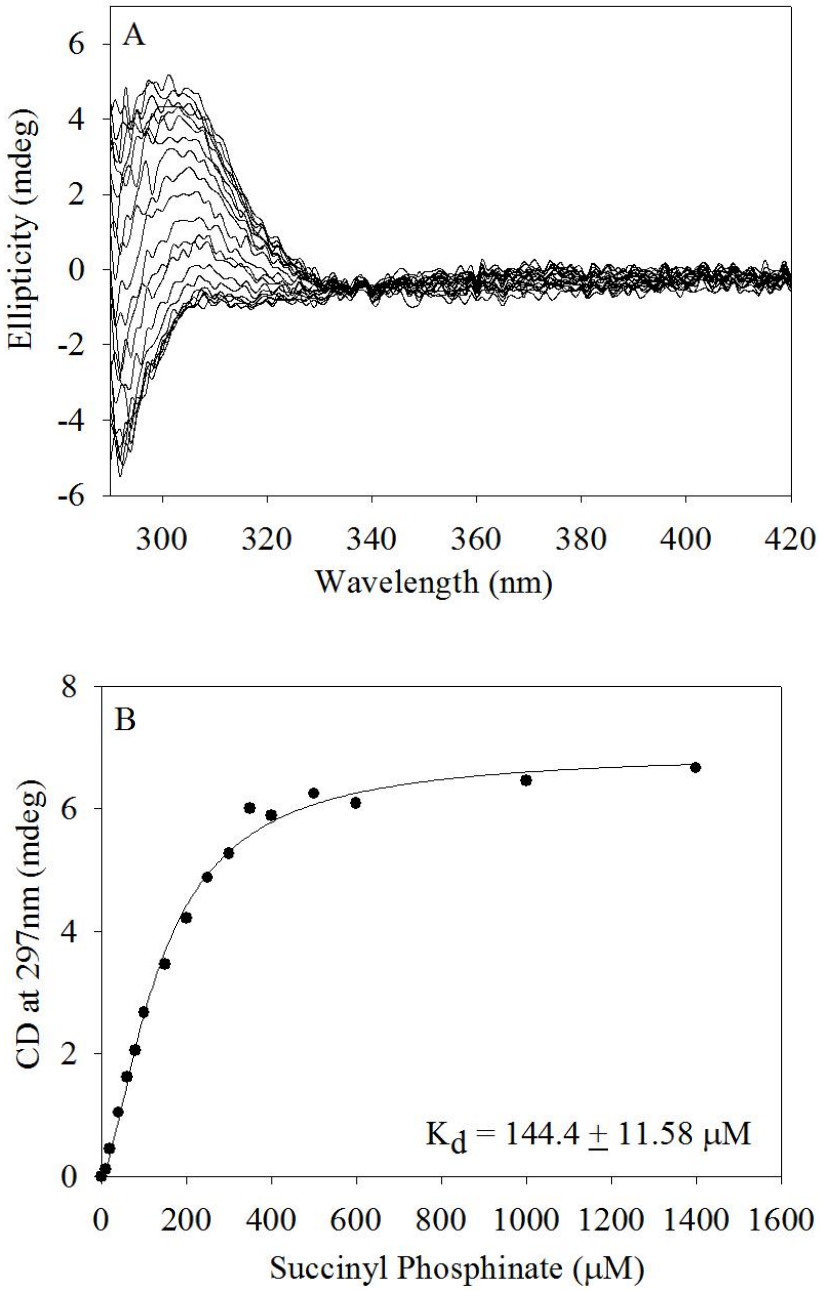


Figure A.4 CD titration of His298Val with Succinyl Phosphonate Methyl Ester and Dependence of Ellipticity at 297 nm on the Concentration of Succinyl Phosphonate Methyl Ester.

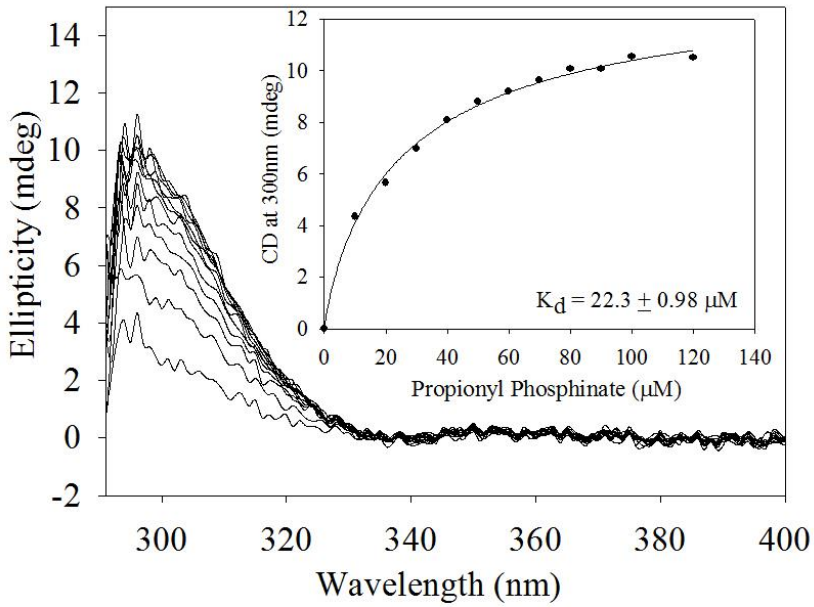


Figure A.5 CD titration of His298Leu with Propionyl Phosphinate and Dependence of Ellipticity at 300 nm on the Concentration of Propionyl Phosphinate.

