Biochemical studies of enzymes in insect cuticle hardening

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ABSTRACT

In insects, the cuticle provides protection against physical injury and water loss, rigidness for muscle attachment and mechanical support, and flexibility in inter-segmental and joint areas for mobility. As most insects undergo metamorphosis, they need to shred off old cuticle and synthesize new cuticle to fit the body shape and size throughout their life cycles. The newly formed cuticle, mainly composed of cuticular proteins, chitin, and sclerotizing reagents, needs to be hardened through the crosslinks between cuticular proteins and sclerotizing reagents. This dissertation concerns the biochemical activities of several pyridoxal 5-phosphate (PLP)-dependent decarboxylases with most of them involved in insect cuticle hardening. Herein, we first present a detailed overview of topics in reactions and enzymes involved in insect cuticle hardening. Aspartate 1-decarboxylase (ADC) is at the center of this dissertation. β-alanine, the product of ADC-catalyzed reaction from aspartate, is the component of an important sclerotizing reagent, N-β-alanyldopamine; the levels of β-alanine in insects regulate the concentrations of dopamine, therefore affecting insect sclerotization and tanning (collectively referred as cuticle hardening in this dissertation).

Biochemical characterization of insect ADC has revealed that this enzyme has typical mammalian cysteine sulfinic acid decarboxylase (CSADC) activity, able to generate hypotaurine and taurine. The result throws lights on research in the physiological roles of insect ADC
and the pathway of insect taurine biosynthesis. Cysteine was found to be an inactivator of several PLP-dependent decarboxylases, such as ADC, glutamate decarboxylase (GAD) and CSADC. This study helps to understand symptoms associated with the abnormal cysteine concentrations in several neurodegenerative diseases. A mammalian enzyme, glutamate decarboxylase like-1 (GADL1), has been shown to have the same substrate usage as insect ADC does, potentially contributing to the biosynthesis of taurine and/or β-alanine in mammalian species. Finally, the metabolic engineering work of L-3, 4-dihydroxyphenylalanine decarboxylase (DDC) and 3, 4-dihydroxyphenylacetaldehyde (DHPAA) synthase has revealed that the reactions of these enzymes could be determined by a few conserved residues at their active site. As both enzymes have been implicated in the biosynthesis of sclerotizing reagents, it is of great scientific and practical importance to understand the similarity and difference in their reaction mechanisms. The results of this dissertation provide valuable biochemical information of ADC, DDC, DHPAA synthase, and GADL1, all of which are PLP-dependent decarboxylases. ADC, DDC, DHPAA synthase are important enzymes in insect cuticle hardening by contributing to the biosynthesis of sclerotizing reagents. Knowledge towards understanding of these enzymes will promote the comprehension of insect cuticle hardening and help scientists to search for ideal insecticide targets. The characterization of GADL1 lays groundwork for future research of its potential role in taurine and β-alanine metabolism.

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List of Abbreviations

5-HT  5-hydroxytryptamine
5-HTP  5-hydroxytryptophan
AAAD aromatic amino acid decarboxylase
AANAT arylalkylamine N-acetyltransferase
AAS aromatic acetaldehyde synthase
ABAM antibiotic antimyotic
ADC Aspartate 1-decarboxylase
AeADC aspartate 1-decarboxylase from Aedes aegypti
AeGAD glutamate decarboxylase from Aedes aegypti
AnADC aspartate 1-decarboxylase from Anopheles gambiae
AnGAD glutamate decarboxylase from Anopheles gambiae
AMD α-methyl DOPA
AMD-r α-methyl DOPA-resistant
ATP adenosine-5’-triphosphate
CDO cysteine dioxygenase
CDS coding sequences
CSADC cysteine sulfenic acid decarboxylase
DDC L-3, 4-dihydroxyphenylalanine decarboxylase
DHPAA 3, 4-dihydroxyphenylacetaldehyde
DMEM Dulbecco’s Modified Eagle Medium
DOPAC 3, 4-dihydroxyphenylacetic acid
DrADC aspartate 1-decarboxylase from Drosophila melanogaster
DrGAD glutamate decarboxylase from Drosophila melanogaster
ED electrochemical detection
FBS fetal bovine serum
GABA γ-aminobutyric acid
GAD glutamate decarboxylase
GADLI glutamate decarboxylase-like 1, glutamate decarboxylase-like protein 1
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
<table>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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Chapter 1

Introduction

Pyridoxal 5-phosphate (PLP)-containing enzymes generally play important roles in amino acid metabolism in living species. Typical reactions catalyzed by PLP-containing enzymes include transamination, decarboxylation and deamination (1,2). Among the reactions catalyzed by PLP-containing enzymes, PLP always form an internal aldimine with a conserved active site lysine residue and such internal aldimine is replaced by an external aldimine formed between the PLP and the incoming substrate (2,3). The conformation at the active site of PLP-containing enzymes dictates the C\textsubscript{\alpha}-proton stabilization mechanisms and substrate orientation, both of which are proposed to be closely relevant to the reaction types (2). The versatility of reactions catalyzed by PLP-containing enzymes and their unifying mechanistic principle expose some of the most interesting questions to biochemists and structural biologists as to what are the determining factors of reaction types and substrate selectivity. In addition, PLP-containing enzymes play crucial roles in diverse cellular processes;
they compose an ideal group for studies concerning the structure-function relationship of enzymes.

In this dissertation, the cellular process with the participation of PLP-containing enzymes is insect cuticle hardening. Insects continuously shred off their old cuticle and form a new one as they go through either hemimetabolous or holometabolous development. The newly formed cuticle is soft and needs to be hardened to provide protection and support. Instead of calcium, insects utilize chitin and proteins for the hardness that is mainly accomplished by the crosslinks of cuticular proteins (4,5), thereby evolving a unique set of protein-crosslinking reactions in insect cuticle formation (often collectively called sclerotization and tanning). The extent of rigidity is determined by the need to accommodate living conditions at different stages of the same species or for different species. The genes and proteins actually represent a huge and unique gene/protein family. The absence of these genes and/or proteins in other species could shed light on potential insecticide design.

