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PURIFICATION OF LYSINE DECARBOXYLASE: A MODEL SYSTEM FOR PLP ENZYME INHIBITOR DEVELOPMENT AND STUDY

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PURIFICATION OF LYSINE DECARBOXYLASE: A MODEL SYSTEM FOR PLP ENZYME INHIBITOR DEVELOPMENT AND STUDY

By

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Pyridoxal phosphate (PLP) dependent enzymes have the ability to manipulate amino acid substrates, serving variously as (i) racemases, transaminases, and beta- or gamma eliminases (all involving Cα-H bond cleavage); (ii) decarboxylases (Cα-CO2- bond cleavage), or (iii) retroaldolases (Cα-Cβ bond cleavage). Dunathan posited that stereoelectronics govern the key C-X bond cleavage step across the class of PLP enzymes; namely by aligning the scissile bond of the substrate with the extended pi system of the substrate-PLP imine that bond is weakened. A mechanistic understanding of electron flow in this enzymatic class has motivated many groups, including the Berkowitz group, to develop mechanism-based enzyme inactivators for specific PLP enzymes. Most relevant to this thesis is the finding that L-alpha-(2'Z- fluoro)vinyllysine, designed as a "suicide substrate" is, indeed, an efficient irreversible inactivator ($t_{1/2}$ ~ 3 min, Ki ~ 100 uM, partition ratio ~ 16) of lysine decarboxylase from *Hafnia alvei* (K. R Karukurichi et al. *J. Am. Chem. Soc.* **2007**, *129*, 258-9) while the D-antipode is a slow substrate.

This thesis is motivated by the desire to better understand this interesting result at the molecular level. Described is a streamlined protocol for the purification of this useful model enzyme from the native source, *Hafnia alvei*.. The ultimate goal is prepare homogeneous protein of sufficient quality and quantity to permit its successful crystallization to yield diffraction quality crystals. This thesis details and documents an improved purification procedure of LDC,

as well as presents preliminary data toward its crystallization. The thesis will also review related key precedents in the field, both for the successful mechanism based inactivation of PLP-dependent enzymes, and for the structural inactivation, principally involving with protein crystallography

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μM	micromolar
AADC	amino acid decarboxylase
Arg	arginine
BSA	bovine serum albumin
Cu^{+}	copper (I)
Cu^{2+}	copper (II)
$CuSO_4$	copper sulfate
Da	Dalton
DAP(DC)	*meso*-diaminopimelate decarboxylase
DC	decarboxylase
dd	doubly distilled
DEAE	diethylaminoethyl
DFMO	α-difluoromethyl ornithine
DOPA	dopamine
E. Coli	*Escherichia coli*
FVL	fluoro-vinyl lysine

GABA	γ-amino butyric acid
GABA-AT	γ-amino butyric acid amino transferase
H. Alvei	*Hafnia Alvei*
K_2CO_3	potassium carbonate
kDa	kilo Dalton
Lactobacillus sp.	*Lactobacillus strain 30A*
LDC	lysine decarboxylase
Lys	lysine
mass spec	mass spectroscopy
MES	methyl ethyl sulphate
mg	milligram
$MgSO_4 \bullet 7H_2O$	magnesium sulfate heptahydrate
mM	millimolar
Na_2CO_3	sodium carbonate
nm	nanometer
NaOH	sodium hydroxide
ODC	ornithine decarboxylase

PEG	poly-ethylene glycol
PLP	pyridoxal phosphate
SDS	Sodium dodecyl sulfate
TNBS	2,4,6-trinitrobenzenesulfonic acid
TRIS	tris(hydroxymethyl)aminomethane
U	enzyme activity units
UV-VIS	ultraviolet-visible spectroscopy
w/v	weight per volume

I. Background

a) Pyridoxal Phosphate

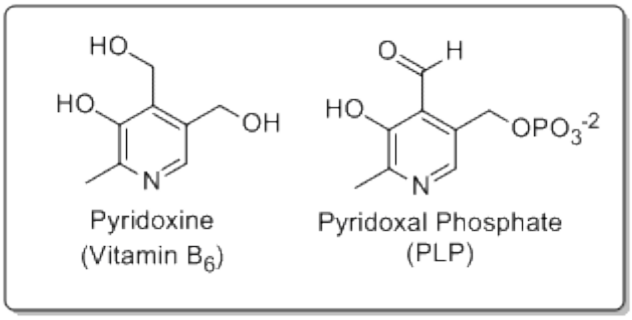


Figure 1.1 Vitamin B_6 and PLP

Pyridoxal phosphate (PLP) is the active form of Vitamin B_6[1]. PLP serves as an essential cofactor for a variety of enzymatic processes.[2] The beauty of PLP is its ability to serve a variety of functions. PLP-dependent enzymes generally perform chemistry on amino acids, and can cleave bonds to the α-carbon in transamination, racemization, decarboxylation, and retroaldolase reactions.[1-3] At the β- and γ-positions, PLP enzymes have the ability to facilitate eliminations or replacements.[3]

Harmon Dunathan (Haverford College) proposed a now classic model to explain how PLP-dependent enzymes are able to use a single cofactor for so many transformations, and yet maintain specificity for a particular reaction within a given active site.[4] He proposed that a given PLP-enzyme active site is able to dictate which bond to the α-carbon is broken by aligning that C-X bond with the extended pi-system of the cofactor imine. The Dunathan hypothesis implies that the ensuing electron delocalization is able to lower the energy of the transition state for bond

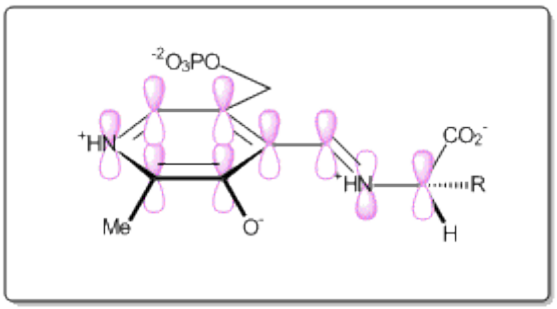


Figure 1.2 The Dunathan Hypothesis (illustrated for a decarboxylase active site)

breaking, thereby increasing the rate of the reaction. This stereoelectronic argument has found general acceptance among PLP chemists, although there is some discussion on the generality of the concept.[4-6] Both computational[7,8] and kinetic[9,10] studies are also consistent with the Dunathan hypothesis. Figure 1.2 depicts a C_α-CO_2^- bond aligned with the pi-system, as expected for a decarboxylase enzyme. By rotating around the C_α-N bond in 120° intervals, one arrives at the predicted transition state geometries for a racemase (C_α-H bond aligned), or a retroaldolase (C_α-C_β bond aligned).

The Dunathan hypothesis is discussed as it proposes that there are certain electronic and geometric constraints upon PLP catalysis, and elements of this hypothesis are testable by x-ray crystallographic examination of PLP enzymes. This thesis is concerned with the purification of lysine decarboxylase, toward its eventual crystallization, by those who will continue on the project. As will be discussed below, identification of an active site proton donor in the vicinity of the cofactor's pyridine nitrogen is a key element in considering the likelihood of complete electron delocalization (i.e formation of a quinonoid intermediate) along the PLP enzyme reaction coordinate. Solution of the three dimensional structures of either an internal aldimine or (better) an external aldimine (e.g. with a bound substrate analogue or inhibitor) allows one to better

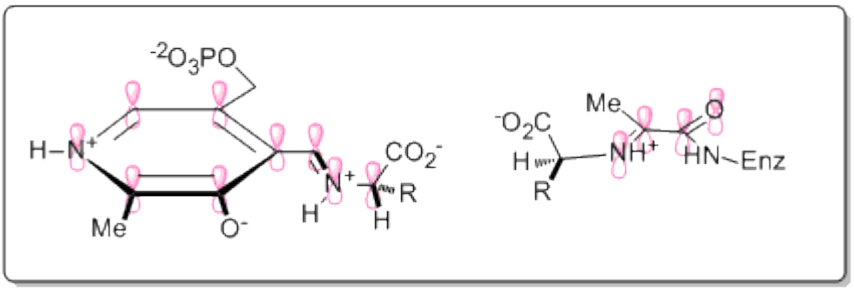


Figure 1.3 p-orbital alignment of PLP-dependent enzymes (left) versus pyruvamide dependent enzymes (right).

identify such active site residues.

On the other hand, the fact that nature has also evolved pyruvamide dependent enzymes that are able to catalyze some of the same reactions as PLP-dependent enzymes suggests that extended electronic delocalization is not always essential for such

Table 1.1 k_{cat} and K_m data for various strains of PLP and pyruvamide dependent histidine decarboxylases, compared to other PLP-Dependent amino acid decarboxylsses

Pyridoxal 5'-Phosphate Dependent Histidine Decarboxylases					
Strain	Morganella AM-15[11]	M. morganii[13]	K. planticola[13]	E. Aerogenes[13]	Fetal Rat Liver[19]
K_m (mM)	1.3	1.1	2.4	2.2	0.5
k_{cat} (sec^{-1})	52	107	101	92	.9

Pyruvamide Dependent Histidine Decarboxylases						
Strain	Lactobacillus 30a[14,17]	Lactobacillus buchneri[17,18]	C. perfringens[14,18]	Micrococcus sp. N[14,16,18]	Tetragenococcus muriaticus[15]	Oenococcus oeni[12]
K_m (mM)	0.4	0.6	0.3	0.8	0.74	0.33
k_{cat} (sec^{-1})	45	39	4	33	11.8	11.6

Argnine Decarboxylase			
Strain	*M. Jannaschi*	*E. Coli (inducible)*	*E. Coli (biosynthetic)*
Cofactor	Pyruvamide	PLP	PLP
Km (mM)	7.1	.065	.03
k_{cat} (sec^{-1})	2.65	704	18.5

Other PLP Dependent Decarboxylases				
Enzyme	ODC	ODC	ODC	LDC
Strain	*T. Brucei (pH 7.0)*[21]	*T. Brucei (pH 8.0)*[21]	*T. Brucei (pH 9.0)*[21]	*H. Alvei*[23]
Km (mM)	0.36 ± 0.01	0.44 ± 0.03	0.280 ± 0.030	1.35 ± 0.12
k_{cat} (sec^{-1})	12.7	15.1	3.1	180
Enzyme	ODC	Aromatic AADC	Aromatic AADC	Aromatic AADC
Strain	*Lactobacillus 30a*[22]	*Dirofilaria immitis*[20]	*Dirofilaria immitis*[20]	*Dirofilaria immitis*[20]
Substrate	ornithine	phenylalanine	tyrosine	tryptophane
Km (mM)	1.7	0.032	0.035	0.029
k_{cat} (sec^{-1})	383	188	122	112

chemistry. For example, bacterial histidine decarboxylase converts L-histidine to

histamine by utilizing PLP (Gram-negative species) or pyruvamide (Gram-positive species)[11] For a pyruvamide enzyme, the presumed intermediate upon decarboxylation, would delocalize the resultant electron density across three centers and four p-orbitals, as opposed to 5 centers and 9 p-orbitals for a fully delocalized PLP-based system. Table 1.1[11-19] depicts a variety of PLP- and pyruvamide-dependent histidine decarboxylases, along with their kinetic constants. By analyzing other PLP-only-dependent amino acid DC's, it is seen that kinetic data for histidine DC are not atypical for such enzymes. Specifically, aromatic amino acid DC displays kcat values of 188, 122 and112, as compared with 52, 92, 107,0.9 and101 for the PLP-dependent histidine DC. For arginine DC, the difference between the PLP-dependent (k_{cat} =18.5 and 704) and pyruvamide-dependent (k_{cat} = 2.65) enzymes is larger suggesting that, perhaps as much as an order of magnitude in rate enhancement may be gained via the increased conjugation in PLP vs. pyruvamide [20] Other kinetic data from other PLP-dependent AADC's such as ornithine decarboxylase[21,22] and lysine decarboxylase[23] supports this theory,

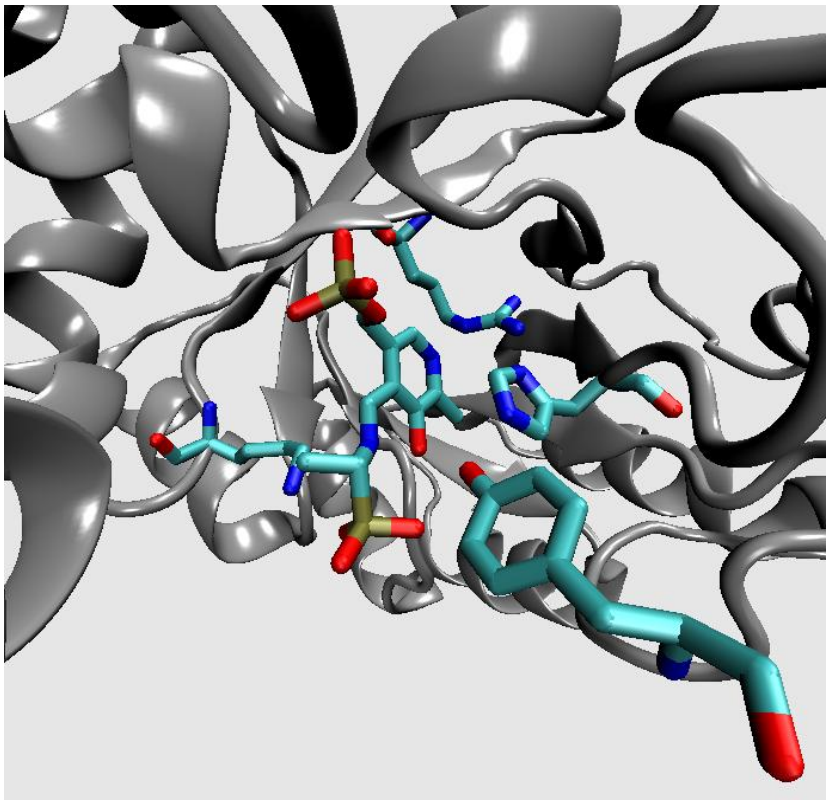


Figure 1.4 WT alanine racemase with alanine phosphonate

seeing k_{cat} values ranging from 3.1 to 383 sec^{-1}. Interestingly, overall it appears that there is not a large nor a general correlation between catalytic efficiency and cofactor used across the family of AADC enzymes. This observation increases interest in studying the validity and generality of the both the bond alignment and the pi-delocalization tenets of the Dunathan hypothesis.

Another enzyme that has been widely studied in debate over the Dunathan hypothesis is alanine racemase. According to the crystal structure of alanine racemase, there are three essential residues that give insight to the mechanism. In the conversion of L-alanine to D-alanine, tyrosine-265 serves as the essential base to deprotonate the α-proton on the si-face of bound L-alanine. This is inferred from the crystal structure of a bond phosphono-analogue of L-alanine (pdb 1BD0). Reprotonation by the active site lysine (Lys 39) residue then likely occurs on the re-face, allowing for conversion of L-alanine to D-alanine (Figure 1.5).

Toney uses this crystal structure to argue that even in the absence of a quinonoid intermediate, a PLP-enzyme is still able to catalyze a racemase reaction at good rates.

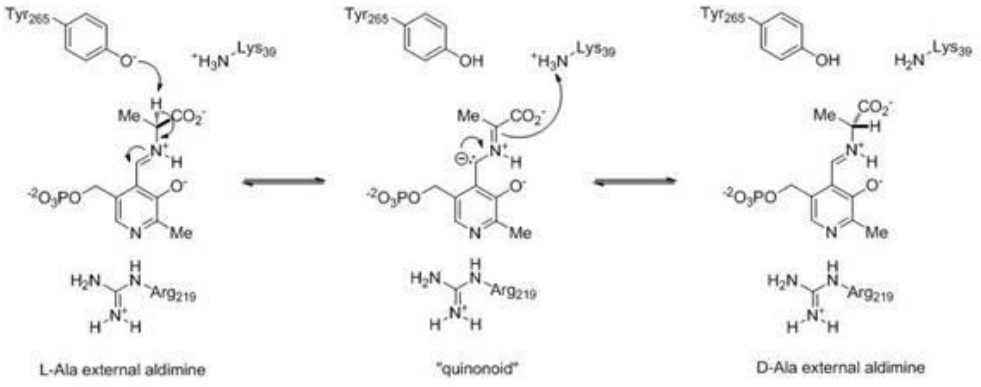


Figure 1.5 Proposed mechanism of alanine racemase

His argument is based on the presence of arginine-219 near the pyridine nitrogen. Given the generally lower acidity of a guanidium functionality (pKa ~ 12.5 for free Arg) compared to a carboxylic acid functionality (pKa~ 3-5 in solution), Toney hypothesizes that the formation of a quinonoid intermediate is less likely in this enzyme. Inspecting this structure, I find that the pyridine-N/Arg-219-N distance is measured at 2.93 Å, in contrast to 2.6 Å, for the aspartate oxygen-pyridine nitrogen in the crystal structure of ornithine decarboxylase from Lactobacillus (pdb ID 1ORD), hinting that hydrogen bonding is still present but there may not be a complete transfer of the proton. Toney utilizes a pKa argument, stating that in solution the arginine has a pKa of 12.6 is unlikely to protonate the nitrogen within the pyridine ring with a pKa of 5. It is well to note that active site bases can have dramatically lowered pKa's in appropriate active sites. Perhaps the classic case here is that the active site lysine in acetoacetate decarboxylase, estimated by Westheimer and co-workers to be 5.9, based upon active site titration of 2,4-dinitrophenyl propionate with acetoacetate decarboxylase. [24]

These initial results gave insight to study the mechanism of alanine racemase.[25-28] Toney mutated the active site arginine to a variety of amino acids to study the effect on reaction rates.[26] As Table 1.2 depicts, the weaker proton donors have large affects on the