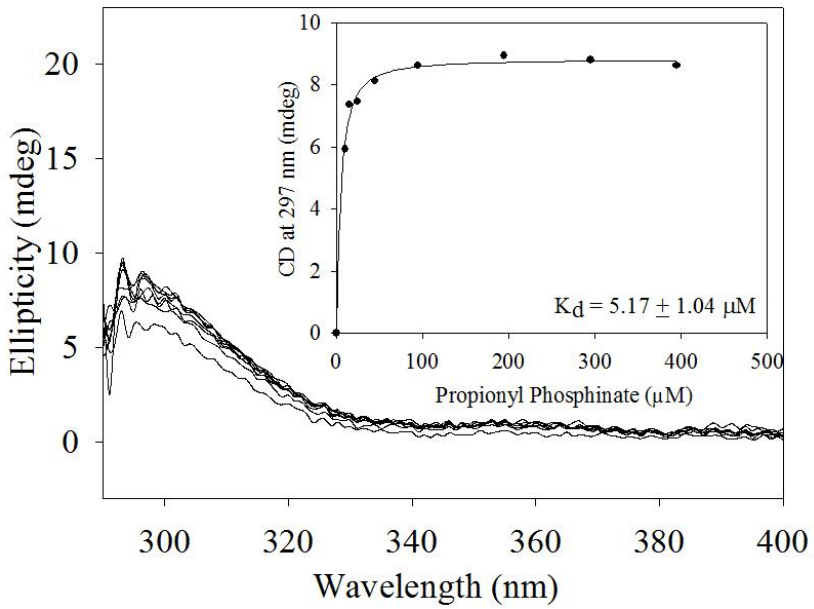


Figure A.6 CD titration of His298Val with Propionyl Phosphinate and Dependence of Ellipticity at 297 nm on the Concentration of Propionyl Phosphinate.

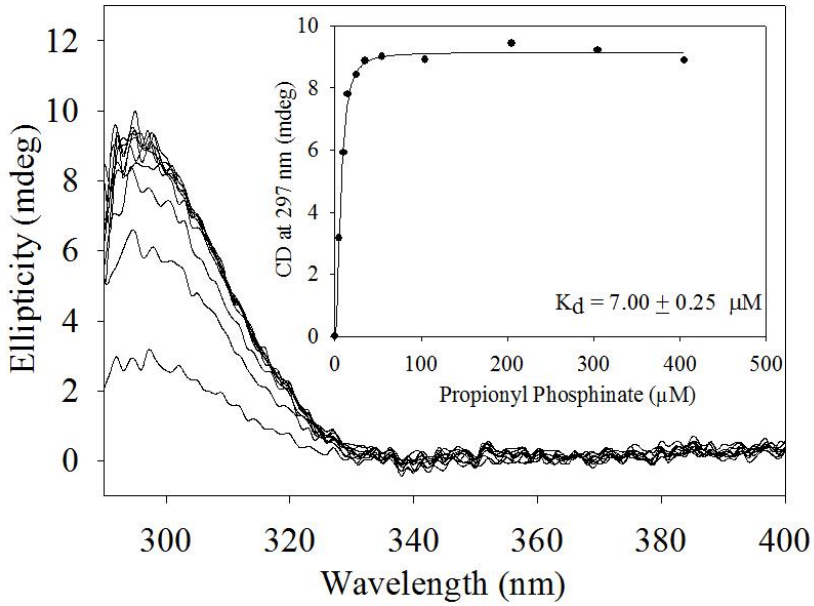


Figure A.7 CD titration of His298Thr with Propionyl Phosphinate and Dependence of Ellipticity at 297 nm on the Concentration of Propionyl Phosphinate.

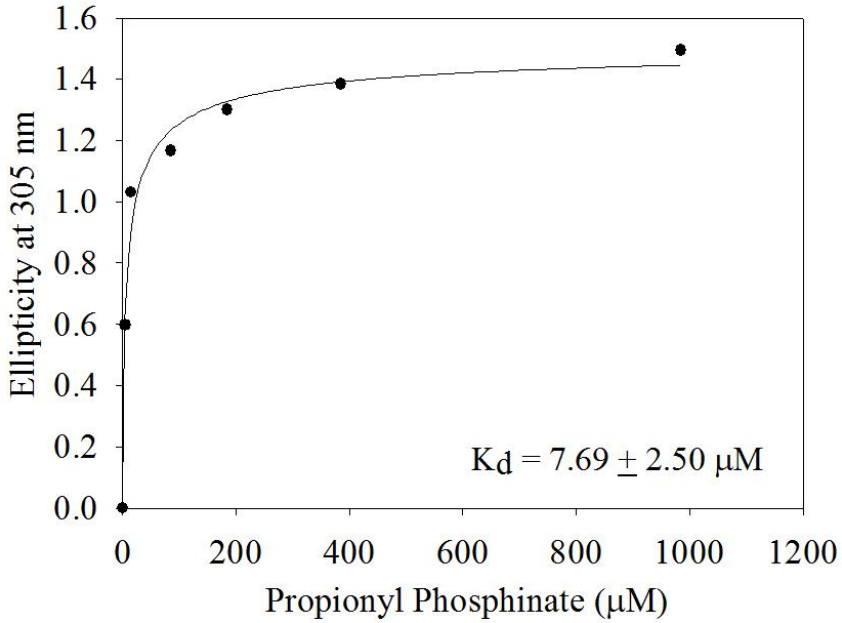


Figure A.8 CD titration of His260Glu/His298Asn with Propionyl Phosphinate and Dependence of Ellipticity at 305 nm on the Concentration of Propionyl Phosphinate.

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