Insect cuticle hardening is reviewed in Chapter 2. Topics of the review include: 1. the components, functions, and variation of insect cuticle; 2. the reagents, reactions, and timing of insect cuticle hardening; 3. the physiological roles and biochemical/structural aspects of enzymes in insect cuticle hardening; and 4. the latest research progress made on three PLP-containing enzymes in the insect cuticle hardening process.

The first PLP-containing enzyme studied in the dissertation is aspartate 1-decarboxylase (ADC). ADC and its product, \( \beta \)-alanine, control the level of melanization as the cuticle hardens, leading to the formation of black cuticle when there is not enough \( \beta \)-alanine or
the ADC activity is compromised (6,7). Detailed physiological roles of ADC are reviewed in Chapter 2. With the success of recombinant ADC expressed from different insect species, biochemical studies of ADC have shown that ADC is able to work on cysteine sulfinic acid and cysteic acid to generate hypotaurine and taurine respectively (Chapter 3). Typical mammalian cysteine sulfinic acid decarboxylase (CSADC), on the other hand, could not work on aspartate. With the availability of crystal structure information of an ADC-similar enzyme, subsequent mechanistic studies have proposed and later proved that a conserved glutamine residue of insect ADC gives it an advantage over mammalian CSADC to stabilize aspartate at the active site for reaction (Chapter 3).

As an endeavor to better characterize biochemical properties of insect ADC, cysteine is unexpectedly discovered to be a potent inactivator. The cysteine-caused inactivation of insect ADC has been proven due to the formation of a stable complex between cysteine and the PLP at the active site (Chapter 4, Fig 4.11 D). This inactivation mechanism is found in several other PLP-dependent decarboxylases, such as glutamate decarboxylase (GAD) and CSADC, with a general conclusion regarding cysteine-caused inactivation of PLP-dependent decarboxylase being proposed (Chapter 4).

Still related to insect ADC, in an attempt to find similar enzymes in other living species, a mammalian enzyme glutamate decarboxylase like-1 (GADL1) draws our attention, with around 40% identity to other identified PLP-dependent decarboxylases and its unknown biochemical activities. Through expression and characterization, it has been shown to catalyze similar reactions as insect ADC does (Chapter 5). Subsequent studies of mRNA/protein
distribution and biochemical assays with tissue samples have confirmed the presence of this enzyme and pinpointed where it may play a role (Chapter 5).

Back to insect cuticle hardening, the other two PLP-containing enzymes studied are called L-3, 4-dihydroxyphenylalanine decarboxylase (DDC) and 3, 4-dihydroxyphenylacetaldehyde (DHPAA) synthase. DDC catalyzes the decarboxylation of L-3, 4-dihydroxyphenylalanine (L-DOPA) to dopamine, the central chemical in insect cuticle hardening. On the other hand, little is known about DHPAA synthase. This enzyme has long been proposed to be related to insect cuticle formation and its catalyzed reaction was recently identified (8). Both enzymes work on L-DOPA and share good sequence similarity. DHPAA catalyzes decarboxylation-deamination of L-DOPA versus decarboxylation of L-DOPA by DDC. Inspired by similar protein engineering work done in insect ADC (Chapter 3) and plant aldehyde synthases (Appendix A), a few residues of DDC were chosen as candidates for mutagenesis and the mutated DDCs were tested after expression and purification (Chapter 6). Two sites have been shown to have a minor presence of DHPAA synthase activity. This research project is now actively going on and the up-to-date knowledge is summarized in Chapter 6.

The whole work is done on several PLP-dependent decarboxylases, with most of them directly participating in insect cuticle hardening. It is important to develop our understanding of the substrate selectivity and the reaction mechanisms of this group of enzymes. This work also aids in the establishment and development of several research projects beyond the scope of insect cuticle hardening.
1.1 References

Chapter 2

Insect cuticle hardening: reactions
and enzymes involved

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Abstract

There have been over one million identified insect species in the world and the total number of identified plus unidentified insect species was estimated to be as many as 10 million. It has commonly been accepted that the highly protective cuticle is one of the primary reasons for the success of insects on earth throughout evolution. As a result, the biochemistry and physiology of insect cuticle, particularly its production and structural characteristics, have been attracting considerable attention. As continuous growth along with metamorphosis, insects need to routinely shred off their old cuticle and synthesize a new one to fit the increased body size and different shapes. The newly formed cuticle is soft and vulnerable to adverse environmental conditions; therefore, it must be hardened shortly after formation. Chitin and cuticular proteins are the two major components of insect cuticle and the mechanism, leading to the formation of solid and protective insect cuticle, appears to be primarily due to the crosslinks of cuticular proteins. The physical appearances and characteristics of insect cuticle differ greatly in different species, reflecting the extent of crosslinking as well as the diverse types of cuticular proteins and crosslinking reagents in the process. Our general perception about insect cuticle often was its rigidness and the major emphasis in the production of protective cuticle has been reactions, leading to the formation of rigid cuticle. For the vast majority of insects, however, most areas of their cuticle are covered with highly flexible cuticle. Recent studies suggest some differences in mechanisms that produce rigid and flexible cuticle, respectively. It is necessary to be more inclusive in terms of mechanisms and reactions that result in the production of protective cuticle in insects. Herein, we sum-
marize different biochemical mechanisms involved in the formation of insect cuticle with an emphasis on their functions and variation, particularly in the formation of protective, yet flexible cuticle. We believe that insect melanization plays an important role in insect cuticle hardening, which has been overlooked in the past.