Table 1.2 Catalytic efficiencies of alanine racemase mutants

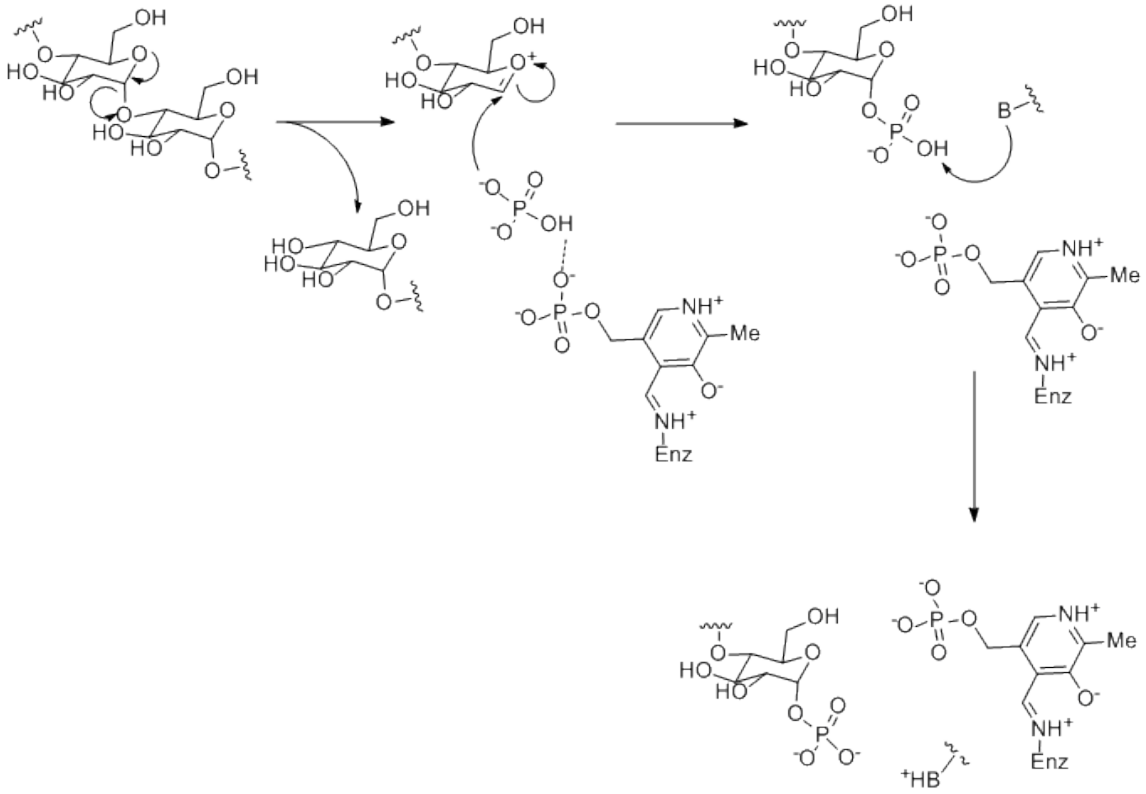
	D → L		L → D	
	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
WT	1.1×10^3	4.0×10^5	1.4×10^3	4.7×10^5
R219K	3.5×10^2	9.5×10^4	7.5×10^2	9.5×10^4
R219A	0.97	3.2×10^3	1.3	3.7×10^3
R219E	0.32	6.0×10^2	0.42	7.7×10^2

rate of reaction, decreasing the catalytic efficiency of the enzyme. On the other hand, one actually observes the quinonoid intermediate for the R219E by UV analysis. Moreover, the R219E mutant had an increased propensity to give transamination. WT alanine racemase favors racemization over transamination 10^6:1, where R219E alanine racemase favors racemization over transamination 185:1. This suggests that, in general, (N-protonated) quinonoid intermediates may have a higher propensity for C4'-protonation, relative to Cα-protonation, than their non-delocalized aza-allylic anion counterparts. Accordingly, Toney has suggested while this may prove to be an example that disfavors the production of the quinonoid intermediate, it also suggests that specificity of the reaction catalyzed may be dependent on formation of the quinonoid intermediate, or lack thereof.

b) Lysine Decarboxylase

Although the three dimensional structure of lysine decarboxylase (LDC) has yet to be determined, the structures of numerous PLP-dependent enzymes have been solved crystallographically. Despite the vast functionality of PLP-dependent enzymes, all of these can be categorized into one of five fold types[29]: aspartate amino transferase family (Fold Type I), tryptophan synthase family (Fold Type II), alanine racemase family (Fold Type III), (*D*)-alanine aminotransferase family (Fold Type IV), and starch and glycogen phosphorylases (Fold Type V).[29]

One of the most interesting of the fold types is Fold Type V, which consists of only glycogen phosphorylase and maltodextrin phosphorylase. These enzymes are necessary to break and phosphorylate a glycosyl linkage, with PLP bound to an active



Scheme 1.1a Proposed mechanism of glycogen phosphorylase

site lysine.[30] Withers, as well as others, have seen that PLP is necessary for the reaction to proceed, however, there is no Schiff base formation as seen in all other PLP-dependent mechanisms.

In order to better understand the mechanism of glycogen phosphorylase, Withers developed a series of PLP analogues.[31-33] Three mechanisms for glycogen phosphorylase have been proposed. The first mechanism proposes that PLP phosphate could attack the C-1 carbon while an active site histidine could serve as a general acid to protonate the leaving glycosidic oxygen.[31] The second hypothesis is that the phosphate group of the PLP serves directly as a general acid to protonate the leaving oxygen. Evidence has been obtained by the Withers group that speaks against the second hypothesis. Namely, this research group was able to replace the cofactor in glycogen phosphorylase with both the

phosphonate ($pK_a(2) \sim 8$) and the α,α-difluorinated phosphonate ($pK_a(2) \sim 5.5$) analogues of PLP. Though these are poorer and better Bronsted acids than the native PLP-phosphate ($pK_a(2) \sim 6.5$) respectively, only minor changes seen in reaction rate are seen. This in turn suggests that leaving group protonation by cofactor does not occur, at least not in the rate determining step.

The third proposed mechanism that has been suggested is that the PLP phosphate (or phosphonate) group can accept a hydrogen bond with inorganic phosphate (HPO_4^{2-}) which can facilitate transfer of this phosphate group to glycogen, resulting in α-glucose-1-phosphate, as seen in Scheme 1.1a.[31] Studies have shown that PLP functions primarily as an essential bridge to facilitate transfer of an inorganic phosphate to the sugar through this hydrogen bond.[32]

This interaction was seen utilizing the natural pyridoxal phosphate, as well as seeing a comparable rate with the α-difluorinated phosphonate analogue of PLP.[31-33] Interestingly, since the mono-fluorinated phosphonate is also able to facilitate the phosphorylation of α-glucose-1-phosphate, it may not be essential to assist in formation of the hydrogen bond. In order to test this proposed mechanism, Withers and coworkers synthesized 5'-pyridoxal-1-diphospho-α-*D*-glucose (Figure 1.5b, α-PLPPG), and its rate of reconstitution was compared to the rate of

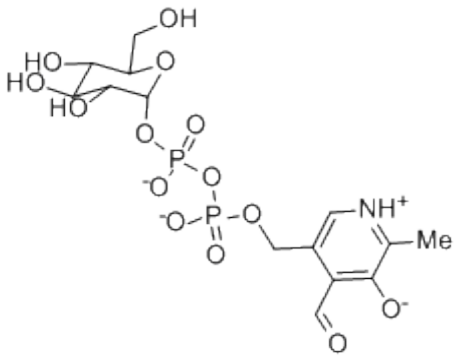


Figure 1.5b 5'-pyridoxal-1-diphosphono-α-*D*-glucose

reconstitution of 5'-pyridoxal-1-phospho-β-*D*-glucose (β-PLPG). It was found that the α-PLPPG analogue had a rate of reconstitution that was one third less of PLP, yet was 38 fold better than β-PLPG .[31] This difference in rate shows that the pocket of the holozyme has space sufficient to hold PLP and an additional phosphate group, though it is unlikely that an actual pyrosphosphate linkage actually forms along the reaction coordinate.

Also, glycogen phosphorylase was found to catalyze cleavage of the alpha-glucosidic linkage to PLPP, thus releasing glucose-centered oxocarbenium ion-like species that ultimately gives glucose and leaves PLPP covalently bound to the enzyme. This finding is consistent with glycogen phosphorylase using the PLP as a catalyst by hydrogen bonding with inorganic phosphate, thereby facilitating its attack upon a similar cationic glucosyl center. The decrease in rate of reconstitution from the pyrophosphate-linked cofactor-glucose construct suggests that the two are likely unattached. Crystal structures also are unable to unambiguously confirm/refute this hypothesis, although continued studies along these lines are needed.[32,33] Glycogen phosphorylase is a rare case where chemistry is created without formation of the Schiff base with the substrate. Chemistry occurs with PLP remaining in the form of the internal aldimine. PLP enzymes of the other four fold types, however, appear to perform the three basic functions of general PLP-dependent enzymes.

Fold Type I (Aspartate aminotransferase Family) is the most well studied of all PLP-enzyme fold types based on the number of solved crystal structures, and also accounts for a majority of the PLP dependent enzymes.[29] Members of this fold type are found to be active as homo-dimers, in general, with subunits comprising a large domain and a small domain. Out of 18 crystal structures identified for Fold Type I, the position of the cofactor bound appears to be nearly identical in all cases, with the PLP attached to a lysine side chain in the large domain.[29] The Fold Type I family contains many enzymes, including those breaking three different bonds to the α-carbon, such as aminotransferase subclass 1 and 2 (alpha-C-H), tyrosine-phenol lyase (alpha-C-H), serine hydroxymethyltransferase (alpha-C-C(side chain)), bacterial ornithine decarboxylase (alpha-C-CO_2-), as well as others.[29] Because LDC has such a high homology with ODC from *Lactobacillus sp.*, having 34% sequence identity and 51% sequence homology over

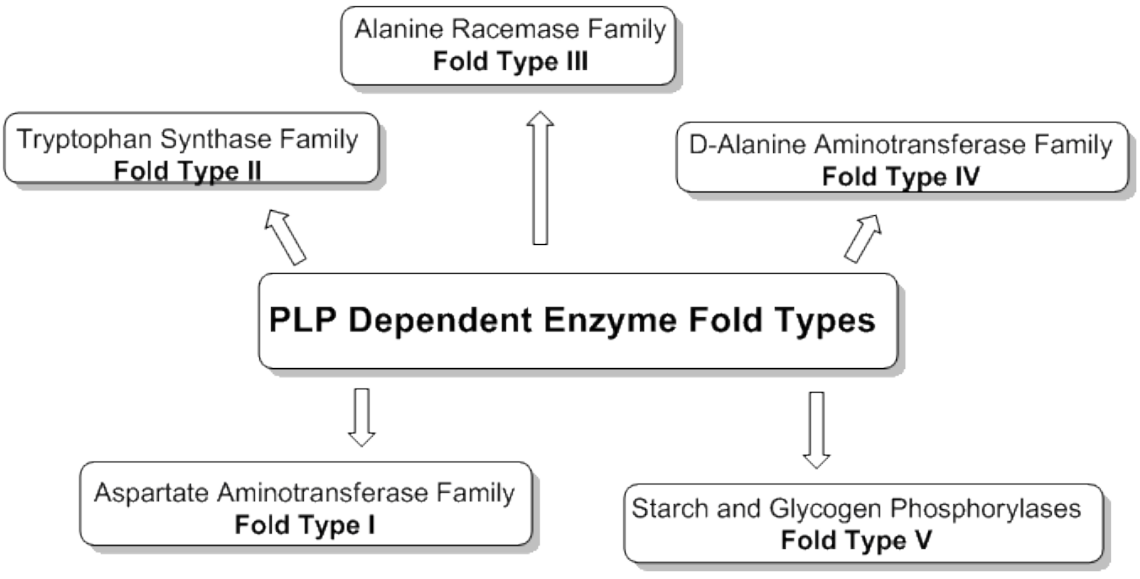


Figure 1.6 PLP Dependent Enzyme Fold Types

731 amino acids (vide infra), it is inferred that LDC likely exists in the Fold Type I family.

Schneider had developed a table of crystallized enzymes, but since 2000, over 100 crystal structures of PLP-dependent enzymes (without substrates, inhibitors, or mutations), so his fold type classification was in need of expansion. Again, the table was developed by looking at protein folds seen in the crystal. Table 1.3 presents my effort to establish an up to date version of the Schneider table, taking into account the very significant body of structural data that has since been deposited in the pdb.

Table 1.3 Fold types of PLP-dependent enzymes

Fold Type I	Enzyme	*Species*	Function	Year discovered	PDB Code
	Aspartate Amino Transferase	*chicken*[34]	Aminotransferase	1982	1AAT
		E. coli[35]	Aminotransferase	1994	1ARS
		Pig[36]	Aminotransferase	1997	1AJR
		Saccharomyces cerevisiae[37]	Aminotransferase	1998	1YAA
		Thermus Thermophilus [38]	Aminotransferase	1998	1BJW
		Rhodobater sphaeroides[39]	Transferase	2010	3NRA
		Plasmodium falciparu(unpublished)	Transferase	2010	3K7Y
		mouse[40]	Transferase	2010	3PD6
	Dialkylglycine Decarboxylase	*Pseudomonas cepacia*[41]	Decarboxylase	1994	1DGD
	Tyrosine Phenol-Lyase	*Citrobacter freundii*[42]	Lyase	1997	2TPL
		Erwinia Herbicola(unpublished)	Lyase	2003	1C7G
	Ornithine Aminotransferase	*Human*[43]	Aminotransferase	1998	1OAT
	Aromatic Amino Acid Aminotransferase	*Paracoccus denitrificans*[44]	Aminotransferase	1998	1AY4
	Ornithine Decarboxylase	*L. bacillus*[45]	Decarboxylase	1995	1ORD
	Glutamate-1-Semialdehyde Aminomutase	*Synechococcus sp.*[46]	Aminotransferase	1997	2GSA
	Aromatic Amino Acid Aminotransferase	*Pyrococcus Horikoshii OT3*[47]	Aminotransferase	2001	1DJU
	Tyrosine Aminotransferase	*E. coli*[48]	Aminotransferase	1999	3TAT
		Typanosoma Cruzi[49]	Aminotransferase	1999	1BW0
		Human(unpublished)	Transferase	2008	3DYD
		Mouse [50]	Transferase	2010	3PDX

Phosphoserine Aminotransferase	*Bacillus circulans (unpublished)*	Aminotransferase	1998	1BT4
	E. coli[51]	Aminotransferase	1998	1BJM
	Bacillus Alcalophilus[52]	Aminotransferase	2004	1W23
	Yersinia pestic CO92(unpublished)	Transferase	2011	3QBO
	Cytophaga Hutchinsonii (unpublished)	Transferase	2008	3FFR
Cystathionine Beta-Lyase	*E. coli*[53]	Lyase	1996	1CL1
	arabidopsis thaliana[54]	Lyase	2001	1IBJ
Cystathionine Gamma-Synthase	*Nicotiana tabacum*[55]	Lyase	1999	1QGN
	E. coli[56]	Lyase	1999	1CS1
Serine Hydroxymethyl Transferase	*Rabbit*[57]	Transferase	1999	1CJ0
	Human[58]	Transferase	1999	1BJ4
	B. stearothermophilus[59]	Transferase	2002	1KKJ
3-amino-5-hydroxybenzoic acid synthase	*Amycolatopsis mediterranei*[60]		1999	1B9H
Adenosylmethionine-8-amino-7-oxonanoate aminotransferase	*E. coli (unpublished)*	Aminotransferase	2000	1DTY
7,8-diaminopelargonic acid synthase	*E. coli* [61]	Transferase	2000	1QJ5
	Mycobacterium tuberculosis[62]	Transferase	2008	3BV0
	Bacillus subtillis[62]	Transferase	2009	3DOD
gamma-aminobutyrate aminotransferase	*pig*[63]	Aminotransferase	2004	1GTX
	E. coli[64]	Aminotransferase	2004	1SF2
	Sulfolobus tokodaii strain 7 (unpublished)	Transferase	2007	2EO5
2-amino-3-ketobutyrate CoA ligase	*E. coli*[65]	Transferase	2001	1FC4
2-aminoethylphosphate transaminase	*Salmonella typhimurium*[66]	Transferase	2002	1M32
3,4-dihydroxyphenyl alanine decarboxylase	*Drosophila*[67]	Lyase	2010	3K40
Acetylornithine aminotransferase	*Thermus thermophilus hb8(unpublished)*	Transferase	2005	1VEF
	Thermotoga maritime (unpublished)	Transferase	2007	2ORD
	Aquifex aeolicus VF5(unpublished)	Aminotransferase	2007	2EH6
Acyl-CoA Synthase	*E. coli*[68]	Transferase	1999	1BS0
Alanine Aminotransferase	*Pyrococcus furiosus(unpublished)*	Aminotransferase	2004	1XI9
	Human(unpublished)	Transferase	2009	3IHJ
Alanine Glyoxylate Aminotransferase	*Human*[69]	Aminotransferase	2003	1H0C
	Nostoc sp.[70]	Aminotransferase	2004	1VJO
Alliinase	*Allium Sativum*[71]	Lyase	2007	2HOR
Alpha-aminodipate aminotransferase	*Thermus thermophilus hb27 (unpublished)*	Transferase	2008	2EGY
alpha-amino-epsilon-caprolactam racemase	*Achromobater obae*[72]	Isomerase	2009	3DXV

Arginine Decarboxylase	*E. coli*[73]	Decarboxylase	2009	2VYC
ArnB Transferase	*Salmonella typhimurium*[74]	Lyase	2002	1MDX
Aspartate-Beta-Decarboxylase	*Pseudomonas dacunhae*[75]	Decarboxylase	2009	3FDD
CsdB	*E. coli*[76]	Lyase	2000	1C0N
Cystalysin	*Treponema denticola*[77]	Transferase	2000	1C7N
Cystathionine gamma-Lyase	*Saccharomyces cerevisiae*[78]	Lyase	2002	1N8P
	Human[79]	Lyase	2006	2NMP
Cysteine desulfurase	*E. coli*[80]	Lyase	2003	1P3W
DOPA Decarboxylase	*Pig*[81]	Decarboxylase	2001	1JS6
GDP-4-keto-6-deoxy-D-mannose-3-dehydratase	*E. coli*[82]	Transferase	2006	2GMS
GDP-perosamine synthase	*Caulobacter crescentus cb15*[83]	Transferase	2008	3BN1
Glutamate Decarboxylase	*E. coli*[84]	Decarboxylase	2004	1PMM
Glutamate oxaloacetate transaminase	*Human(unpublished)*	Transferase	2009	3II0
Glutamate-1-Semialdehyde Aminomutase	*Thermus thermophilus hb8(unpublished)*	Isomerase	2007	2E7U
	Aeropyrum pernix(unpublished)	Isomerase	2007	2EPJ
Glutamine Aminotransferase	*Thermus thermophilus*[85]	Aminotransferase	2004	1V2D
Glutamine-2-deoxy-scyllo-inosose aminotransferase	*Bacillus circulans*[86]	Transferase	2006	2C7T
Glycine Decarboxylase	*Thermus thermophilus hb8*[87]	Decarboxylase	2005	1WYU
Histidinol-phosphate aminotransferase	*E. coli*[88]	Aminotransferase	2001	1GEW
	Corynebacterium glutamicum[89]	Transferase	2008	3CQ4
Hydroxykyrnurenine Transaminase	*Anopheles Gambiae*[90]	Transferase	2006	2CH1
Kynureninase	*Pseudomonas Fluorescens*[91]	Hydrolase	2003	1QZ9
	Human[92]	Hydrolase	2004	1W7L
	Aedes Aegypti[93]	Aminotransferase	2005	1YIY
L-aspartate beta-decarboxylase	*Pseudomonas sp. atcc 19121*[94]	Decarboxylase	2009	2ZY2
L-Cysteine Lyase	*Synechocystis sp. Pcc 6714*[95]	Lyase	2000	1ELQ
LL-Diaminopimelate aminotransferase	*arabidopsis thaliana*[96]	Transferase	2008	3EI5
L-methionine alpha-, gamma-lyase	*Pseudomonas putida(not published)*	Lyase	2004	1PG8
	Citrobacter freundiia[97]	Lyase	2005	1Y4I
L-Threonine Aldolase	*Thermotoga maritime*[98]	Lyase	2002	1M6S
L-Threonine-O-3-Phosphate Decarboxylase	*Salmonella enteric*[99]	Decarboxylase	2002	1LKC
Lysine Aminotransferase	*Mycobacterium tuberculosis*[100]	Transferase	2006	2CIN

Methionine Gamma-Lyase	*Trichomonas Vaginalis(unpublished)*	Lyase	2001	1E5E
Multiple Substrate Aminotransferase	*Thermococcus profundus(to be published)*	Transferase	2005	1WST
N-acetylornithine aminotransferase	*Salmonella typhimurium*[101]	Transferase	2008	2PB0
O-Acetyl homoserine sulfhdrylase	*Thermus thermophilus hb8(unpublished)*	Transferase	2005	2CTZ
Ornithine delta-aminotransferase	*Plasmodium falciparum*[102]	Transferase	2010	3NTJ
O-succinylhomoserine sulfhydrylase	*Mycobacterium tuberculosis(unpublished)*	Lyase	2010	3NDN
Phenylserine Aldolase	*Pseudomonas putida(unpublished)*	Lyase	2005	1V72
Phosphoserine Aminotransferase	*Mycobacterium tuberculosis(unpublished)*	Transferase	2007	2FYF
	Human(unpublished)	Transferase	2008	3E 77
PseC aminotransferase	*Helicobacter pylori 26695*[103]	Transferase	2006	2FNI
Selenocysteine Lyase	*E. coli*[104]	Lyase	2001	1JF9
	Rattus norvegicus[105]	Lyase	2010	3A9X
Serine Palmitoyltransferase	*Pseudomonas paucimobilis*[106]	Transferase	2007	2JG2
	Sphingobacterium multivorum[107]	Transferase	2009	3A2B
Tryptophanase	*E. coli*[108]	Lyase	2006	2C44
Ureidoglycine-gloxylate aminotransferase	*Bacillus subtillis(unpublished)*	Transferase	2010	3ISL
1-aminocyclopropane-1-carboxylate synthase	*Malus x domestica*[109]	Lyase	2000	1B8G
Fold Type II				
Tryptophan Synthase	*Salmonella typhimurium*[110]	Lyase	1996	1TTP
Threonine Deaminase	*E. coli*[111]	Lyase	1998	1TDJ
	Thermus thermophilus hb8(unpublished)	Lyase	2005	1VE5
	Salmonella typhimurium[112]	Lyase	2006	2GN0
5,6-aminomutase	*Clostridium sticklandii*[113]	Isomerase	2004	1XRS
1-aminocyclopropane-1-carboxylate deaminase	*Psuedomonas sp. Acp*[114]	Hydrolase	2004	1TYZ
Serine Dehydratase	*Human*[114]	Lyase	2004	1P5J
Serine Racemase	*S. pombe (unpublished)*	Isomerase	2005	1V71
	Rattus norvegicus[115]	Isomerase	2010	3HMK
Threonine Synthase	*Thermus thermophilus*[116]	Lyase	2003	1UIM
	E. coli (unpublished)	Lyase	2005	1VB3
	arabidopsis thaliana[117]	Synthase	2005	2C2B
	Mycobacterium tuberculosis[118]	Lyase	2006	2D1F
Fold Type III				
Alanine Racemase	*Geobacillus stearothermophilus*[119]	Isomerase	1997	1SFT
	Enterococcus faecalis[120]	Isomerase	2009	3E5P
	Mycobacterium tuberculosis[121]	Isomerase	2005	1XFC
	Corynebacterium glutamicum(unpublished)	Isomerase	2007	2DY3
	Pseudomonas Fluorescens(unpublished)	Isomerase	2008	2ODO

		Oenococcus oeni (unpublished)	Isomerase	2008	3CO8
		Bacillus Anthracis [122]	Isomerase	2008	2VD8
		E. coli[123]	Isomerase	2008	2RJG
	Ornithine Decarboxylase	*Mouse*[124]	Decarboxylase	1999	7ODC
		Trypanosoma Brucei[125]	Decarboxylase	1999	1QU4
		Human[126]	Decarboxylase	2007	2OO0
	O-Acetylserine Sulfhydrylase	*Salmonella typhimurium*[127]	Lyase	2000	1OAS
		Thermus thermophilus hb8(unpublished)	Lyase	2005	1VE1
		arabidopsis thaliana[128]	Transferase	2005	1Z7W
		Entamoeba histolytica[129]	Lyase	2008	3BM5
	Arginine Decarboxylase	*Paramecium bursaria Chlorella virus*[130]	Decarboxylase	2007	2NV9
		Campylobacter jejuni[131]	Decarboxylase	2010	3NZP
	Diaminopimelate Decarboxylase	*Helicobacter pylori(unpublished)*	Decarboxylase	2008	2QGH
		Mycobacterium tuberculosis[132]	Lyase	2003	1HKV
	Cystathionine Beta-Synthase	*Human*[133]	Lyase	2001	1JBQ
		Drosophila[134]	Lyase	2010	3PC2
	Cysteine Synthase	*E. coli*[135]	Transferase	2005	2BHS
		Mycobacterium tuberculosis[136]	Transferase	2008	3DWI
	O-phosphoserine sulfhydrylase	*Aeropyrum pernix K1*[137]	Transferase	2005	1WKV
Fold Type IV					
	D-amino acid aminotransferase	*L. bacillus*[138]	Aminotransferase	1995	1DAA
	Branched-chain Amino Acid Aminotransferase	*E. coli*[139]	Aminotransferase	2001	1I1K
	4-amino-4-deoxychorismate lyase	*haemophilus somnus 129py(unpublished)*	Lyase	2008	3CEB
		Thermus thermophilus hb8[140]	Lyase	2008	2ZGI
	Aminodeoxychorismate Lyase	*E. coli*[141]	Lyase	2000	1ET0
Fold Type V					
	Glycogen Phosphorylase	*Oryctolagus cuniculus*[142]	Phosphorylase	1991	1A8I
	Maltodextrin Phosphorylase	*E. coli*[143]	Phosphorylase	1997	1AHP

One of the most interesting data points in the table is that ornithine decarboxylase is found in both Fold Type I and Fold Type III, depending on whether the prokaryotic

form (Fold Type I) or the eukaryotic form (Fold Type III) is under review. Another interesting fact is that certain traits are conserved across different fold types. As expected, all of the enzymes have the cofactor bound by an internal lysine. Also, the internal lysine is always found in an alpha-helix. This may help explain the observation of relatively similar chemistry for a great difference in fold types. By utilizing these similarities and trends seen in fold types, we could predict the fold type of LDC. Namely, we would predict Fold Type I for this enzyme, based mostly on the sequence similarity to bacterial ornithine decarboxylase.

Table 1.4 Results of Dali lite alignments of tyrosine aminotransferase

Enzyme Aligned	PDB ID	Z	rmsd	%id
Aspartate Aminotransferase	1AJR	53.1	1.4	39
Threonine Deaminase	1TDJ	1.1	3.1	17
O-Acetylserine Sulfhydrylase	1OAS	0.3	4.6	5
D-Amino Acid Transferase	1DAA	0.1	8.4	9
Glycogen Phosphorylase	1A8I	1.9	3.5	7

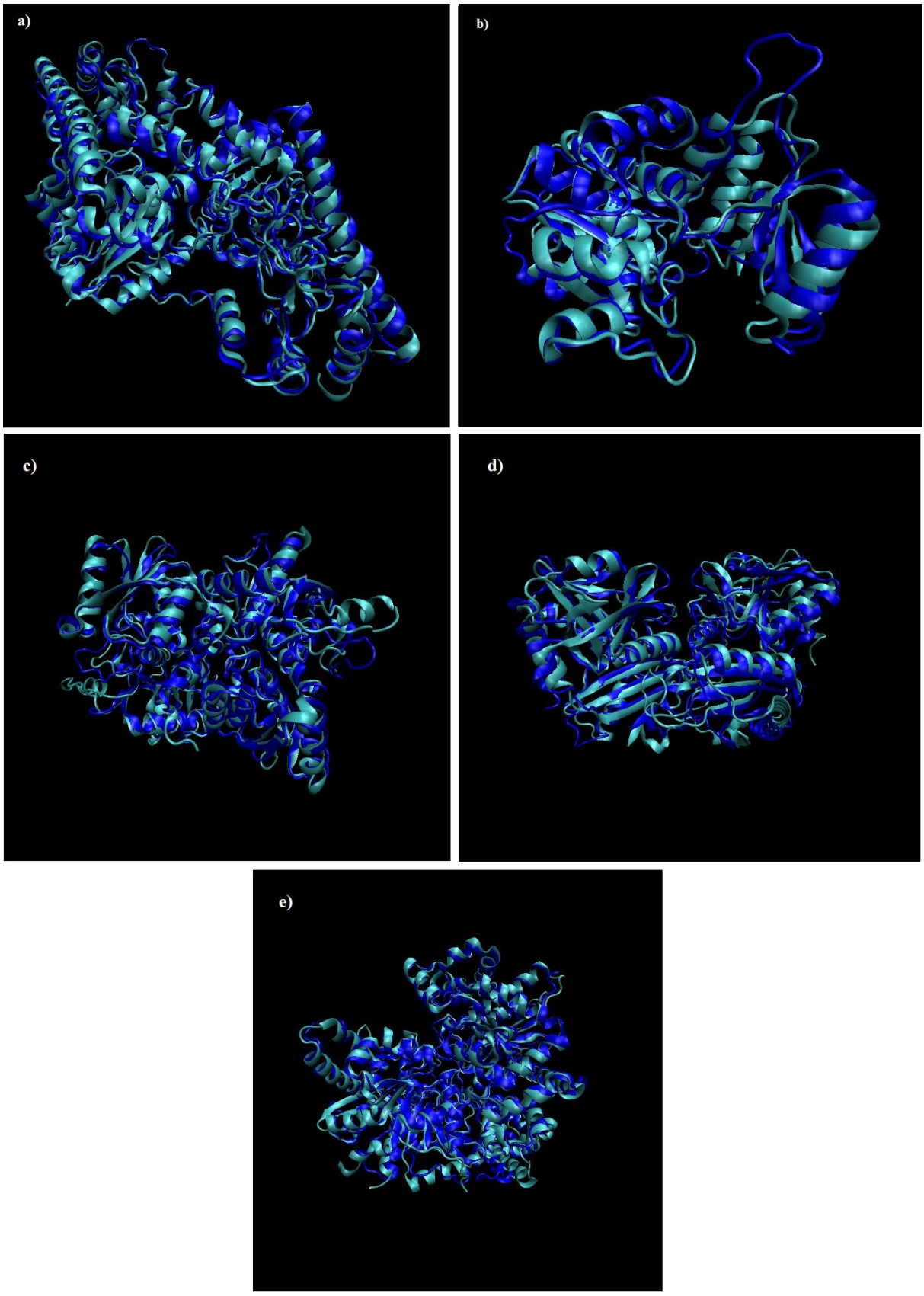


Figure 1.7 DALI overlay of a) Aspartate Aminotransferase (1AJR), cyan, with Tyrosine Aminotransferase (3TAT), blue (Fold Type I), b) Threonine Deaminase (1TDJ), cyan with Serine Racemase (3HMK), blue (Fold Type II), c) O-Acetylserine sulfhydrylase (1OAS), cyan, with Cysteine Synthase (2BHS), blue, d) D-amino acid transferase (1DAA), cyan, with 4-amino-4-deoxychorismate lyase (2ZGI), blue, and e) Glycogen phosphorylase (1A8I), cyan, with Maltodextrin phosphorylase (1AHP), blue.

done comparing the pdb entry for the enzyme in question to those for established members of each fold type: Fold Type I (1AAT – aspartate aminotransferase), Fold Type II (1TTP-tryptophan synthase), Fold Type III (1SFT-alanine racemase), Fold Type IV enzyme (1DAA-D amino acid transaminase), and Fold Type V enzyme (1A8I –glycogen phosphorylase). Enzymes were characterized by fold type by choosing the pairing that gave the highest E value (> 15) and low rmsd score (< 2). An example of a typical case is provided in Table 1.4 for the classification of tyrosine aminotransferase. Figure 1.7 shows examples of these DALI overlays. This general method allows for characterization of fold type, and thus will likely help provide a menu of appropriate reference enzymes to investigators for the construction of homology models.

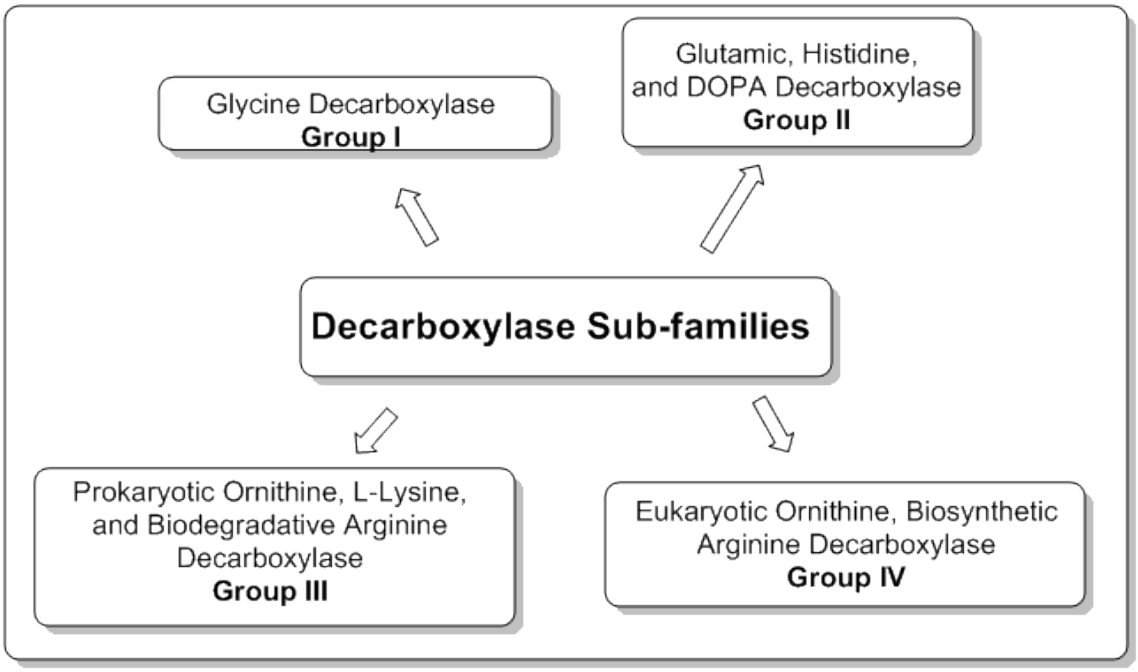


Figure 1.8 Decarboxylase Subfamilies

Sandmeier and coworkers separately developed a bioinformatic classification of amino acid decarboxylases (AADC's).[145] They took sequences of 54 amino acid decarboxylases and performed a sequence alignment. The sequence alignment found that there were four decarboxylase families. Essentially, each group is separated by sequence alignments. Group I contains a single enzyme, glycine decarboxylase. Group II contains the aromatic AADC's such as tryptophan DC and tyrosine DC. Group III contains the prokaryotic forms of ornithine, lysine, and arginine DC, which is interesting, because the

Figure 1.9 Sequence alignment of LDC (top) and bacterial ODC (bottom)

last and final group, Group IV, contains the eukaryotic versions of ornithine and arginine DC.[145]

One should take note of the correlation between fold type and decarboxylase group. Groups I, II, and III are all related to Fold Type I, while Group IV is related to the Fold Type III family.[145] As seen in the sequence alignment of LDC to bacterial ODC (Figure 1.9), the two proteins are highly homologous, with 34% sequence identity and 51% sequence homology from a sequence alignment across approximately 731residues, using Clustal W, on the two proteins.[146] Based on the homology to bacterial ODC, LDC