Keywords

cuticle hardening, sclerotization, tanning, dopamine, N-acetyldopamine, N-β-alanyldopamine, β-alanine, aspartate 1-decarboxylase, pyridoxal 5-phosphate, and decarboxylase

2.1 Introduction

Insect is a class of invertebrate within the arthropod phylum. It represents the most diverse group of animals and contains around half of all living organisms (1,2). The ability to adapt to different environments leads to the success of insects in evolution and their widespread presence on earth, which is attributed considerably to the protection of insect cuticle. Insect cuticle comes in a wide range of toughness and elasticity. Compared to other arthropods whose cuticle generally contains high levels of calcium (3,4), it seems that insects utilize cross-linked chitin and cuticular proteins as the substitute with levels of rigidness/flexibility being affected primarily by 1) the degree of crosslinking and 2) the mechanism(s) of crosslinks. Cuticle tanning and cuticle sclerotization have been commonly used to describe the biochemical processes, leading to the formation of hard and rigid cuticle in insects. These two processes involve protein crosslinking reactions mediated by N-acetyldopamine (NADA) and
N-β-alanyldopamine (NBAD)- derived reactive quinones. The chemical reactions and mechanisms have been described and cited in numerous articles (3-5). Although intermediates and crosslinking structures remain to be established, the proposed reactions of NADA- and NBAD- quinones with cuticle proteins likely occurring in the cuticle of many insects and may well be the major biochemical reactions occur during the formation of rigid cuticle in many Coleoptera species. However, the reactions and intermediates involved in insect cuticle sclerotization are far from clear. In many other species, the currently proposed cuticle sclerotization reaction could not well explain the cuticle formation in those species. As a result, the currently proposed cuticle sclerotization reaction may be one of the primary biochemical pathways or even a minor pathway toward the formation of their protective cuticle. Cuticle melanization often is a major biochemical event in many insects during cuticle production, but the contribution of cuticle melanization to the formation of rigid cuticle is not clearly addressed. Our recent studies dealing with mosquito and Drosophila 3, 4-dihydroxyphenylacetaldehyde (DHPAA) synthase suggest that the DHPAA (a reactive acetaldehyde)-mediated protein crosslinking is indispensable for the formation of flexible protective cuticle (5). Therefore, there is a need to be more inclusive in terms of overall reactions happening during the course of protective cuticle formation.

In this communication, we present a panorama of insect cuticle hardening, including the components of insect cuticle, biochemical reactions during hardening and enzymes involved in the biosynthesis of sclerotizing reagents. We also discuss possible current/future research directions of this field. In this review, the phrase cuticle hardening is frequently used to
collectively describe the currently known biochemical pathways leading to the formation of protective cuticle, including quinone tanning and sclerotization. Although less definitive, cuticle hardening seems to be able to cover the complexity of reactions involved in the formation of protective cuticle in insects.

2.2 Insect cuticle: functions and components

The insect cuticle is important because of: 1) the flexibility and elasticity that allow movement and growth; 2) the hardness that supports the muscle attachment and provides a protective covering; 3) the impermeability that prevents water from evaporation and allows insects to survive in dry environments; and 4) the physical barrier that provides the first line of defense against pathogen invasion. Based on physical characteristics insect cuticle could be classified as soft (flexible) cuticle and hard (rigid) cuticle or classified as larval cuticle, pupal cuticle, and adult cuticle based on developmental stages. Cuticle materials, including lipids, chitin, cuticular proteins and catecholamines (crosslinking precursors), are synthesized by a single layer of epidermal cells that are on the surface of insects (Fig. 1). Although calcium is an essential cuticle component in crustaceans and other arthropods, calcification of insect cuticle is rare (3,4). The mechanical properties of insect cuticle are influenced by the contents of chitin, cuticular proteins, and crosslinking agents that are involved in cuticle protein crosslinking (6-8).

Structural lipid and free lipid are the two types of lipids found in the insect cuticle (9). The
structural lipid, often called cuticulin, is actually a lipoprotein and a major component of the inner cuticle (10,11) and often crosslinked by reactive quinones after its deposition (9). Structural lipid is insoluble in organic solvents and it can only be released by destructive oxidation (12). Structural lipid participates in the formation of rigid and hard insect cuticle and its amount and distribution have been proposed to determine the different stiffness of the insect cuticle across species and layers (12). Free lipid, on the other hand, is present in the epicuticle and is extractable with organic solvents (9). It forms a barrier that is resistant to desiccation (13). It varies in composition and may contain several chemical classes such as alcohols, aldehydes, esters, fatty acids, glycerides, hydrocarbons and ketones (14-16).

Chitin, with an estimated annual production equal to that of cellulose (17), is the most widespread amino polysaccharide in nature. It is a long-chain polymer of $N$-acetylglucosamine and found in the cell walls of fungi, the exoskeletons of insects and other arthropods (18) (Fig. 2). In insects, chitin is presented as long microfibrils, approximately 20 chitin molecules in each microfibril (19,20). The microfibrils, imbedded within the cuticular proteins, are about
2.8 nm thick and have indefinite length (19). Chitin is partly responsible for the tough and elastic properties of insect cuticle (the other contributor is protein crosslinking). The ratio of chitin to cuticle proteins depends on the types of cuticle, the insect species, the developmental stages of insect cuticle, and the regions of insect cuticle. Typically, soft cuticle contains roughly equal amounts of chitin and protein with 40%-75% water, while rigid cuticle contains only 15%-20% chitin and 12% water (21). Coordination of chitin biosynthesis and its degradation in living species is mainly regulated by chitin synthases and chitinases (22-24). Other degrading enzymes include β-N-acetylglucosaminidases (22,25). Both chitinases and β-N-acetyl-glucosaminidases catalyze the hydrolysis of β-(1-4)-glycosidic bonds of chitin polymers and oligomers (22).

Cuticular proteins are the other major components of insect cuticle besides chitin. They are either synthesized by the epidermal cells or transported from the hemolymph (4,26). Compared to the minor variations of chitin, reflected by chain length and degree of acetylation, cuticular proteins constitute a much more diverse group (7). Cuticular proteins are associated with the flexibility, rigidness, and plasticity of insect cuticle. The types and amounts of insect cuticle proteins show differences among developmental stages, regions, and species. Scientists have identified several conserved sequences that often appear in cuticular proteins. A large cuticular protein family has been defined by the presence of a Rebers and Riddiford (R&R) consensus that was proposed to have affinity to chitin (27,28). Subsequently, cuticular proteins from this family were further separated into three subfamilies, named as RR-1, RR-2, and RR-3 (29,30), respectively. Cuticular proteins with RR-1 type domain
are considered as soft (or flexible) cuticular proteins and those with RR-2 type domain are predicted as hard (or rigid) cuticular proteins (31). RR-3 has only been identified in a few cuticular protein sequences (32) and their role in cuticle formation remains to be established (30). RR-1 type and RR-2 type domains have been used to classify soft and hard cuticular proteins in sequenced insect species in cuticleDB website (33).