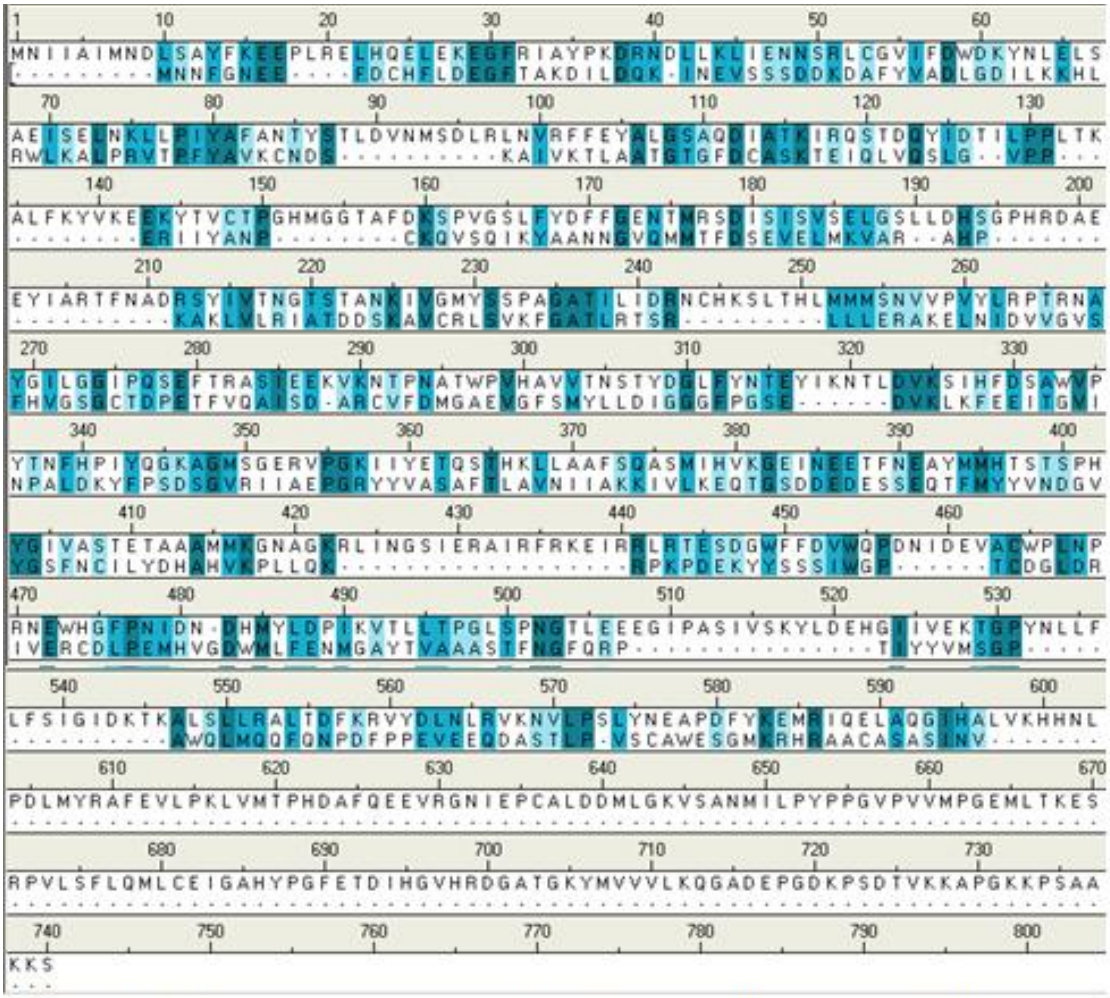


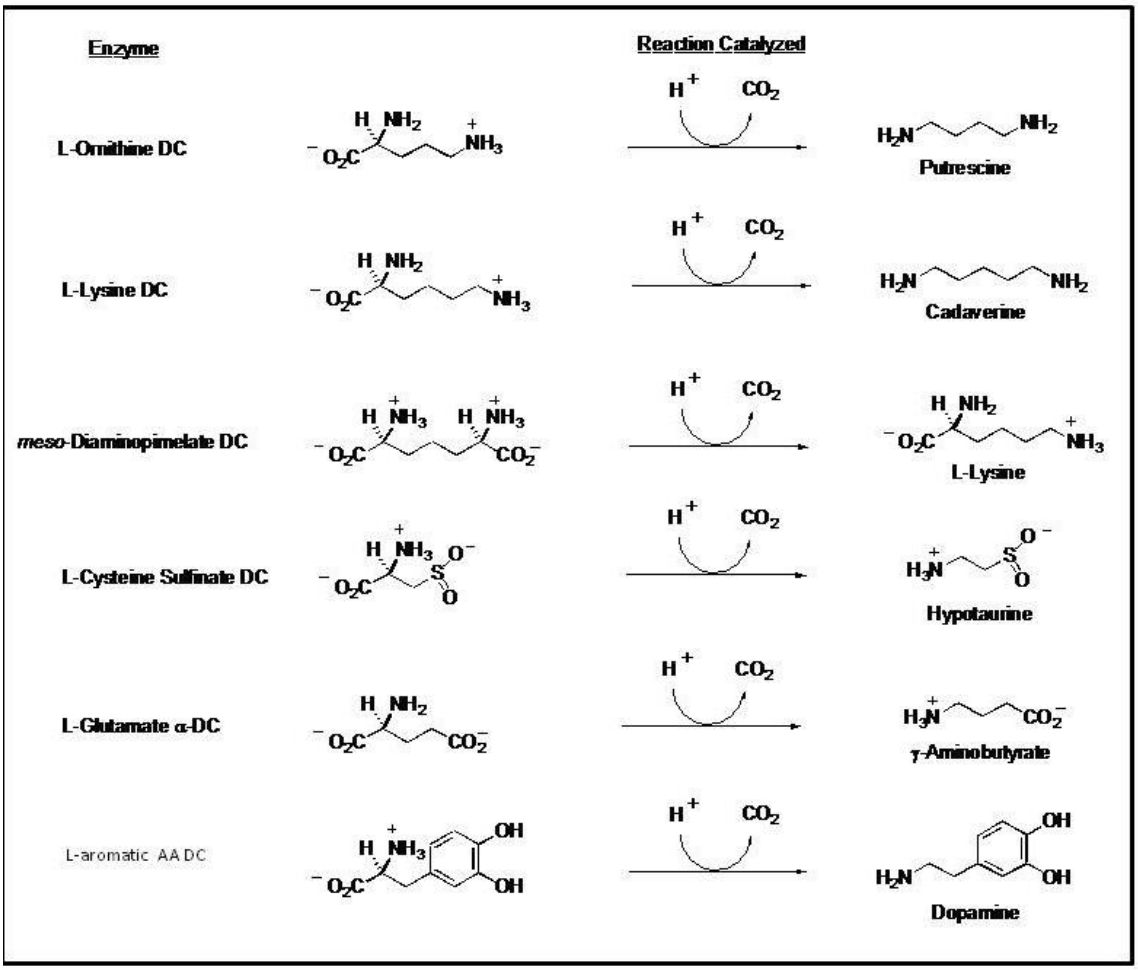
Figure 1.10 Sequence alignment with LDC (top) with human ODC (bottom)

from *Hafnia alvei* can be placed in Group III and, as mentioned, most likely will have a Fold Type I structure. The work described herein builds toward solving that structure.

Even more importantly, Figure 1.6 shows the importance on the strain of ODC used. A second sequence alignment, by comparing LDC (top) with human ODC (bottom), shows the distinct differences in homology of LDC with bacterial ODC and human ODC. Therefore, based on the high similarities with bacterial ODC, we can continue to make the prediction that LDC will display a Type I fold.

c) Amino Acid Decarboxylases

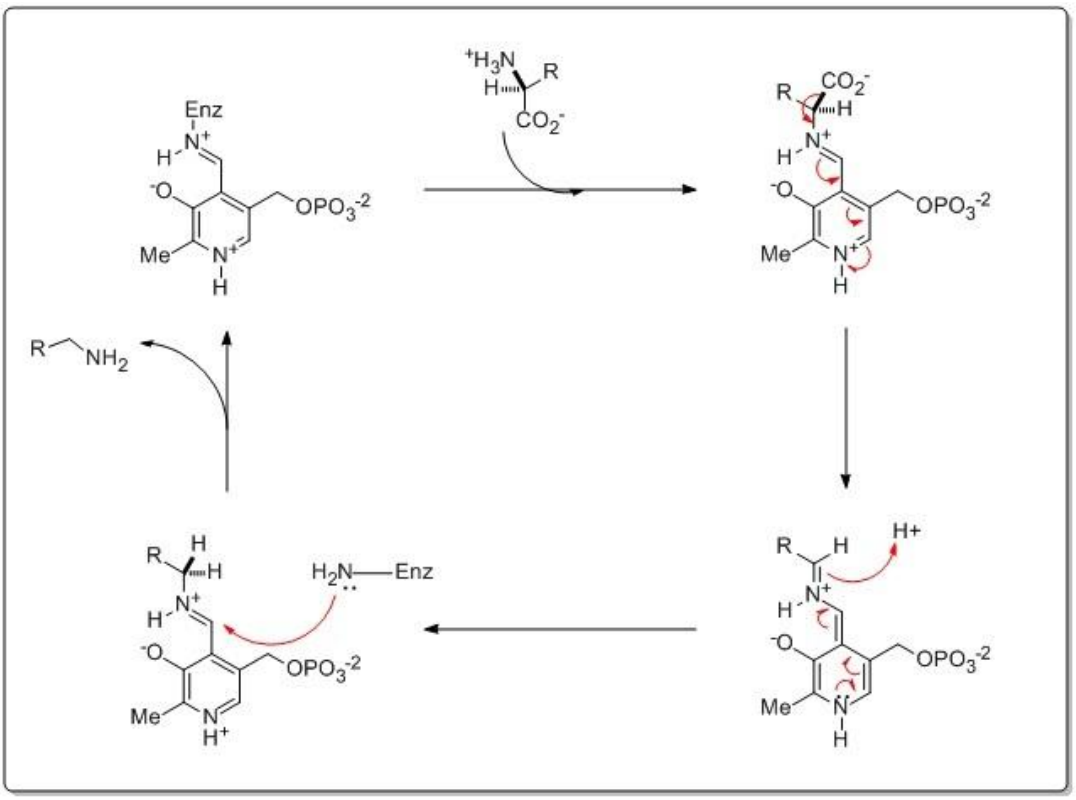
An AADC (Amino Acid Decarboxylase) performs the decarboxylation of an



Scheme 1.1 Representative examples of some PLP-Dependent Amino Acid Decarboxylases

amino acid to form the respective amine. These enzymes have been found to employ either PLP or pyruvamide as a cofactor.[1,2,147] For some AA substrates, both types of AADC's are known. Scheme 1.1, which shows the large spectrum of amino acid targets that can be decarboxylated, does not even cover the full substrate range that AADC's exhibit. For example, 5-hydroxytryptophan DC is also decarboxylated by Aromatic Amino Acid DC and specific PLP-dependent AADC's are known for arginine, glycine, and histidine. Interestingly, both LDC and Diaminopimelate Decarboxylase (DAP DC) have thus far not been found in mammalian sources.[2,132] The latter has obvious implications from the point of view of antibiotic development.

PLP-dependent decarboxylases are believed to proceed through the same basic general mechanism: 1) transimination, 2) decarboxylation, 3) α-protonation, and 4) a second transimination.[2] The first transimination step proceeds by the attack of the amino

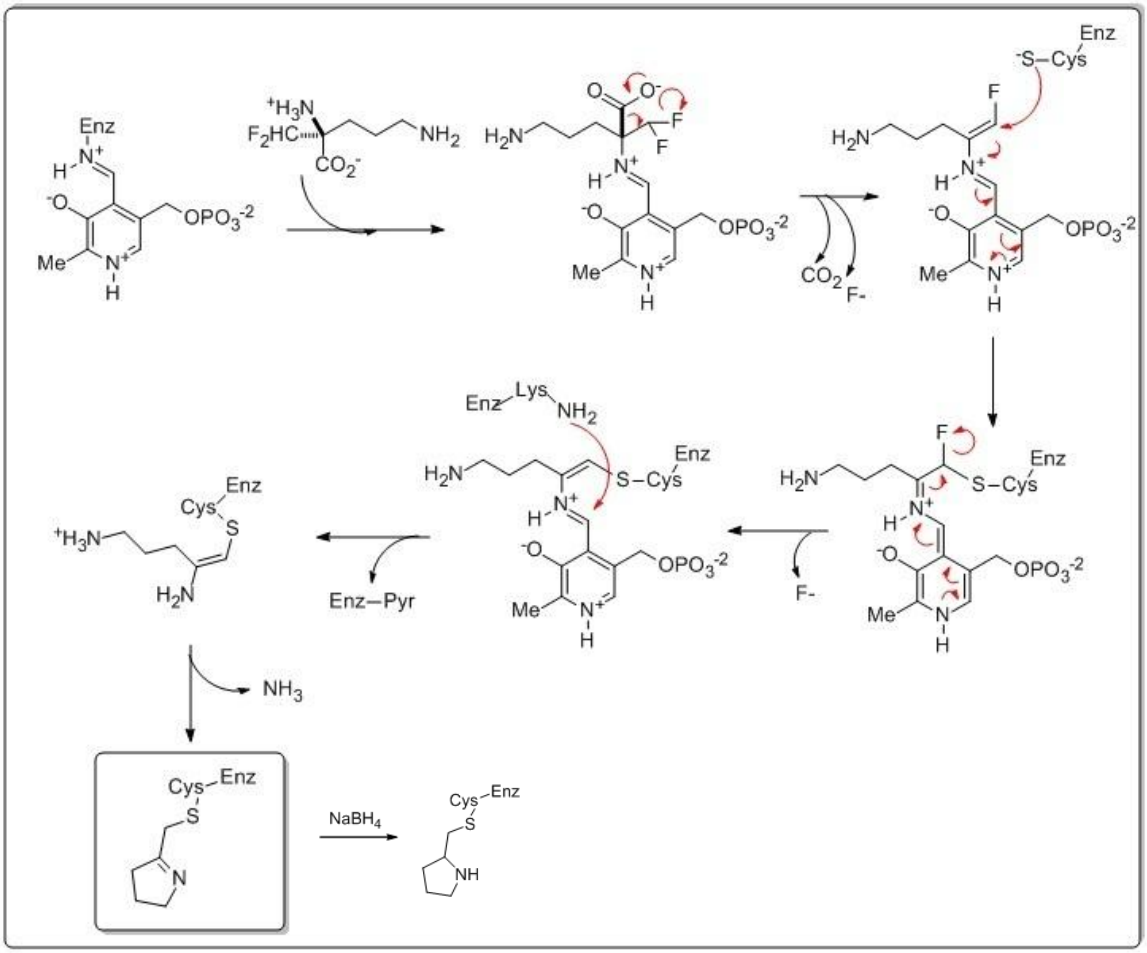


Scheme 1.2 Mechanism of Decarboxylation

acid substrate on the internal aldimine (lysine bound) to form the substrate bound external aldimine and release the enzymatic lysine. Next, with the carboxyl group aligned with the conjugated pi system, decarboxylation is favored and carbon dioxide is released, resulting in formation of a quinonoid intermediate. This highly conjugated intermediate is then protonated at the C_α position. The final step occurs when the active site lysine attacks the external product aldimine, thereby releasing the final product and regenerating the internal aldimine.

d) Inhibition of PLP-dependent decarboxylases

There are many useful applications to inhibiting some PLP-dependent AADC's. Two enzymes in particular, *Trypanosomal brucei* ODC[148,149] and (*L*)-DOPA DC,[150] are the target of inhibitors that serve as useful clinical therapeutics. *(DL)*-α-



Scheme 1.3 Proposed inactivation mechansim of DFMO in ODC

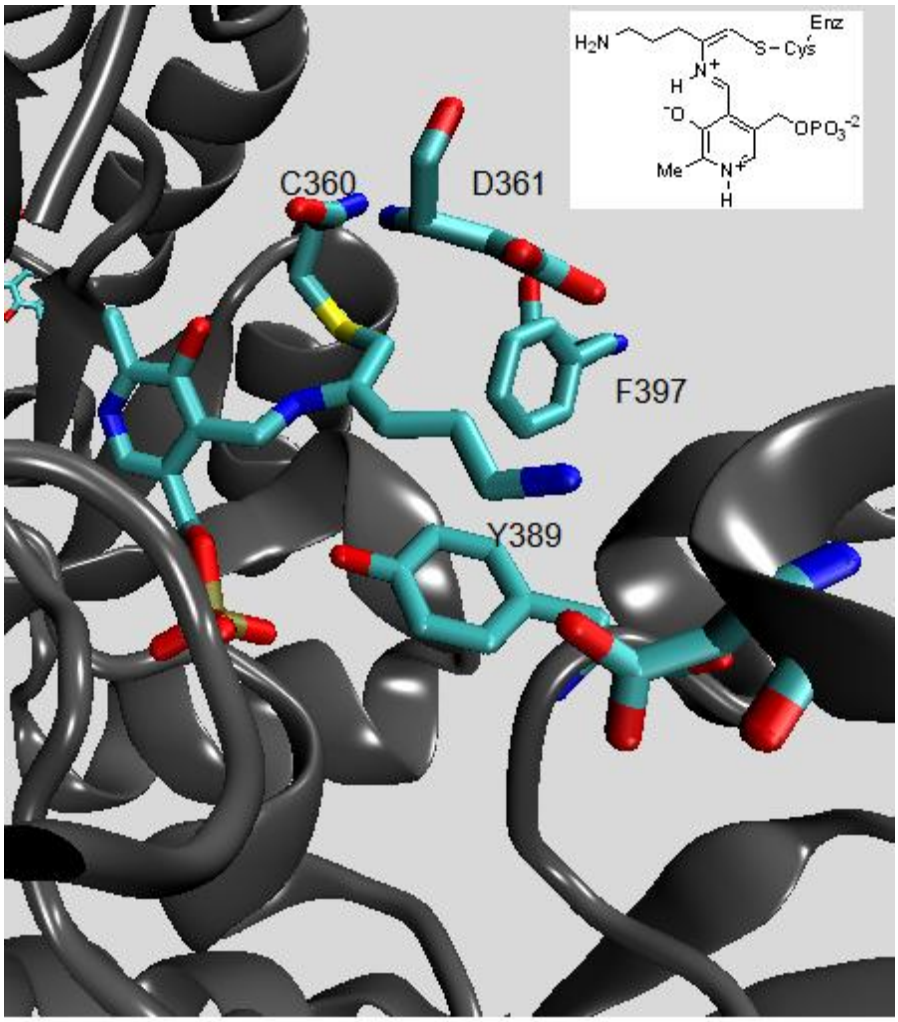


Figure 1.11 Crystal structure of inhibited ornithine decarboxylase

difluoromethylornithine (DFMO) is an irreversible, mechanism-based inhibitor of ODC from *Trypanasoma brucei*, the parasite responsible for African sleeping sickness in humans.[151] African sleeping sickness is caused when a human is bitten by an infected fly. Symptoms begin with a painful swollen lesion, and can worsen with fever, swelling, anemia, and discomfort. If untreated, the trypanosomal infection can spread to other organs, spinal cord, and the central nervous system.[152] DFMO has been developed as an intravenously delivered drug. The originally proposed mechanism (Scheme 1.3) is based on the interactions of the inhibitor with mouse ODC.[149] Following decarboxylation, DFMO undergoes fluoride expulsion as opposed to α-

protonation. Next, an internal cysteine is believed to attack the β-position, via an extended conjugation addition mechanism, resulting in the release of fluoride.[149] Reformation of the internal aldimine leads to a covalent modification of cysteine, resulting in inactivation. In 1999, this inactivation mechanism was further supported by a crystal structure of the inhibitor bound to the enzyme.[148] The crystal structure depicted in Figure 1.8 shows the covalently bound adduct.

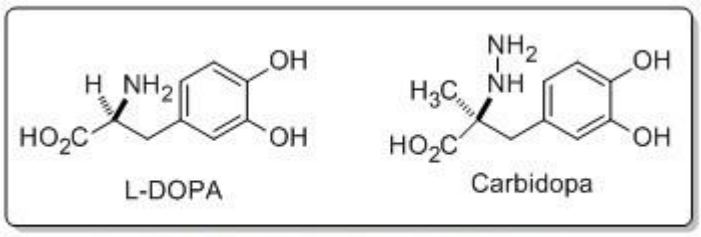


Figure 1.12 L-DOPA and Carbidopa

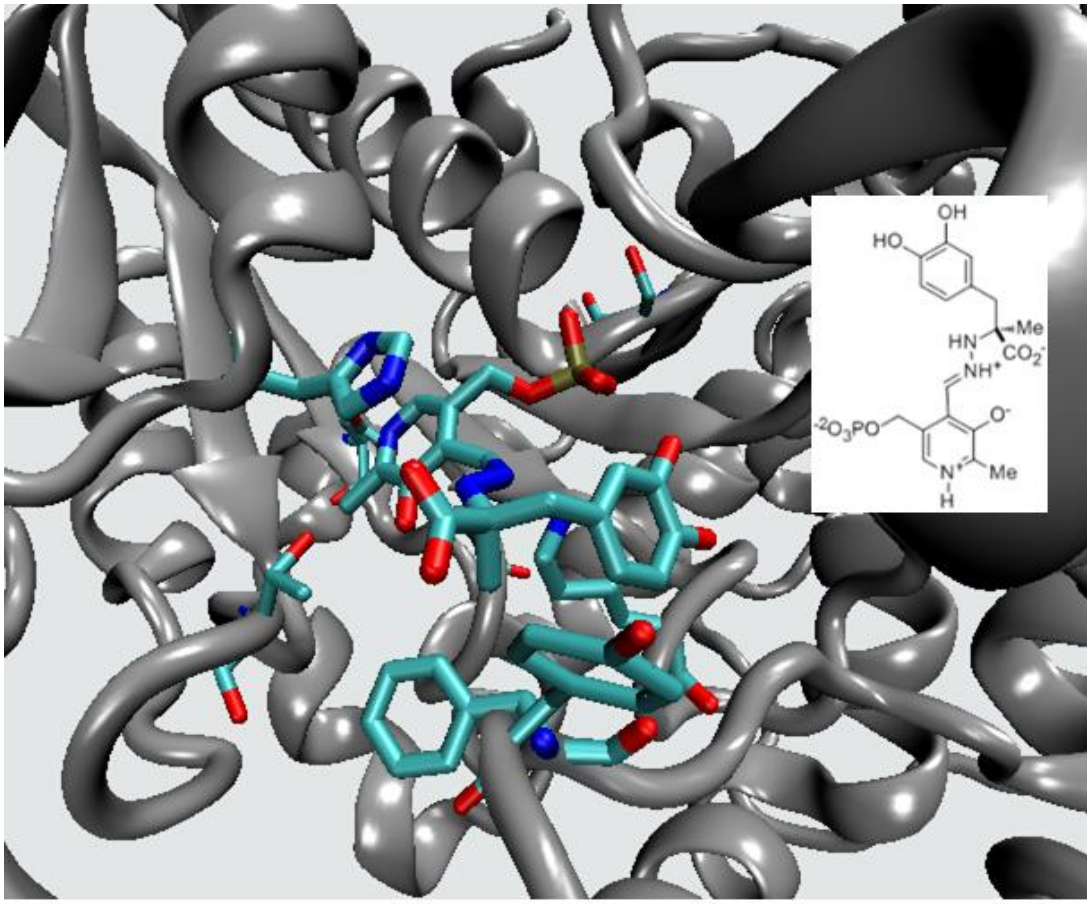
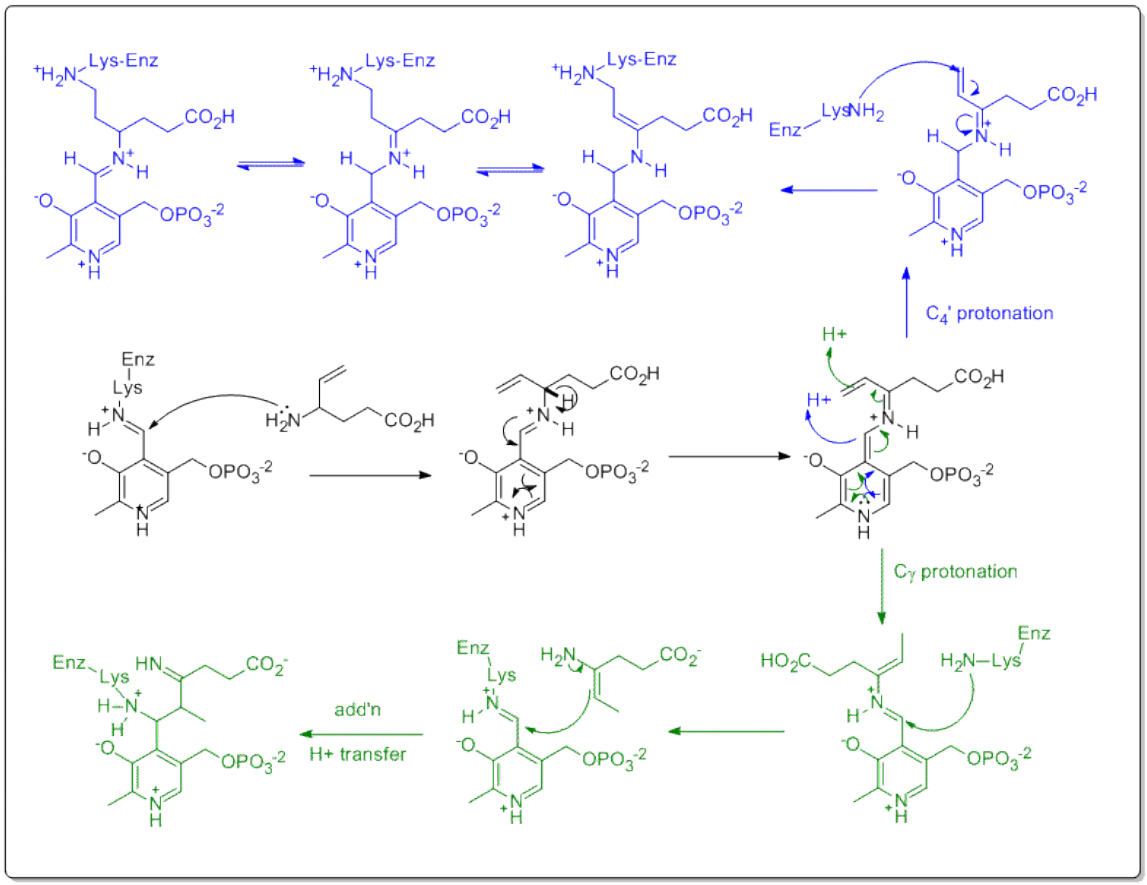


Figure 1.13 Crystal structure of Carbidopa in the active site of DOPA decarboxylase

The second drug discussed is Carbidopa, a drug used in combination therapy for the treatment of Parkinson's disease.[150,153] Carbidopa is used in combination with L-DOPA to cause a dramatic increase of dopamine levels as needed in the substantia nigra of the brain for those afflicted with this syndrome. Dopamine, an active therapeutic for Parkinson's, cannot cross the blood-brain barrier. Carbidopa limits the decarboxylation of L-DOPA and allowing it to then cross the blood-brain barrier, where it undergoes decarboxylation to dopamine in the brain. Unlike DFMO, Carbidopa does not become covalently attached to the enzyme, but rather forms a tightly bound cofactor hydrazone, as seen in the crystal structure.[154] As such, Carbidopa might be regarded as an affinity reagent, rather than as a mechanism-based inactivator. These two examples show the effectiveness of both affinity reagents and irreversible, mechanism-based inhibitors on PLP-dependent enzymes and, also their potential as valuable clinical therapeutic agents. These examples have inspired our lab to develop new motifs for the mechanism-based inactivation of AADC's.

e) Inactivation using vinyl amino acids

Though well established in Europe,[155] Vigabatrin is a relative newcomer to the United States. Vigabatrin, the γ-vinylated analogue of γ-amino butyric acid (GABA), reduces GABA transaminase activity. By increasing basal levels of the inhibitory neurotransmitter, GABA, Vigabatrin mediates against epileptic seizures.[156] Because of the added conjugation from the vinyl substituent, the usual quinonoid intermediate can undergo protonation at either C_γ- or the C_4'- carbon. Whereas the former pathway can lead to inactivation via Michael addition, the latter might lead to adduction via a Mannich condensation ("Meztler enamine mechanism").[157] Scheme 1.4 illustrates both possible



Scheme 1.4 Possible inactivation routes of Vigabatrin

mechanisms. The structure of an inhibited enzyme suggested that inactivation proceeds via conjugate addition of the active site lysine, following azallylic isomerization, as seen in molecular detail in the E-I structure. This study gives us insight to the potential mechanism by which an α-vinyl amino acid might inactivate a decarboxylase enzyme.

Investigations by Silverman and coworkers suggest that fluorinating the inhibitor slightly modifies the mechanism of inhibition.[158] While the same basic routes involving C_4' or C_γ inhibition are still available, the mechanistic possibilities are altered. Instead of stopping immediately after nucleophilic addition of the active site lysine into the vinyl group during the C_4' protonation step, (2'-fluoro)vinyl-GABA is thought to go one step further than the non-fluorinated inhibitor by releasing fluoride after the conjugate

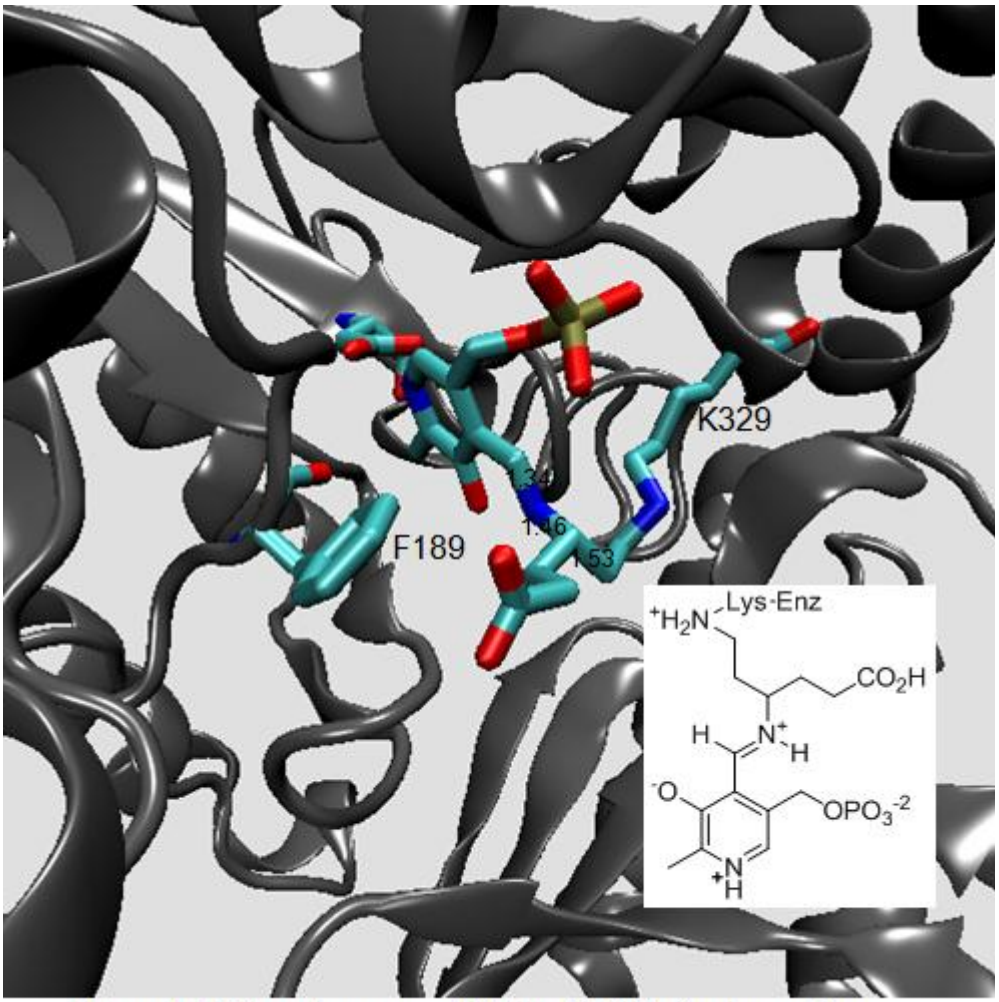
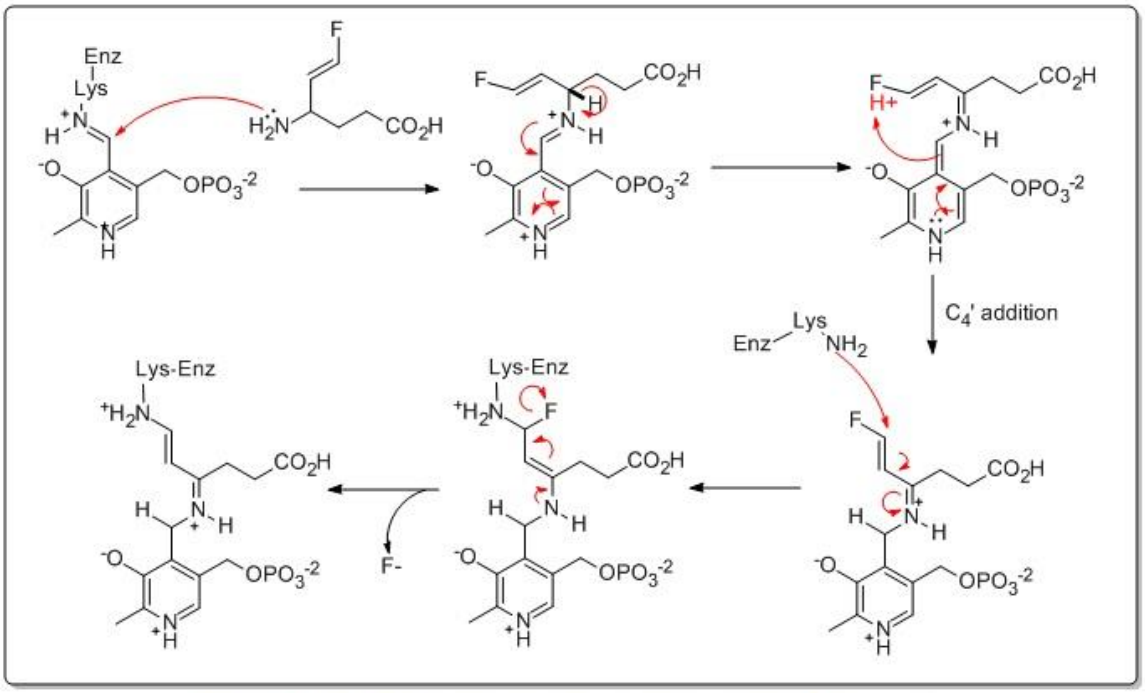


Figure 1.14 Crystal sturcture of inhibited GABA-Aminotransferase

addition. Interestingly, no significant difference was observed in effectiveness of inhibition between the *E* or *Z* fluoro alkene used, although the mechanism of inactivation was altered, due to the ability for fluoride to be utilized as a leaving group.[159] The Silverman studies on florovinyl inhibitors served as some of the key motivations behind the design of "quaternary", α-(2'-fluoro)vinyl AA's in the Berkowitz lab.[160] The quaternary amino acids are expected to be inert to PLP-enzymes that require α-deprotonation, such as GABA transaminase, but may act as covalent inactivators of



Scheme 1.5 Mechanisum of inactivation using fluorinated vinyl glutamtate

AADC enzymes, if decarboxylated in such active sites.

f) Conclusions

Understanding the mechanism of action of an enzyme permits the design, synthesis, and evaluation of mechanism-based inhibitors. Based on the generalities of PLP-dependent enzymes[155], a viable new "trigger" for the inhibition of LDC was developed. This approach was inspired by examples such as DFMO and γ-(2'-fluoro)vinyl-GABA, for both of which latent electrophiles are unveiled in the target PLP-enzyme active sites, ODC and GABA transaminase, respectively, resulting in inactivation of these target enzymes.[161]

After successful synthesis of the L-α-(2'Z-fluoro)vinyllysine (FVL) by Karukurichi and de la Salud Bea,[23,162] inhibition of *H. alvei* LDC was found to be irreversible. On the other hand, the D-antipode functioned as a slow substrate. These

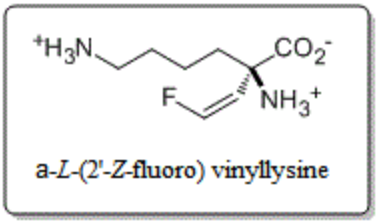


Figure 1.15 Inhibitor of lysine decarboxylase

provocative results within a new AADC inactivation motif motivated the research described in this M.S. thesis. It became a central goal to obtain high titres (i.e. multi milligram) of homogeneous *H. alvei* LDC so that this model AADC enzyme might be crystallized and its structure determined for the first time. In principle, this could then lead to the interrogation of covalent LDC inactivation by X-ray crystallography, as well.

II. Purification of LDC

a) Previous Purification method

In order to attempt enzyme crystallization, LDC must first be purified. Since previous attempts to clone LDC from *Hafnia Alvei (H. Alvei)* have proven ineffective[163], isolation of the native protein was pursued. Attempts at purification of LDC have been seen[164,165], so the process of purification has been built upon a foundation of previous purification reports.