Compared to the RR-1 consensus sequence that is varied in length and sequence, the RR-2 consensus sequence, approximately 64 amino acid residues, is well conserved (34). Physiologically, it has been proposed that cuticular proteins with RR-1 consensus sequence or RR-2 consensus sequence differ in their affinity to chitin (34). The presence of RR-1 or RR-2 type domain in deduced proteins helps identify them as cuticle proteins (cuticle protein-like), provides structural basis to further separate them into subgroups and to give some insight towards predicting their possible functional differences in cuticle formation and hardening. The notion that the distribution of different types of cuticular proteins is tissue-specific was proposed and later backed with some experimental evidence (29,32,35). Although earlier studies considered that stage-specific genes underwrote metamorphic stages, it appeared that the physical properties of cuticle (rigid or flexible) were more important than metamorphic stages in determining which cuticular proteins would be present (29,36). In addition, most cuticular protein genes were shown to express at more than one stage (29,37). Hence, the regulatory expression of given cuticular proteins may play a dominant role in metamorphosis.

Blast search of sequenced insect genomes revealed that a larger than expected portion of total
genes code for cuticular proteins, which is particularly apparent in mosquitoes. There has been no clear explanation for the presence of the large number of cuticular proteins in some insects. In mosquitoes, it was suggested that the rapid development of mosquitoes requires massive synthesis of cuticular proteins over a short period and the only way to get more proteins rapidly expressed would be to increase gene copy number (29). Rapid production of very similar eggshell proteins by multiple genes also occurs during mosquito egg development (38-40). In *Aedes aegypti*, three major chorion proteins, highly similar to one another, are produced at large quantity during 12-36 hr after blood feeding and utilized to form egg chorion (39). Conceivably, the presence of adequate amount for any one of them should serve for the same purpose of the three. Conceivably, the expression of three highly similar chorion proteins at the same time reflects the need to reach sufficient quantities of the same type of protein during a short period (39). Further studies of cuticular proteins indicated that some cuticular proteins could be clustered together based on high sequence identity (41) and the temporal transcriptional patterns and levels are similar within each sequence cluster (29). Studies of hard cuticular proteins (those with RR-2 type domain) from several insect species (34) have reported that RR-2 sequence clusters generally favor the concerted evolution (an evolving manner characterized by unequal crossing-over and intergenic gene conversion) (42-44) to birth-and-death evolution (an evolving manner characterized by strong purifying selection and a background level of gene turnover) (43).

Catecholamines, indispensable components in insect cuticle, function as precursors in cuticular sclerotization and tanning. These include dopamine, NADA, NBAD, and their glucoside
or sulfate conjugates (Fig. 3). During cuticle hardening, these catecholamines or catecholamine derivatives are oxidized to electrophilic intermediates that react with nucleophilic groups on the chitin-cuticle protein matrix of insect cuticle. The presence of specific catecholamines/quinones and their relative abundance appear to be related to different types of cuticle hardening reactions, which is often reflected by the noticeable color changes of the hardened cuticles and which may explain the different descriptions/terminologies utilized to describe the cuticle hardening reactions, including cuticle sclerotization, cuticle tanning, cuticle melanization, and so forth. The next section discusses the production, reactions, and regulations of catecholamines and quinones in insect cuticle hardening.

Figure 2.3: Chemical structures of dopamine, N-acetyldopamine, and N-β-alanyldopamine. (a) dopamine, (b) N-acetyldopamine, and (c) N-β-alanyldopamine.


2.3 Insect cuticle hardening: definition, reagents, reactions, and timing

A sclerite (also called sclerotin) is a hardened body part, mostly referring to the arthropod exoskeleton. Insect cuticle is often considered a type of sclerite. The difference between insect cuticle and cuticle from other arthropods is the lack of calcium (4). Sclerotization is often used to describe the formation of sclerite by the hardening and stabilization of epicuticle proteins and chitin through crosslinks by reactive quinones derived from catechols or catecholamines that are mainly derived from tyrosine (45). Although the hydrophobicity of cuticular proteins contributes to the stiffness of insect cuticle to some extent, it is believed that much of the stiffness is caused by the modification of cuticular proteins reacting with oxidation products of catecholamines. The types and abundance of these sclerotizing reagents differ greatly by insect species. Tanning refers to the pigmentation of newly formed sclerite, which is often accompanied by sclerotization. Cuticle tanning is essential for most insects. This phenomenon includes two distinct while interrelated processes, sclerotization and pigmentation. Sclerotized cuticular proteins displayed colors from colorlessness to brownish. Melanization generates melanin that protects species from DNA damage caused by ultraviolet light. Colors of melanin vary from brown-black (eumelanin) to reddishness (pheomelanin) (46-48) (Fig. 4). Melanization pathway has been shown to actively participate in the hardening and pigmentation of insect cuticle; the detailed reactions and participating intermediates will be explained in later paragraphs (49-51). Ketocatechols have so far been
obtained from all insect species investigated but not from any other arthropods (52). This indicates that insects have evolved the sclerotization mechanism discussed here and that the other arthropods either use only quinones to stabilize their cuticle or they may have evolved an alternative mechanism for hardening their cuticle.

Figure 2.4: Overview of the general reactions for formation of pheomelanin and eumelanin. (a) pheomelanin and (b) eumelanin. 5-S-cysteynlidopa is the major compound in pheomelanin biosynthesis.

The available sclerotizing reagents in each insect species affect distinct insect sclerotization and tanning. NADA and NBAD are generally considered the major components involved in sclerotization and involved in cuticular tanning (through sclerotizing cuticular proteins). It is generally considered that NADA and NBAD are not involved in melanization. Dopamine, the primary component in melanin synthesis, has been studied in its roles in melanization as well as sclerotization. Quinone methides were suggested to cross-link chitin and cuticular proteins as well. Recently, 3, 4-dihydroxylphenylacetaldehyde (DHPAA) has been proposed
to be a sclerotizing reagent specifically associated with soft cuticle formation (Fig. 5). DH-PAA is the decarboxylation-deamination product of 3, 4-dihydroxyphenylalanine (L-DOPA). Its $\beta$-histidine derivative ($\beta$-histidine-3, 4-dihydroxyphenylacetaldehyde) has been extracted from hydrolysates of sclerotized cuticle from various insect species (53). DHPAA has been shown to react with lysine $\textit{in vitro}$ (5). In addition, the identification of DHPAA supports the existence of an alternative pathway for 3, 4-dihydroxyphenylacetic acid (DOPAC) biosynthesis (54) (Fig. 5). DOPAC was identified in some insects decades ago (55, 56), but its role as sclerotizing precursor remain unclear.