Bacterial LDC can be found in a variety of strains including: *H. alvei*, *E. coli*, and *Vibrio parahaemolyticus*. Out of all three bacteria, *H. alvei* produces the greatest amount of LDC, compromising 4% of the total soluble protein.[164] For this reason, our lab, as well as others has chosen to isolate LDC from *H. alvei*. The first published protein purification of LDC, from Soda and coworkers,[164] employed *Bacterium cadaveris (*also known as *H. alvei* - strain IFO 3731), grown up in a controlled medium (L-lysine, glycerol, peptone, ammonium sulfate, potassium phosphate, magnesium sulfate, and yeast extract). Cells were broken open by sonication in the presence of protease inhibitors and PLP. Following a heat treatment step (50 °C), ammonium sulfate cuts were made, with the first cut having 30% ammonium sulfate saturation and the second cut with 55% saturation. Once the pellet was re-suspended in buffer, it was loaded on an ion exchange DEAE-Sephadex column. The fifth step was a phenyl sepharose column, followed by a second DEAE-Sephadex column. The final step to isolate the protein was said to be performed by "crystallization," obtaining small yellow rod-like crystals that were not tested with an x-ray, with a final purification factor of 61 and a yield of 5.9%,

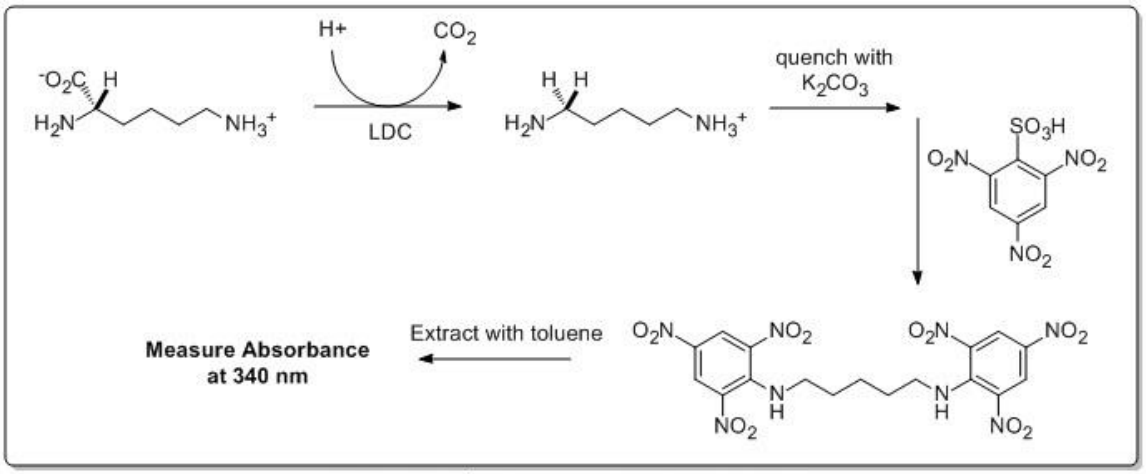
with a final specific activity of 86 U/mg. This laborious procedure and modest yield motivated our lab to seek a streamlined purification procedure.

Berlin and coworkers, the first group to publish a sequence for LDC from *H. alvei*, employed a different purification, which also involved size exclusion and an ion exchange chromatography, and which provided LDC with a supposedly higher specific activity of 110 U/mg, but the difference may reflect experimental uncertainty or variation.[163,165] The surprising finding in Berlin's purification was that this specific activity resulted from a protein that displayed two bands (88 and 66 kDa) on an SDS-denaturing gel.

In our own lab, Karukurichi developed his own purification method for LDC, which involved sonicating crude cells, a 60% saturated ammonium sulfate precipitation, phenyl sepharose chromatography, ion exchange (DEAE Sephadex), and finally size exclusion (S-300) chromatography.[23] My research investigated a modified version of this basic procedure, as described below.

b) Activity Assay

To measure the activity of LDC, de la Salud-Bea and Karukurichi adapted the previously published Lenhoff assay.[23,162] The Lenhoff assay measures the turnover or lysine to cadavarine by spectroscopically measuring the amount of cadaverine reacting with picryl sulfonic acid (TNBS).[166] The assay works by allowing the enzyme to actively convert L-lysine to cadaverine in the presence of lysine (8 mM) for a set time. After the time has passed, enzyme activity is quenched with base and heat. The quenched solution is then heated (47 °C for 7 minutes). After cooling, TNBS is added to each solution, and



Scheme 2.1 Lenhoff Assay Basic Chemistry

the reaction is again heated (47 °C for 7 minutes). This allows for nucleophilic aromatic substitution to occur, covalently attaching cadaverine to the aromatic ring, and presumable releasing sulfite. Interestingly, lysine in solution will not react with TNBS, presumably because the amine becomes basic in cadavarine. Lastly, the derivatized cadaverine is extracted with toluene and its absorbance was measured at 340 nm. The absorbance measured is then compared to that obtained for a set of standards. For more information, please refer to the experimental section.

c) Protein determination

From the published sequence of LDC from *H. alvei*, the molecular weight of LDC appeared to be 82 kDa (or 82,000 g/mol). Utilizing the Lowry method[167], we were able to compare absorbance against a common standard, Bovine Serum Albumin (BSA), which has a monomeric molecular weight of 66 kDa. The Lowry method works in two steps: 1) Reductino of Cu^{2+} to Cu^{+1}, and 2) Reduction of the Folin reagent.[167] The first step proceeds via a Biuret reaction, where copper is complexed with with amide bonds of the protein, specifically with aromatic amino acids.[167] Potassium hydroxide, $CuSO_4$,

sodium carbonate, sodium tartrate, and sodium dodecylsulfate are added to the protein mixture, oxidizing peptide bonds to convert Cu^{2+} to Cu^{+}.[167] The second step involves the Folin-Ciocalteu reagent being reduced by the copper complex, and the mixtures changes from a light purple color to a deep blue color, depending on the concentration of protein in solution, which is measured at 650 nm. A stronger absorbance is related to higher protein concentration. After setting a calibration curve with BSA weight standards, LDC protein concentration could easily be estimated.

d) Protein purification

With assays for activity and protein concentration in hand, we next set out to optimize LDC purification from the native source. Cells were grown in a lysine-supplemented medium (5 g bactopeptone, 5 g yeast extract, 1 g $MgSO_4 \bullet 7H_2O$, 3 g of L-lysine monohydrochloride per 1 L of solution), and disrupted via sonication in the presence of protease inhibitors. The crude extract was then centrifuged and to the supernatant was added a 60% ammonium sulfate saturation. Although ammonium sulfate is generally used in two separate cuts for an increase in purification, preliminary attempts to improve specific activity in this manner showed little benefit. Instead, the ammonium sulfate precipitation served as an excellent means for storage, with no activity loss detected for up to a year or more of storage. This method is a simpler means of storage as compared to glycerol stocks, because instead of having to use dialysis to remove glycerol, the resuspended pellet can be immediately loaded onto the next column. The high ammonium sulfate concentration precipitate insures retention of nearly all LDC activity in the stored precipitate.

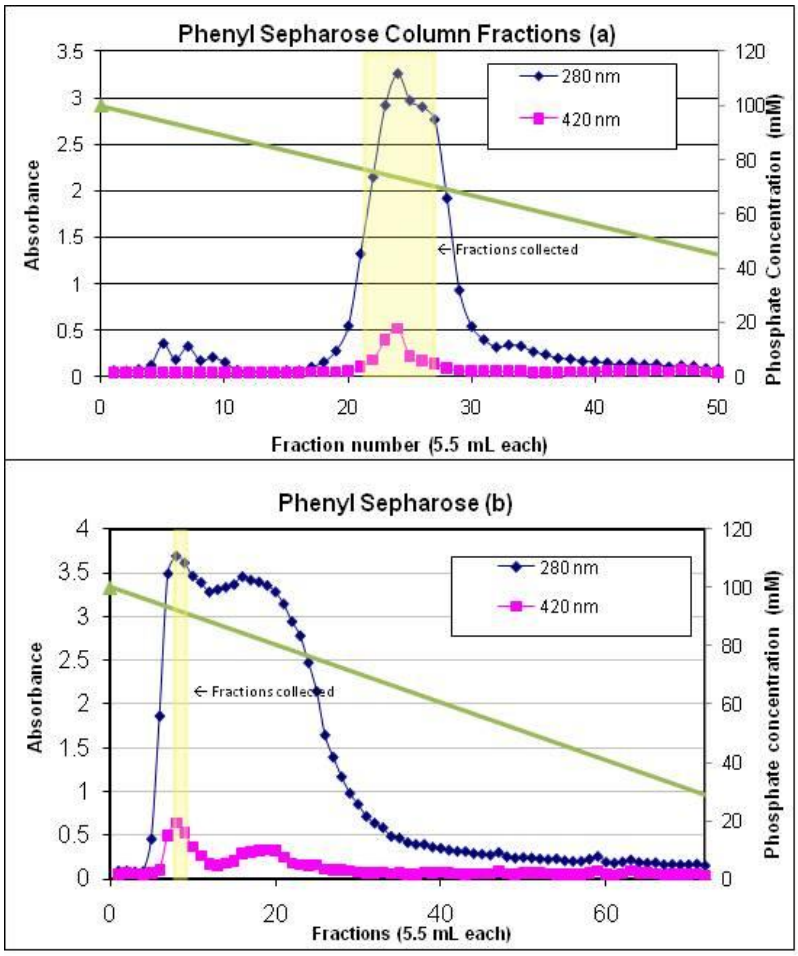


Figure 2.1 Two column fractions from the phenyl sepharose columns (a and b correlate to the purification table in Table 2.1)

The first column used was a phenyl sepharose column. Phenyl sepharose is a hydrophobic stationary phase that separates based on hydrophobicity. Elution was performed by using a linear gradient from high (100 mM) to low (10mM) concentration of phosphate buffer (with 15 μM PLP at pH 7.0). By decreasing phosphate concentration, and hence decreasing ionic strength, one effects the elutions of proteins as a function of increasing hydrophobicity, with the most hydrophobic eluting last. An elution plot is shown in Figure 2.1. Fractions displaying high absorbance at 280 nm and some absorbance at 420 nm were further tested for activity using the Lenhoff Assay, as described before. The fractions collected in Figure 2.1 led to an purification factor of 2.5

(seen in Table 2.1a), but in pooling fewer fractions (i.e. two fractions versus the 8 seen in Figure 2.1) the purification factor was optimized to 12 (Tabel 2.1b), with a yield of 38.9%.. The fractions collected had the highest specific activity as determined by the Lenhoff and Lowry assays, as described above. Table 2.1b represents the fully optimized purification table in the purification of lysine decarboxylase.

Active fractions from the phenyl sepharose column were taken on to the next step. While Karukurichi previously used DEAE-Sephadex as an anion exchange column, this resin was found to swell dramatically, and yield poor separation.[23] Initially, a more basic quaternary anion exchange column, Q-Sepharose, was used, with very good results in separation of the protein. This resin showed less dramatic swelling and led to more

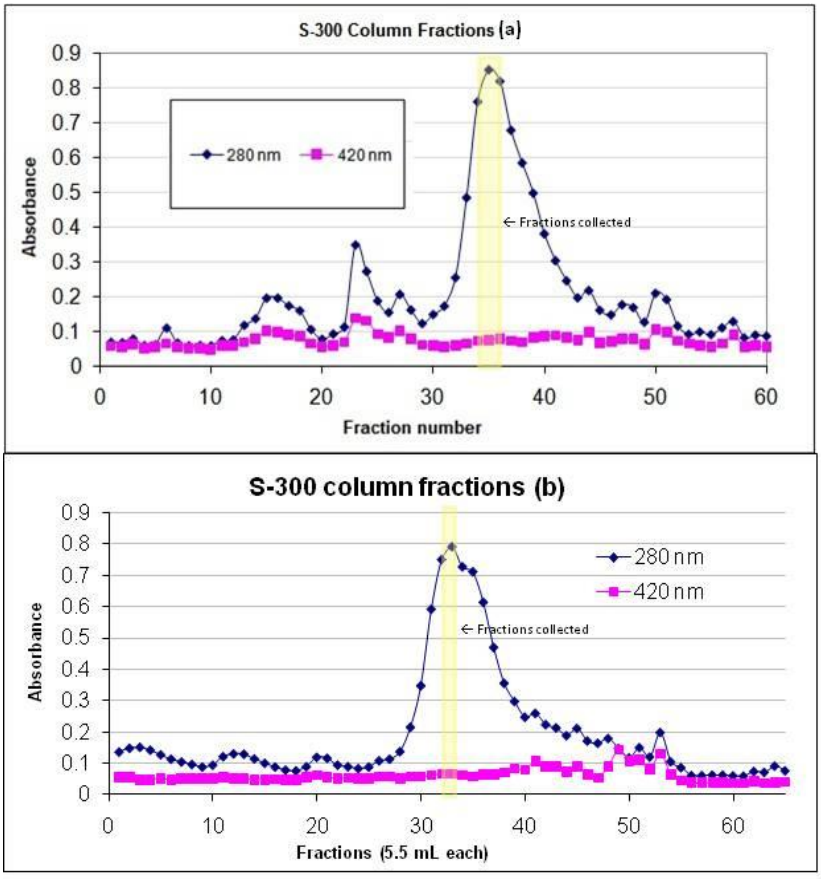


Figure 2.1 Two column fractions from the S-300 columns (a and b correlate to the purification table in Table 2.1)

effective protein fractionation. However, the purification achieved via phenyl sepharose was so significant that it seemed unnecessary to use the Q-sepharose step. Therefore, this column was eliminated, and the active fractions were loaded onto an S-300 size exclusion column. This final column simply separates based on size. Larger macromolecules rapidly traverse the column, while proteins in the size regime of the pores are partially retained, and elute more slowly. Using this streamlined protocol, homogeneous LDC was obtained in 13% overall yield with a final specific activity of 91 U/mg. These results were consistent with previously published data from Karukurichi's protein purification.[23]

Table 2.1 Purification tables of LDC when **a**) Q-sepharose column was used (based on 84 g) and **b**) without Q-sepharose column (based on 80 g). Figures 2.1 and 2.3 relate to purification table **a**

a)	Volume (mL)	Total Units (U) Lenhoff	Units/mL	Total Protein (mg) Lowry Assay	Specific Activity (U/mg)	%Yield	Purification Factor
Crude	120	3018	25	2902	1.04	100.00	1.00
Ammonium Sulfate	60	2977	50	2706	1.10	98.6	1.06
Phenyl Sepharose	9	1874	208	722	2.60	62.1	2.5
Q Sepharose	3	983	328	50	19.7	32.6	18.9
S-300	0.2	362	1810	3.8	95.3	12.0	91.6

b)	Volume (mL)	Total Units (U) Lenhoff Assay	Units/mL	Total Protein (mg) Lowry Assay	Specific Activity (U/mg)	%Yield	Purification Factor
Crude	50	2874	57	2499	1.15	100	1
Ammonium Sulfate	27	2833	104	2380	1.19	98.6	1.03
Phenyl Sepharose	4.5	1118	248	81	13.8	38.9	12
S-300	1	382	382	4.2	91	13	79

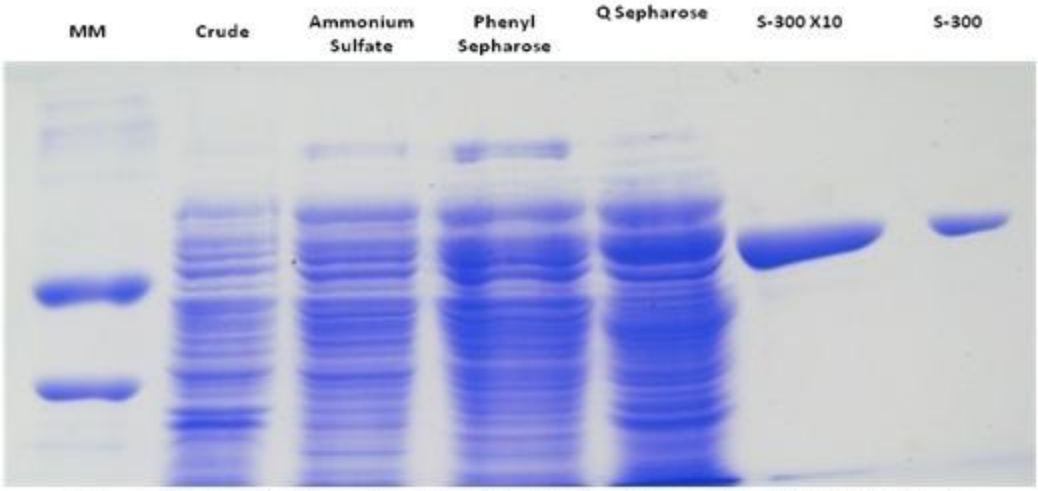


Figure 2.3 Lysine Decarboxylase Purification (Denaturing SDS-PAGE gel)

e) The Next Step: Effectiveness of Mass Spectroscopy and Crystallography

Both mass spectrometry and crystallography have been used as effective methods for the analysis of enzyme sequencing as well as understanding the mechanism of inhibition of certain enzymes.[63,168] In some instances, mass spectrometry has proved more efficient than crystallization, and vice versa. The methods may be viewed as complementary. Mass spectrometry has the advantage of allowing one to more easily examine multiple parallel covalent inactivation mechanisms, because homogeneous crystals are not needed. Moreover, differential sites of alkylation can be examined a single "bottom-up" analysis. The price one pays for this is the need to establish significant sequence coverage, which often requires the use of multiple digestion enzymes, and which becomes more difficult with larger enzymes. The "bottom-up" MS method has the disadvantage of likely missing non-covalent inactivation mechanisms (e.g. Carbidopa) or covalent inactivation mechanisms that can lead to release of inhibitor upon workup (e.g. Metzler enamine mechanism[169,170] – release of the "Schnackerz intermediate"[171,172] under basic conditions.

Crystallography, on the other hand, is able to give the complete sequence of the enzyme in its entirety, showing all contributing residues to the active site, without any digestion. Moreover, non-covalently bound inactivators (e.g. CarbiDOPA) are easily seen by this method. However, crystallizing a protein can be time consuming in the attempts to optimize conditions, as well as in learning to growing crystals of a size and quality to give an acceptable X-ray diffraction pattern. It is worthy to note that this may soon change with the advent of "free electron laser"-based crystallography. [173,174]

Ideally, our lab would try to crystallize LDC to obtain absolute certainty of the enzyme's structure, and would then use crystallography or mass spectrometry to identify the mechanism of inactivation from α-*L*-(2'-*Z*-fluoro) vinyllysine on LDC. Studies as shown by Silverman below help to see the importance of both methods in identifying mechanisms of inactivation. [168,175]

(*S*)-4-Amino-4,5-dihydro-2-thiophenecarboxylic acid is an effective inhibitor of GABA-AT.[176] Silverman was able to discover a unique means of inhibition by using electrospray mass spectrometry and tandem mass spectrometry alone to identify the probable mechanism(s). Out of three mechanisms proposed, all three created different adducts: 1) an external aldimine that results from aromatization of the product, 2) an enamine type mechanism, and 3) a Michael addition. All three create inactive forms of PLP that will slowly be removed from within the active site. If a

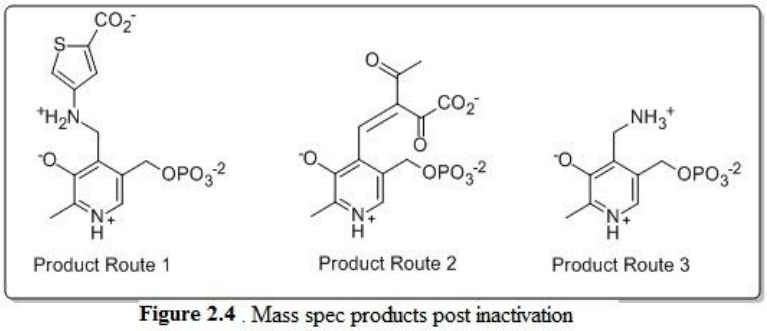


Figure 2.4 . Mass spec products post inactivation

mechanism is proposed and a molecular mass is known, mass spectrometry can help to validate these proposals. The three candidate structures for the inactive forms of PLP all vary substantially in molecular weight. Isolating the modified cofactor product and submitting it to tandem MS/MS allowed for the molecular mass of product 1 to be the primary candidate, leading their group to conclude that the mechanism of inactivation proceeds via aromatization of the adduct.

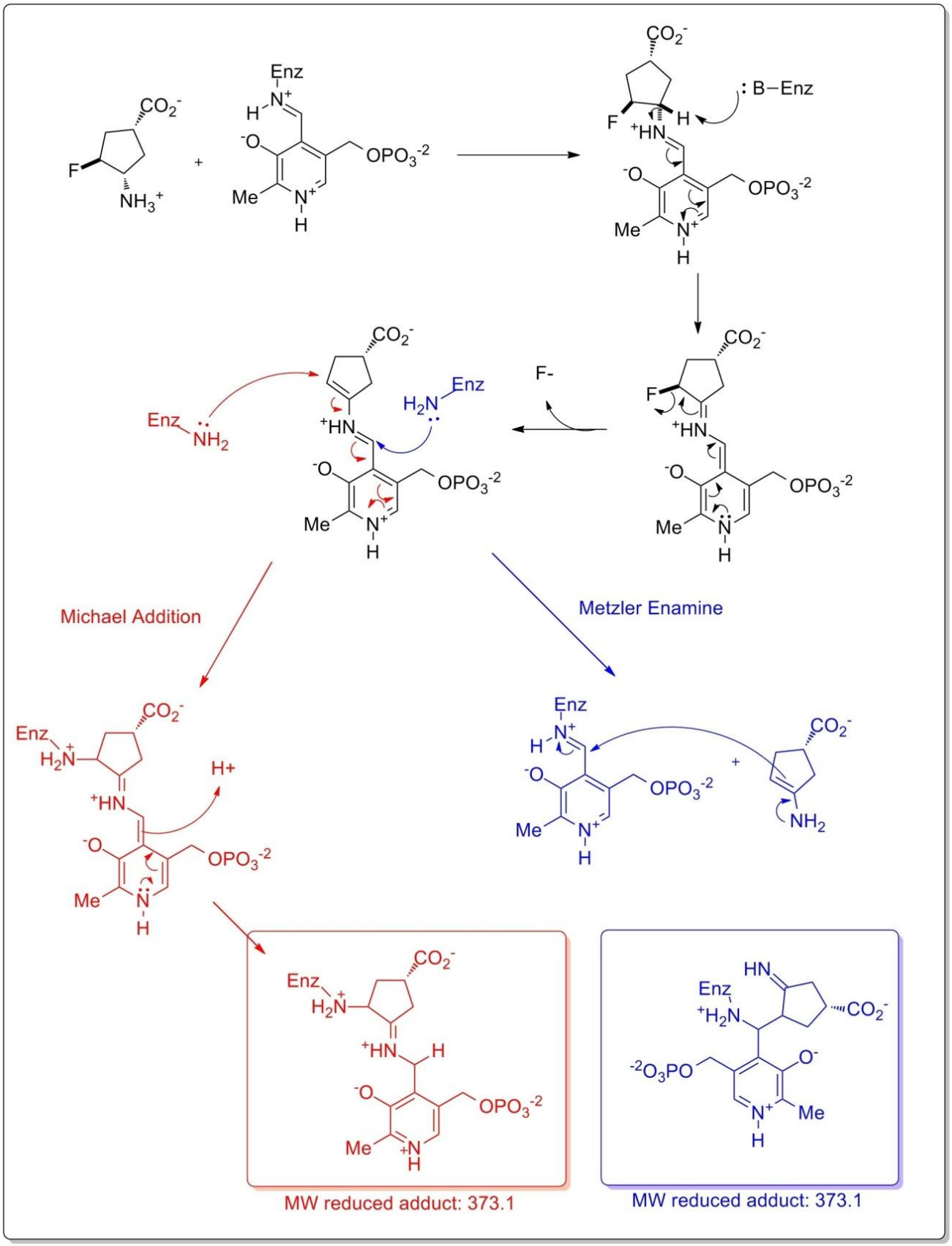
Crystallization offers another means to discovering the path of inactivation. Next to LDC, ODC from *Lactobacillus* is the most highly homologous PLP enzyme known to date. Fortunately, the crystal structure of ODC has been solved by Hackert[177]; who found that ODC existed in the crystalline form as a hexamer of dimers, or a total dodecamer. While the dimer structure seems to fit the fold type 1 of generalized PLP-dependent enzymes[29], the dodecamer is out of the ordinary. It is possible that LDC could crystallize in a similar manner to ODC based on the high homology, or could behave similarly to other fold type 1 proteins.

The beauty of crystallography it that is allows for absolute certainty in identity of each individual amino acid, as well as seeing the identity of the protein as a whole, rather than in fragments as would be seen in mass spectrometry. This would allow the entire quarternary structure of LDC to be fully exposed in its entirety. Obtaining a successful crystal structure would be beneficial in order to have full understanding of the active site, PLP's binding, as well as potential inhibitor interactions.

f) Silverman's Case of Mechanistic Enzymology by Crystallography

Dr. Richard Silverman has employed isotope labeling, mass spectrometry, and crystallography in attempts at depicting mechanistic insights to inactivation of enzymes.[175] While all three give insightful hints into the possible mechanism of inactivation, only crystallization gives scientists a finite structure. Instead of noting radioactivity in the solution or bound to the enzyme, or instead of being given a mass of a fragment, an absolute structure is able to be deciphered. In specific cases, this can finalize the inhibited product, thus giving concrete information regarding mechanism.

Silverman et. al show an extremely useful example to portray the usefulness of mechanistic crystallography. In the case of GABA-AT, the inhibitor (*1R, 3S, 4S*)-3-amnio-4-fluorocyclopentane was developed based on analogy to the structure of GABA.[168] Studies of activity proved this inhibitor to irreversibly inhibit GABA-AT. Because GABA-AT is a PLP- dependent enzyme, both Michael addition or Metzler enamine formation are viable products, as seen in Scheme 3.1. Initially, the mechanism of GABA-AT was proposed to proceed via Michael addition based on the fact that aminotransferases begin with C_4' protonation being a necessary step, as well as other inhibitors, like fluoro vinyl GABA proceed via Michael addition, but experiments for this inhibitor had not been studied. Both mechanisms start in the same fashion. Addition of the substrate generates the external aldimine, which undergoes deprotonation of the α-proton to form the quininoid intermediate, followed by fluoride expulsion. In the Michael addition, the active site Lys will attack the γ-position of the unsaturated iminium, which regenerates the quininoid intermediate and results finally in C_4' protonation. Via the Metzler enamine addition, the active site Lys generates the internal aldimine releasing the small molecule enamine. This enamine is a good nucleophile and is proposed to



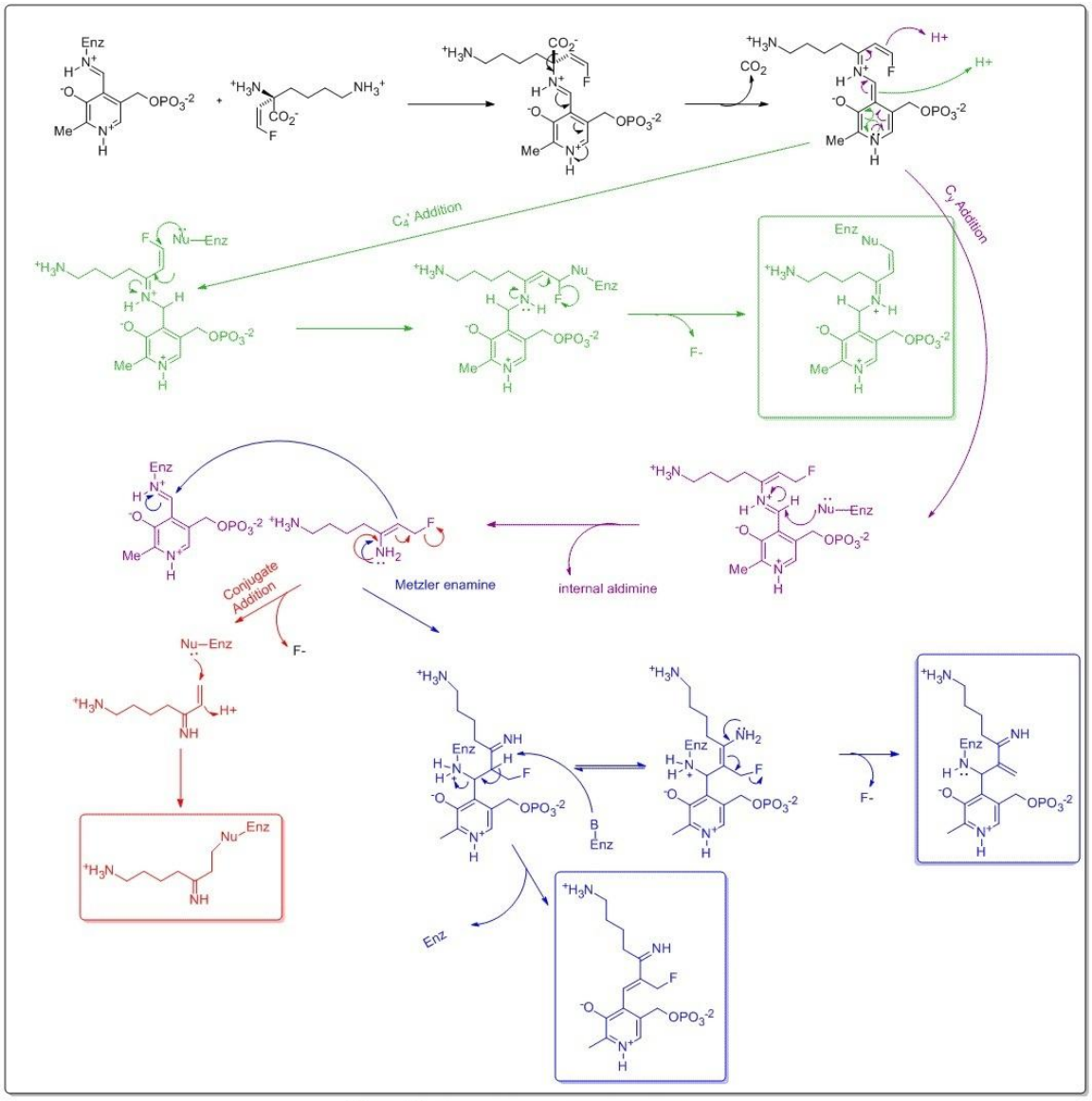
Scheme 2.2 Potential routes of inactivation of GABA-AT

attack the C_4' carbon, resulting in the final adduct. Interestingly, both mechanisms result in fluoride expulsion, thereby eliminating radioactive assays as a means of distinguishing the two. Similarly, if the Metzler enamine does indeed result of an addition of the small molecule back into PLP for formation of the final adduct, both mechanisms would give products of identical molecular weight.

Silverman was not only able to successfully identify the mechanism of inactivation of GABA-AT, but was able to use crystallography to visualize the active site and see interacting active site residues.[168] Arg 192 is able to stabilize the carboxylic acid within the inhibitor. Lys 329 serves as the active site lysine that is able to form the internal aldimine. From these results, Michael addition is conclusively eliminated from a possible mechanism of inactivation. Formation of the adduct seen gives adequate information for scientists to hypothesize that the method of inactivation of GABA-AT proceeds via the Metzler enamine pathway.

g) Possible Inactivation Pathways of LDC with the New Trigger

Based on Silverman's findings, it was essential that we analyze the potential paths of inactivation for LDC. PLP-based enzymes proceed through three major pathways: α- protonation, which results in normal turnover, and two possible pathways that could result in inactivation: C_4' protonation, or C_γ protonation. Another possible method of inactivation, though highly unlikely, is addition directly into the quninonoid intermediate. Indeed, careful consideration of the possibilities suggests that there could be more than one means of inactivation is possible based on C_4' protonation, or C_γ protonation. Because of previous findings within our own lab from Karukurichi



Scheme 2.3 Proposed routes of inactivation of LDC

and de la Salud-Bea, we know that the fluoride expulsion accompanies inactivation.[23,162] Of the three most likely inactivation mechanisms, two (nucleophilic Metzler enamine mechanism and the electrophilic conjugate addition mechanism) follow from C_γ protonation, followed by enamine formation/release.[157,178] The third possible mechanism was a conjugate addition/elimination following C_4' protonation. Scheme 2.3 depicts these three potential inactivation routes.

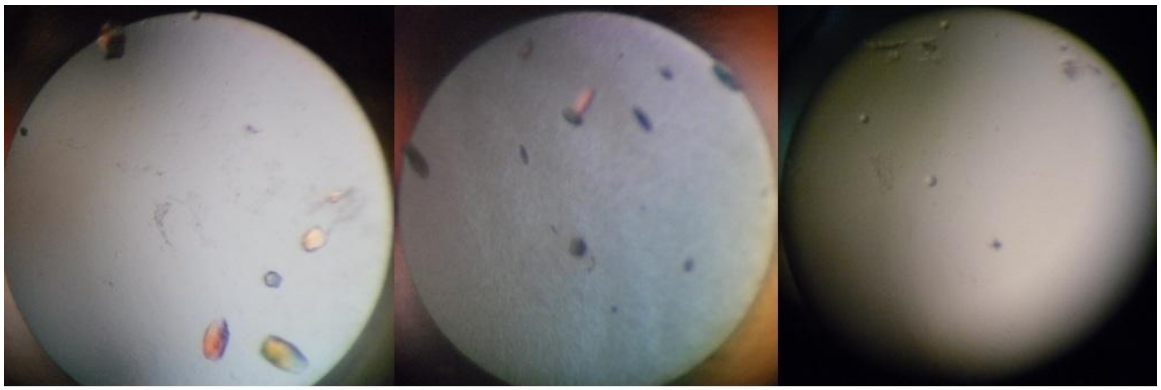


Figure 2.6 Three wells of crystallization results

h) Crystallization attempts

Based on Silverman's findings, our group seeks to obtain a crystal structure of LDC. This effort is collaborative with Professor Mark Wilson (UNL Biochemistry Department). Previous attempts by Karukurichi in our lab led to the successful crystallization of LDC, but the crystals were of insufficient size for diffraction experiments. Here, preliminary crystallization was performed using 10 mM phosphate buffer pH 7.0, 15μM PLP, and 15mg/mL LDC, with no positive results. In the second attempt, the buffer was changed to 10 mM malonate buffer pH 7.0, 15μM PLP, 25 mg/mL LDC, and received two positive hits using the Hampton Screening Kits I and II. After proper growth conditions were developed (in lab notebook), crystals were obtained, as pictured in Figure 2.6. This data suggests that crystallization of LDC is indeed possible.

i) Conclusions

As results have shown, LDC was successfully purified from the native source, in moderate to good yields, with acceptable specific activity. Modifications have been made to the original protocol to eliminate one unnecessary step in purification. From the original purification as published by Soda with seven total steps, the final purification has

been reduced to only four steps, with an increased purification factor, initially at 61 and improved to 79. The more streamlined protocol also provides for an improved yield (13%) over Soda's method (5.9%)[164]

We have a developed a protocol efficient for production of LDC from *H. Alvei*. LDC isolation is now streamlined, and future efforts in the group will be directed toward finding suitable conditions for its effective crystallization. There are numerous benefits to obtaining a crystal structure. First, a crystal structure would provide the absolute sequence of the protein, which would be a valuable piece of information. This is particularly so, given Berlin's unsuccessful attempts at cloning the enzyme, suggesting a possible problem with the sequence. The crystal structure would help to resolve several such outstanding questions about the protein. Next, obtaining a crystal structure would give us the oligomeric state of LDC in crystalline form. We predict to see a homo-dimer structure, but it could have some variance, as seen in ODC from *L. bacillus*.

If the holozyme is able to be crystallized, there is also potential for inhibitors or substrates to be crystallized as well. Within our own lab, α-D-(2'-Z-fluoro) vinyllysine was found to be a substrate with slow turnover, so there is a possibility that a crystal structure could be obtained with the substrate in the active site. This could potentially provide some insight to the Dunathan hypothesis, if the alignment of the C_{α}-CO_2^- is seen to be aligned with the extended pi system. The second target that was synthesized within our lab, α-*L*-(2'-*Z*-fluoro) vinyllysine, was found to be an irreversible inhibitor of the enzyme. If we were able to crystallize the inactivated protein with the processed inhibitor bound, similarly to DFMO in ODC and vinyl-GABA in GABA transaminase, the crystal structure would give useful insight to the mechanism of inactivation.

Alternatively, it may be possible to diffuse such a suicide substrate into the crystal of the holoenzyme, provided that the motion associated with the transamination and inactivation chemistry would not significantly disrupt the crystals.

Currently, a successful and improved purification has been presented, with preliminary results showing that crystallization is an obtainable goal. With our lab's new purification protocol, crystallization of LDC is within our grasp.