Figure 2.5: Biosynthesis of DHPAA and DOPAC in insects. DHPAA, 3, 4-dihydroxyphenylacetaldehyde. DOPAC, 3, 4-dihydroxyphenylacetic acid. DHPAA has been shown to react with histidine and lysine in different studies.

The biosynthetic pathway for sclerotization and tanning precursors primarily involves a series of enzymatic and non-enzymatic reactions: (a) hydroxylation of phenylalanine to tyrosine, (b) hydroxylation of tyrosine to L-DOPA, (c) decarboxylation of L-DOPA to dopamine, (d)
acylation of the dopamine amino group with either acetate or \( \beta \)-alanine, or (e) oxidation of dopamine to dopaquinone (Fig. 6). Although other compounds, such as DOPA, DOPAC, \( N \)-acetylnorepinephrine, and \( N \)-\( \beta \)-alanylnorepinephrine, could be detected from insect cuticle, their contribution to insect cuticle sclerotization and tanning is less defined (6,57).

![Figure 2.6: Overview of the reactions involved in the biosynthesis of major sclerotizing reagents.](image)

Melanization generally is composed of a series of reactions, consisting of the hydroxylation of phenylalanine to tyrosine, the hydroxylation of tyrosine to L-DOPA, the enzymatic oxidation of this catecholamine to o-quinones (dopaquinone), to the spontaneous cyclization to leucoaminochrome (leukodopachrome), and the enzymatic oxidation of leucoaminochrome to p-quinone imine (dopachrome) (51,58) (Fig. 4). The oxidation of catecholamines and leucoaminochromes to o-quinones and p-quinone imines are catalyzed by diphenol oxidases. Even though NADA and NBAD are preferred substrates for diphenol oxidation compared to dopamine, the o-quinone derivatives from these catecholamines are much slower to undergo the spontaneous cyclization than that from dopamine or L-DOPA (59). P-quinone imines derived from NADA and NBAD cannot undergo indolization (59). Taking all melaniza-
tion reactions together, dopamine is the major substrate in melanization; L-DOPA can be oxidized to DOPA quinone derivative directly.

Detailed studies have reported the difference of eumelanin biosynthesis in mammalian species and insects. In insects, a heat-labile and protease-susceptible compound has been shown to specifically accelerate the speed of L-DOPA quinone imine (dopachrome) to 5, 6-dihydroxyindole (DHI) conversion but have no apparent effect on the velocity of dopamine quinone imine to DHI conversion (Fig. 7); since the nature of the compound was not well known at that time, it was called DOPA quinone imine conversion factor (QICF) (59). QICF was subsequently characterized as an enzyme catalyzing the decarboxylation of dopachrome, named as dopachrome conversion enzyme (DCE). The enzyme has two isoforms in Drosophila melanogaster; they were shown to catalyze the decarboxylation of α-methyl dopachrome (and the isomerization/tautomerization of DOPA methyl ester chrome to DHI methyl formate (Fig. 7) (60). The reaction of dopachrome decarboxylation significantly accelerates insect melanization and possibly melanin-related sclerotization as well (Fig. 8). In mammalian species, a corresponding enzyme dopachrome tautomerase (DCT) regulates the conversion of dopachrome to 5, 6-dihydroxyindole-2-carboxylic acid (Fig. 8) (61,62). Compared to the melanization process in insects, the mammalian DCT-regulated melanization is a slow process (63).

There are mainly two types of sclerotization, quinone sclerotization and β-sclerotization (Fig. 9) (64). Once the quinone derivatives are formed from catecholamines, the keto group of a quinone molecule can covalently interact or crosslink with the ε-amino or N-terminus of cuticular proteins. The quinone sclerotization is summarized in Fig. 9 (right
Figure 2.7: Reactions catalyzed by insect DCE. DCE, dopachrome conversion enzyme.

column). \( \beta \)-sclerotization, on the other hand, happens on the \( \alpha \)-carbon of the side chain of catecholamines or quinones (Fig. 9, left column). Alternative mechanisms of \( \beta \)-sclerotization have been proposed though there is still no concluding statement whether they are coexisting or mutually exclusive in different insect species. In addition to chitin and cuticular proteins, NADA and NBAD have been shown to conjugate with glucose, phosphate, and sulphate, increasing the complexity of insect sclerotization.

Two types of sclerotization are available depending on the order of the crosslinks on the sclerotizing agents. The first step of quinone sclerotization is the oxidation of catecholamines to \( \alpha \)-quinones. Then the 5 position of the quinone ring will react with the N-terminus or \( \epsilon \)-amino group of cuticular proteins to produce the protein-catechol ring adduct that will then be oxidized to protein-quinone ring adduct by diphenol oxidases. The newly formed protein-quinone ring adduct will cross-link with another N-terminus or \( \epsilon \)-amino group of
Figure 2.8: Overview of the melanization in insects and mammalians species, and the reactions of insect melanization intermediates in sclerotization.

cuticular proteins on its ring. The product depicted in Fig. 9 right column will be stabilized by tautomerization through its conjugated double-bond system and will undergo maximal two additional crosslinks with cuticular proteins at the side chain of ring.