III. Experimental

Hafnia alvei was grown from a purified sample from K. Karukurichi, which was grown from *Hafnia Alvei* strain IFO 3731 was bought from the Institute for Fermentation of Osaka. Centrifugation was completed using the SorvallRC5B. Protein concentration was measured using the Lowry assay. Protein activity was measured using the Lenhoff assay.

Purification of Lysine Decarboxylase:

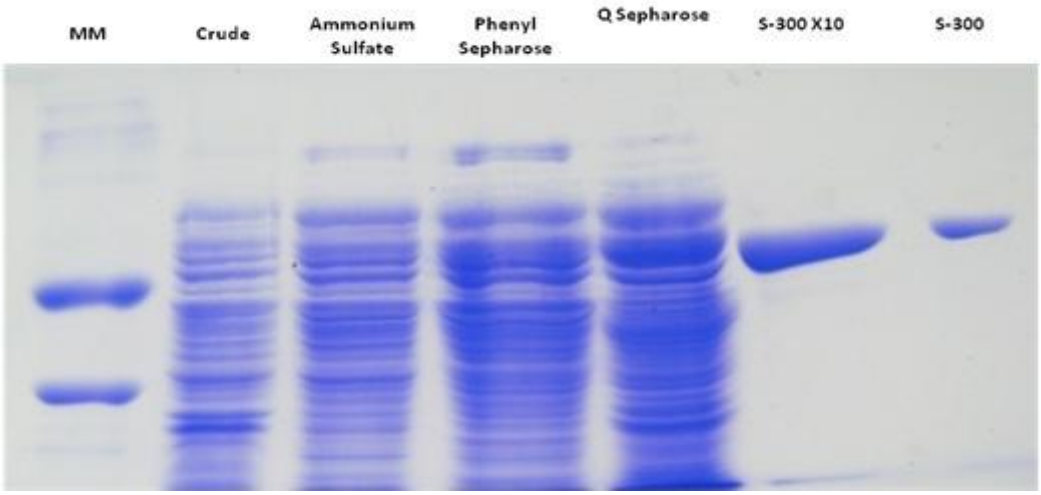
H. Alvei was grown a modified Laemmli broth, consisting of 5 g bactopetpone, 5 g yeast extract, 1g $MgSO_4 \bullet 7H_2O$, and 3 g of L-lysine monohyrdide per 1 L of solution. Initially, the bacteria was grown in a starter culture consisting of 50 mL of the culture broth, and after 12 hours at 43 °C was split into six 4 L flasks, containing 2 L of broth. The solution was kept in a shaker for 3 days at 150 rpm and 43 °C.

To 24 g of wet cells, ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), and 80 mL of 10mM potassium phosphate buffer (Buffer A), and 6 crushed capillary tubes were added to increase surface area, and separated into two 250 mL beakers. The beakers were sonicated on ice five times in thirty second intervals, and was centrifuged at 10,000 rpm for 15 minutes. The supernatant was decanted, and a 500 μL fraction was taken for analysis.

To the remaining liquid, 60% ammonium sulfate saturation was added at 4 °C and was stirred for 20 minutes until completely dissolved. The mixture was spun at 10,000 rpm for 15 minutes. Nearly all activity (99%) was found in the pellet. The supernatant was discarded and the pellet was stored at −70 °C.

The pellet was resuspended in 80 mL of 100 mM potassium phosphate pH 6.0 (Buffer B), and was centrifuged at 15,000 rpm for 15 minutes. The supernatant was decanted and was concentrated down to 60 mL. The solution was loaded onto a 200 mL phenyl sepharose column and eluted with a gradiant of 100 mM (250 mL) to 10 mM (250 mL) potassium phosphate buffer, pH 6.0. These fractions were collected in 5.5 mL increments. To find active protein, 300 μL of each fraction was placed on a 96-well plate and measured the absorbance at both 280 nm and 420 nm. The fractions were then tested by the Lenhoff assay, which will be described later.

Active fractions from the phenyl sepharose column were concentrated to 2 mL and were loaded onto the S-300 column, and was eluted using 10 mM potassium phosphate buffer with 10 μM PLP, and were collected in 5.5 mL fractions. Active fractions were measured by the Lenhoff Assay.



	Volume (mL)	Total Units	Units/mL	Total Protein (mg) Lowry Assay	Specific Activty (U/mg)	% Yield	Purification Factor
Crude	50	2874	57	2499	1.15	100	1
Ammonium Sulfate	27	2833	104	2380	1.19	98.6	1.03
Phenyl Sepharose	4.5	1118	248	81	13.8	62.1	12
S-300	1	382	382	4.2	91	13	79

Activity Measurements

Cadavarine Standard

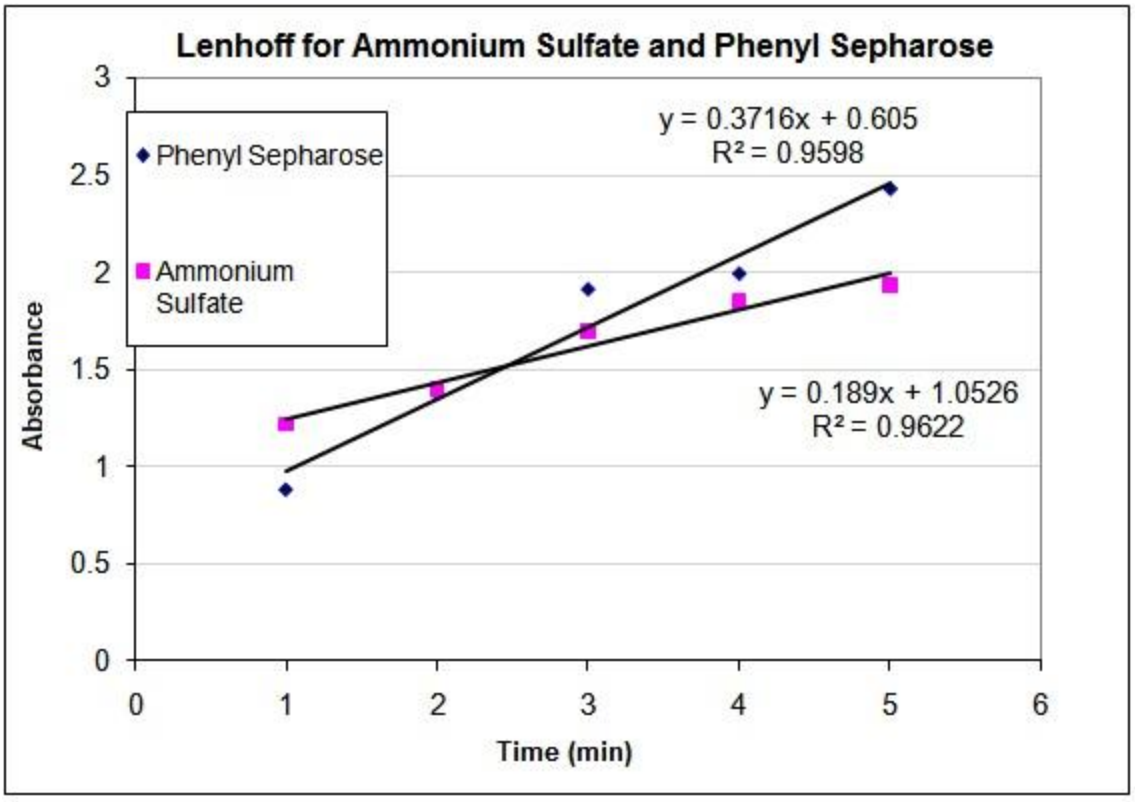
A 2.1 mM solution of cadaverine stock solution was made. 9.5 µL to 95 µL of the cadaverine solution was added to 24 µL of 100 mM L-lysine. The solution was finalized to 300 µL by adding 10 mM phosphate buffer, and the tube was heated at 43°C for 7 minutes. Once brought back to room temperature, 300 µL of 22 mM TNBS was added and the solution was heated at 43 °C for 7 minutes. Once cooled to room temperature, 600 µL of toluene was added, and the solution was mixed on a mini vortex 2 times for one minute. 300 µL of the organic layer was extracted and the absorbance was

measured at 340 nm for a range of concentrations of cadaverine. Absorbance versus concentration was plotted, and the slope gave the absorbance per 1 μM of cadaverine.

Activity of LDC

Each test tube contained 20 μL of selected LDC and 256 μL of 10 mM phosphate buffer pH 7.0. At time point 0 minutes, 24 μL of a 100 mM L-lysine solution was added to the test tube. At the timepoint in which the reaction was desired to be stopped, 300 μL of 1.0 M K_2CO_3 was added to quench the reaction, and the mixture was heated at 43 °C for 7 minutes. After the solution was allowed to cool to room temperature, 300 μL of 22 mM TNBS was added and heated again at 43 °C for 7 minutes. Once the second solution was cooled to room temperature, 600 μL of toluene was added to the solution. The mixture was agitated in the mini vortex two times, each lasting for one minute. The final product resulted in two layers, an orange aqueous layer and a clear to yellow organic layer. Interestingly, measurements of the crude extract also have a frothy bright yellow substance at the top of the aqueous layer.

After completing the reaction, a 300 μL aliquot of the toluene layer was placed in the quartz plate and absorbance was measured at 340 nm. A graph plotting absorbance vs. time was made, with the slope being used to calculate activity. Based on the calibration curve, we can calculate the activity units in the 300 μL solution.



Protein Measurements

For the Lowry Assay, the following solutions can be made ahead of time and used for multiple assays:

Solution A: 0.5 g $CuSO_4$, 1.0g sodium citrate, and 100 mL dd H_2O

Solution B: 20 g Na_2CO_3, 4g NaOH, and 1L dd H_2O

When the assay will be run, the following solutions should be made fresh:

Solution C: 100 mL Reagent B

Solution D: 10 mL Folin-Ciocalteau reagent (SIGMA, 2 N) and 10 mL H_2O

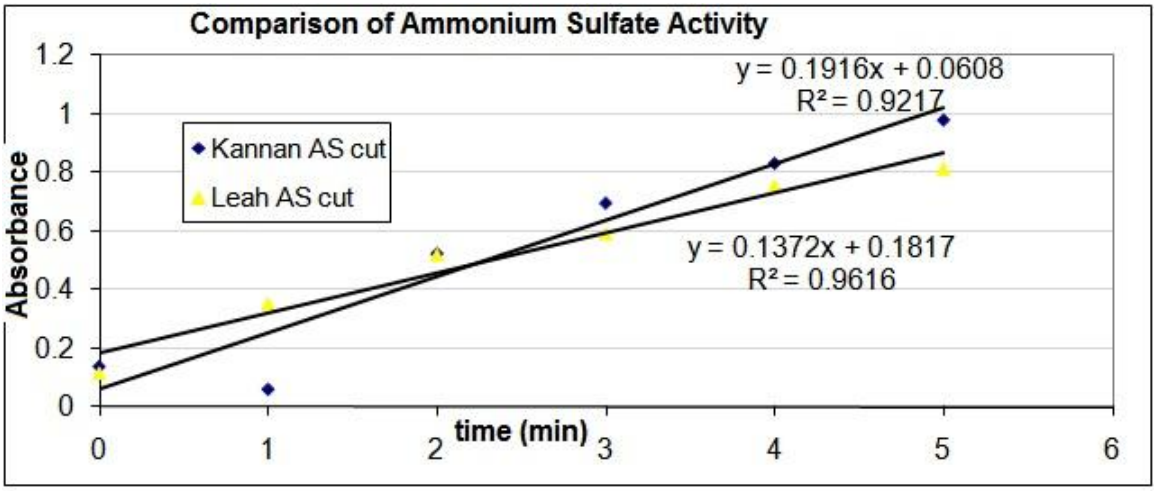
The standard protocol was followed:

1. 10 BSA standards were made in triplicate ranging from 0.1 mg/mL BSA to 1 mg/mL BSA, in increments of 0.1 mg/mL
2. 1.25 mL of solution C and 250 μL of the standard or the protein to be measured were mixed, vortexed at a medium speed, and were let to sit in an incubator at 37 °C for 10 minutes.
3. 125 μL of Solution D were added to the solution, vortexed at a medium speed, and incubated at 37 °C for 25 minutes.
4. Absorbance of the solutions was measured at 650 nm.

A plot of the standard absorbance versus concentration was made, with the slope of the plot giving the Abs/mg of protein. The absorbance of the purified protein could also be used to estimate the amount of protein present in solution.

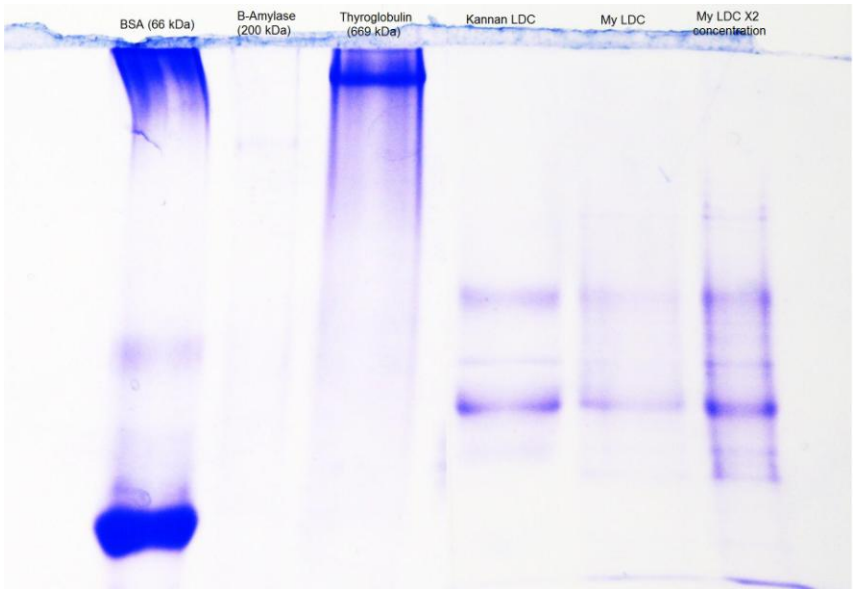
Ammonium Sulfate Test

A test was run to compare the activity of Karukurichi's ammonium sulfate precipitant which had been stored at −80 °C for two years versus a freshly made ammonium sulfate precipitation. A small fraction of each pellet was re-suspended in 1 mL of 10 mM phosphate buffer pH 7.0 in the presence of 15 μM PLP. 20 μL aliquots were taken from both samples, and a Lenhoff activity assay was run on the two samples. Interestingly, the old sample appeared to have slightly higher amounts of activity than the new pellet, with the activities differing slightly. This shows that even after years of storage, ammonium sulfate pellets serve as a suitable storage method.



Native State Determination

Native state determination of LDC was determined by running LDC on a 4% stacking, 9% resolving discontinuous polyacrylamide gel. The gel was run at a constant current of 40 mA and was cooled using flow cooling as set by the Hoeffer Mighty Small II electrophoresis apparatus. BSA was used as a standard. From the gel, it appears that LDC exists as a monomer or a dimer, based on molecular weight measurements.



Sedimentation Equilibrium Experiment

An ultracentrifugation was run to perform a sedimentation equilibrium experiment. If LDC existed as a dimer, the Beckman-Coullter Proteomelab XLI Protein Characterization system was run at 8000 rpm with a protein concentration of 0.4 mg mL^{-1} in a buffer of 10 mM potassium phosphate pH 6.0 at 20 °C for 24 hours, and was run at 11000 rpm under the same conditions to test for a monomer at molecular weight 83 kDa. Data was collected using Micocal Origin software with the Beckman ultracentrifuge data analysis add-ons. The data was analyzed at 280 nm. After analysis, and given a 95% confidence interval, the molecular weight from both runs was shown to be 398 (±22) kDa based on its sequence. The molecular weight suggests LDC existing in a pentameric state, which would correlate to work done by Beier, showing a molecular weight of 422 kDa.

An issue regarding these results is that they do not agree with the results obtained from the gel filtration, however, it may be that LDC is exists as an equilibrium mixture of several different oligomeric forms. In a simple analysis here, one could have equal amounts of tetramer and hexamer, giving an apparent averaged molecular weight of a pentamer.

Crystallization of LDC

1st screening:

LDC in a 30% glycerol stock was loaded onto a G-25 column and was eluted with 10 mM potassium phosphate buffer pH 6.0 in 10 μM PLP. Fractions were collected following UV reading at 280 nm, and protein was concentrated down to 5 mg/mL using a Centricon. Due to the low concentration of protein, trays were set loading 2:1 protein/buffer. Preliminary screening trays were set up utilizing the Hampton I and Hampton II screening kits. Trays were kept at 9 °C.

2nd screening:

LDC in a 30% glycerol stock was loaded onto a G-25 column and was eluted with 10 mM malonic acid buffer pH 6.0 in 10 μM PLP. Fractions were collected following UC reading at 280 nm, and protein was concentrated down to 20 mg/mL using a Centricon. Preliminary optimization trays were performed by utilizing the Hampton I and Hampton II screening kits. Trays were kept at 9 °C.

Mass Spectroscopy

After collecting S-300 fractions, pure and active fractions of LDC were loaded onto a 9% SDS PAGE gel. After destaining had occurred, gels were slices and digestion of LDC was followed by standard protocol (discussed below). The slices were digested with AspN, formic acid, trypsin, and chymotrypsin. The digested protein was extracted and run using a C18-reverse phase LC column (75 micron x 15 cm, Pepmap 300, 5 micron particle size). Results showed multiple hit of various strains of LDC, yet LDC from *H.*

Alvei was not the top hit from any of the digestion results. The following depicts fragments found within the published sequence of LDC from *H. Alvei:*

Tandem MS/MS for protein modification

Upon sequence alignment, it was seen that the active site lysine exists in a highly conserved region of 12 LDC's from various bacterial strains. To a sample of pure LDC (≈1 nmol in 60 μL), sodium cyanoborohydride ($NaCNBH_3$, 1.0 mg, 16 μmol) was added and was incubated at 0 °C for two minutes. The solution was centrifuged at 10,000 rpm for 10 minutes. The solution was then placed on a 4 kDa Microcon filter and was washed 4 times with 300 μL 10 mM Potassium Phosphate buffer pH 6.0, containing 10 μM PLP. After the final rinse, the solution was resuspended in 150 μL of the washing buffer, and was incubated at 25 °C. After 18 hours, 10 μL of the solution was mixed with 7 μL of loading buffer, was denatured at 80 °C for 7 minutes, and was loaded onto a 9% acrylamide SDS-PAGE gel. Bands at approximately 80 kDa were excised and digested and run on LC/MS, as described above.

V. References

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