In β-sclerotization, different mechanisms have been proposed. Only the hydroxyl group of catecholamine ring will be oxidized by diphenol oxidases to form a quinone methide, whose β-carbon at the side chain will then cross-link with protein or chitin. Such a protein-catechol side chain adduct could be oxidized again at the same position by phenol oxidases and another protein could be cross-linked with the quinone methide, resulting in a maximum of two crosslinks at the side chain of catecholamines. The compound can undergo further crosslink reactions at its ring.
Insects generate distinct cuticle in larval, pupal, and adult stages; they tend to use different catecholamines and cuticular proteins for cuticle formation at these developmental stages. The choice of sclerotizing reagents for each cuticle type is reflected by the concentrations of catecholamines in hemolymph and cuticle at larval, pupal, and adult stages. A large portion of catecholamines exist as conjugates in hemolymph and it has been proposed that they will be hydrolyzed before transported into cuticle though catecholamine conjugates could also be detected from cuticle. NADA has been shown to be the major catecholamine in larval and adult cuticle, whereas NBAD has been reported to be dominant in pupal cuticle (65). During larval development, hemolymph catecholamines were only found in high con-
centrations (NADA>dopamine>NBAD) in freshly ecdysed larvae and their concentrations decreased to very low levels during the first twenty-four hours after ecdysis (65). Meanwhile, the concentrations of catecholamines were reported to increase dramatically in larval cuticle three hours after ecdysis (65). NBAD is the major catecholamine in pupal hemolymph (NBAD>dopamine>NADA) and its concentration decreases to very low levels within the first forty-eight hours after pupal ecdysis, during which NBAD was detected to increase steadily but rapidly at pupal cuticles with visible tanning in those regions (65). Compared to NBAD whose hemolymph concentrations stay low throughout adult ecdysis, NADA is the dominant catecholamine in adult hemolymph; its concentrations increase during pharate adult and decrease to very low levels twenty-four hours after adult ecdysis (65). Not surprisingly, NADA was the major catecholamine found in adult cuticle. The exact amount and role of each catecholamine in different regions of insect cuticle may bear some variation and therefore worth case-by-case studying to figure out.

2.4 Catecholamine/DHPAA and their synthesizing enzymes in insect tanning and sclerotization

2.4.1 NADA-synthesizing enzyme

Arylalkylamine N-acetyltransferase (AANAT) catalyzes the transacetylation of acetyl coenzyme A to dopamine to generate NADA. AANAT also works on L-DOPA (Fig. 10). De-
pending on the activities of tyrosine decarboxylase and L-DOPA decarboxylase (DDC), N-
acetylttyramine may be the major compound in some insect species. Using identified AANAT
sequence from *Drosophila melanogaster*, thirteen putative AANAT sequences were found in
*Aedes aegypti* with eight of them successfully expressed and purified (there are more puta-
tive AANAT sequences in mosquito genomes than fly genomes) (66,67); following activity
screening identified two could work on dopamine in addition to several other arylalkylamines
and one could work on histamine and hydrazine (66,67). These thirteen putative AANAT
sequences could be categorized into three clusters: the typical insect AANAT, the polyamine
NAT-like AANAT, and the mosquito unique putative AANAT (67). The typical AANATs
may play diverse roles in insect sclerotization, neurotransmitter catabolism, and/or mel-
atonin biosynthesis as their mRNA developmental stage and tissue distribution profiles are
varied (68-71). Mutagenesis and RNAi interference of typical AANAT both led to the black
phenotype of insects due to accumulated dopamine for melanization, indicating the role
of AANAT in pigmentation (70,72). The exact role of polyamine NAT-like AANAT and
mosquito unique putative AANAT has not been confirmed yet.

Structures from each of the three clusters were recently solved (67); although protein se-
quences share little similarity among the three clusters, their crystal structures superimpose
well with a common fold core of GCN5-related *N*-acetyltransferase superfamily proteins and
a unique structural feature of helix/helices between β3 and β4 strands (67). Comparison
of these AANAT crystal structures revealed that a wider catalytic tunnel might help the
enzyme to adapt larger substrates (67). The surface of the catalytic tunnel of the mosquito
unique putative AANAT is more positively charged than the other two, providing some insight regarding what type of substrate may work on (67). As insect AANATs differ considerably from mammalian AANATs and they play important roles in insect physiology, design of specific insecticide against insect AANATs is promising and achievable.
2.4.2 DHPAA-synthesizing enzyme

The enzyme responsible for DHPAA synthesis was recently characterized in mosquitoes and flies (5). Pyridoxal 5’-phosphate (PLP) is bound to the enzyme as a cofactor. The UV-visible spectrum of the purified enzyme is similar to that of typical DDC, with a peak absorbance at around 330 nm and a shoulder at around 400 nm, corresponding to different tautomers of the internal aldimine between PLP and enzyme (5,73). Compared to DDC, this enzyme catalyzes decarboxylation-deamination of L-DOPA to produce DHPAA (Fig. 5) (5). So the enzyme was named DHPAA synthase for clarification. Due to the fact that the enzyme shares high sequence similarity (~48%) with DDC and the gene locus is next to Ddc (74), DHPAA synthase was considered a DDC-type enzyme. DHPAA synthase was known as α-methyl DOPA-resistant (AMD-r) protein for a long time because mutants at this locus did not affect the DDC activity but the tolerance to α-methyl DOPA (AMD, a chemically synthesized L-DOPA analog) and the varied tolerance to AMD was independent of the DDC activity (75-77). The ability to use AMD as a substrate may explain the observed function of DHPAA synthase in relation to AMD resistance, which was experimentally proved (5). However, the metabolism of AMD by DHPAA synthase is unlikely the physiologically relevant role of this enzyme in insect species. The expression and physiological roles of DHPAA synthase have been extensively studied before its biochemical activity was determined. Mutations of Drosophila melanogaster DHPAA synthase gene exhibited a dual phenotype of recessive lethality and dominant conditional lethality (78). The recessive lethal phenotype was during embryonic hatching, when the dying embryos had abnormal cuticles and necrotic anal organs.
the dominant phenotype is reflected by the lower LD_{50} (76). Moreover, the clear flexible pupal cuticle produced during pupariation would turn fragile and the larvae would die at the larval molts and at pupariation when the activity of the enzyme was inhibited (78). It is speculated that the enzyme, though its reaction was not known at that time, was involved in the biosynthesis of certain catecholamines, as they are important cross-linking reagents in insect sclerotization and cuticle hardening. Particularly, the distribution of this enzyme correlates with the regions of the soft flexible cuticle production (79). DHPAA, the authentic product of this enzyme, is highly unstable and reacts with lysine and its derivative, leading to the formation of DHPAA-Nα-acetyl-lysine complex (5). We have some preliminary data suggesting the evolution of this enzyme in insect species. We are currently working on the crystallization of DHPAA synthases from mosquitoes and flies.

2.4.3 NBAD synthesizing and degrading enzymes

NBAD is produced by NBAD synthase (Ebony protein, as the enzyme is coded by the ebony gene) through transferring β-alanine to dopamine (Fig. 10). NBAD synthase also works on other amines, such as norepinephrine, octopamine, tyramine, serotonin, and histamine, producing other β-alanyl derivatives (80,81). The putative NBAD synthase is 98.5 kDa and has been shown to be stimulated by Mg^{2+} and Mn^{2+} but inhibited by Co^{2+} (80). The enzyme contains three domains: an aminoacyl adenylate-forming domain, a thioester-forming domain, and a dopamine-binding domain. The reaction mechanism is proposed to start with adenosine-5’-triphosphate (ATP)-dependent activation of the adenylating module forming
β-alanyl-adenylate and then a thioester via intramolecular transfer before the thioester activated carboxyl group of β-alanine reacts with dopamine (82). Although a previous study has indicated that there is no participation of coenzyme A as a cofactor (80), it was proposed that coenzyme A was needed in the initial modification of NBAD synthase catalyzed by 4'-phosphopantetheinyl transferase, resulting in an enzyme capable of acyl group activation via thioesterification of the cysteamine thiol of 4'-phosphopantetheine linked to a serine residue (83). NBAD synthase has been reported very unstable and thus very difficult to purify for further structural studies (80). In insect pigmentation, NBAD synthase could suppress the biosynthesis of melanin; loss of NBAD synthase function elevates black pigment (83,84). NBAD synthase is also important in the visual system (81,85), the nervous system (86), and the innate immune system (87,88).

NBAD is synthesized by NBAD synthase and degraded by NBAD hydrolase, which is often called Tan (Fig. 10). The gene coding for the enzyme was identified in a mutant strain that lacks the dark pigmentation of wild type flies, so it was given the name tan. Further biochemical characterization of Tan protein has indicated that the enzyme can hydrolyze NBAD, carcinine (β-alanylhistamine), and β-alaninylnorepinephrine. Tan is synthesized as a 43.7 kD precursor protein and undergoes self-cleavage at its Gly-Cys motif to give two interacting subunits of 29.9 kD and 13.8 kD (89). The enzymatic activity was not stimulated by Mg$^{2+}$ (89). NBAD hydrolase is expressed at epidermis and neural tissues (89). Together with NBAD synthase, NBAD hydrolase regulates the cuticle sclerotization and tanning, in addition to the metabolism of neurotransmitter carcinine (90,91). The absence of NBAD
2.5 The biochemical characterization of aspartate 1-decarboxylase (ADC) and its role in sclerotization and tanning

The PLP containing ADC participates in sclerotization and tanning by supplying β-alanine. PLP is the active form of vitamin B₆. PLP containing enzymes appear early in evolution and catalyze many essential reactions, such as transamination, decarboxylation, racemization, and deamination. Aspartate has complicated decarboxylation reactions. First, aspartate is the only amino acid that can undergo decarboxylation reactions at both its α- and β-carbons. Second, bacterial and insect ADCs use different cofactors. Bacterial ADC uses a covalently bound pyruvoly group as the cofactor, which is produced from enzyme self-cleavage (92,93). Insect ADC, on the other hand, employs PLP as the cofactor and is the only identified enzyme capable of producing β-alanine from aspartate through in α-decarboxylation (94,95).

Although β-alanine in mammals has been investigated extensively as the component of carnosine, in insect species, it is only under studies in sclerotization and cuticle tanning. β-alanine participates in the biosynthesis of NBAD. The concentration of β-alanine also affects the levels of several other important sclerotizing and tanning catecholamines.
The expression profiles of ADC transcript have been investigated in *Drosophila melanogaster* and *Tribolium castaneum* (94,96). In *Tribolium castaneum*, the highest level of ADC transcript was detected in the pharate adult (94). As regarding to its tissue distribution, a study done in adult *Drosophila melanogaster* reported detection in the neuropil of the first optic ganglion and in the motor neuron axons of the thoracic musculature (97). These pieces of information is to understand the regulation of ADC within the individual species. As insects undergo distinct sclerotization, insect ADC is likely to have different development- and tissue-specific distribution.

So far, phenotypes associated with defective ADC have been reported and studied in *Drosophila melanogaster* and *Tribolium castaneum* (94,96), greatly assisting the understanding of the molecular and behavioral characterization of ADC. ADC was originally named Black, as its defects at the gene locus would lead to abnormally dark pigmentation of the adult cuticle (64,98,99). Both black mutants have decreased levels of β-alanine and NBAD (99,100). Supplementation of β-alanine could at least partially rescue the pigmentation phenotype of *Drosophila black* at the larvae stage (101,102). The black mutation in *Drosophila* leads to a frame shift of ADC, producing truncated and null ADC, which corresponded with the absence of ADC and decreased ADC activity in the protein extracts of black larvae at puparium (96).

Compared to studies done in *Drosophila melanogaster*, the molecular and functional analyses of ADC in cuticle tanning in *Tribolium castaneum* were investigated in more detail. During the period between 4-5-day-old pupae and 0-day adults, the black mutant had very low levels
of β-alanine and NBAD but high levels of dopamine compared to a wild type strain (103). Dopamine in excess will go through melanization that results in the black cuticle phenotype; this melanization could be prevented by the supply of β-alanine or NBAD into newly ecdysed black mutants (103). The black phenotype, though not tested in the previous study, could be rescued by supplementation of β-alanine or NBAD at earlier stages in theory. The cuticle of wild type and black beetles eventually had no difference in terms of hardness though it took longer time for black mutants to sclerotize their cuticle (104).

Insect ADC is a PLP-dependent decarboxylase. In animal protein databases, ADC is homologous with and shares about 40% similarity with glutamate decarboxylase (GAD), cysteine sulfinic acid decarboxylase (CSADC), and glutamate decarboxylase-like 1 (GADL1). GAD is the major enzyme in γ-aminobutyric acid (GABA) biosynthesis and CSADC is the major enzyme in taurine biosynthesis (Fig. 11). There has been no study of GADL1 and its substrate usage of glutamate and as its name implies, it has not been experimentally confirmed so far.

Although earlier studies reported that GAD could generate β-alanine from aspartate, they all lacked substantial evidence to support that hypothesis and a more recent study clearly demonstrated that there was no substrate overlap between GAD and ADC using recombinant insect enzymes purified from Escherichia coli (105). A few conserved residues at the active site were targeted for site-directed mutagenesis in hoping to switch the substrate usage of insect ADC (Fig. 12A). Although these mutations affected the spectrum and activity of the enzyme to some extent (Fig. 12B), scientists still have not determined residue(s) that
determines the substrate specificity between GAD and ADC. For a long time, ADC had been considered to solely work on aspartate to produce β-alanine until a recent study reported that it could also use cysteine sulfinic acid and cysteic acid as substrates (Fig. 13) (106). Its kinetic parameters for cysteine sulfinic acid were comparable to those for aspartate (106). In that study, the conserved glutamine residue at position 377 of AeADC was speculated and later confirmed to determine the ability of ADC to work on aspartate (106). It is not known if there are additional factors that can differentiate the substrate usage of ADC and CSADC, but we believe there could be other residues or structural confirmations involved.
Figure 2.12: Sequence comparison and site-directed mutagenesis studies of insect GAD and ADC. A. Partial alignment of AeADC, AeGAD, DrADC, and DrGAD showing conserved active site residues that may determine the substrate selectivity of the enzymes. The amino acid sequences of AeADC (XP 001658435.1), AeGAD (XP 001655801.1), DrADC (NP 476788.1), and DrGAD (NP 523914.2) were aligned using ClustalW program with default parameters. B. The UV-visible spectra of wild type AeADC, AeADC F178S, and AeADC A221S.

Blast search using insect ADC in mammalian species has identified a mammalian protein with unknown activity and function at that time. The protein is named GADL1 and effort has been spent on the protein toward its functional identification. GADL1 has recently been characterized to work on aspartate, cysteine sulfenic acid, and cysteic acid; therefore being an enzyme of ADC and CSADC activity (95). Its transcript and protein have been detected in skeletal muscles in Mus musculus (95). The characterization of GADL1 has suggested its possibility of participating in taurine and/or β-alanine biosynthesis, although more studies are needed for its definitive conclusion.

Earlier studies reported that cysteine would form a thiazolidinecarboxylic acid derivative...
with PLP (Fig. 14) (107,108); hence, cysteine could be an inhibitor of PLP-containing enzymes in theory. A recent study has confirmed the hypothesis and further showed that ADC, GAD, and CSADC could all be inactivated by cysteine (109). The conformation of the active site of these enzymes allowed cysteine to react with the internal aldimine and subsequently form a thiazolidinecarboxylic acid derivative (109). Such cysteine-caused inactivation of PLP-dependent decarboxylases is important to the understanding of the symptoms of neurological diseases as cysteine imbalance is often associated with neurological diseases and these PLP-containing decarboxylases involve in the biosynthesis of neurotransmitters, such as GABA and taurine.
2.6 Future research directions

With the better identification and classification of flexible and rigid cuticular proteins, scientists are working at how they differently participate and are regulated in the insect cuticle hardening process. In addition, scientists are aiming at a chemistry-based explanation to the physiological roles of flexible and rigid cuticle based on the properties of their corresponding component cuticular proteins. As for individual flexible or rigid cuticular proteins, will there be any observable and varied phenotype associated with knockdown of specific cuticular proteins? Due to the uniqueness of insect cuticular proteins, they could offer promising insecticide-design strategies.

The flexible cuticle is present in the majority of insects, providing some extent of elasticity. A flexible cuticle is particularly important for mosquitoes and other blood-sucking insects, whose abdominal cuticle could expand as the insects can take a blood meal that is equivalent to their body weight. Taking blood is an essential action to survive and reproduce.
for mosquitoes and those blood-sucking insects. It is also an important step in vector-borne disease infection. Pathogens would be transmitted from infected individuals to others. The formation of flexible cuticle is as crucial as that of rigid cuticle, although there are fewer studies addressing issues relating to insect flexible cuticle. Our current knowledge of cuticle formation is from studies of cuticle sclerotization and pigmentation that result in the rigid cuticle formation. Recently, there have been more concerted efforts to explore the biochemical processes leading to flexible cuticle formation. There have been data suggesting that the formation of flexible cuticle and rigid cuticle is quite different. We have characterized DHPAA synthase whose mutation has been associated with abnormal flexible cuticle formation. Next, it is important to identify the *in vivo* functions of DHPAA synthase and its relationship with enzymes involved in rigid cuticle formation. It is important to investigate whether there exists specific regulatory mechanism to regulate and control the sclerotization process in flexible cuticle and rigid cuticle. DHPAA synthase behaves as both a lyase and an oxidase and the product by the enzyme is highly toxic. This poses the question as to why such a highly toxic biochemical pathway evolved in mosquitoes. Could there be other functions associated with this seemingly toxic pathway in mosquitoes?

Last but not least, although the reactions, compounds, and enzymes involved in sclerotization and tanning have been studied, there is still huge gap in terms of the regulation mechanism of insect sclerotization and tanning. In mammalian melanogenesis, tyrosine hydroxylase (TH) is regulated by phosphorylation and such regulation controls the velocity of melanogenesis in mammals. There are numerous studies addressing the regulation of TH in
mammalian species. In insects, there is little information regarding what factor(s) and/or pathway may regulate the velocity of sclerotization, the speed of sclerotizing reagent biosynthesis, to be more specific. In mosquitoes, pupal cuticle hardening is rapid and much more easily monitored than in other life cycle stages. Newly formed pupal cuticle undergoes rapid sclerotization, changing from white cuticle to dark cuticle in less than one hour. Therefore, pupal cuticle serves as a good example to study insect sclerotization regulation. Our preliminary studies have suggested phenylalanine hydroxylase and TH are up regulated during the pupal cuticle sclerotization. By comparing the regulatory domain of THs from mammalian and insect species, it is expected that the regulation mechanism should be quite different, as insect THs do not retain the conserved serine residue for phosphorylation as mammalian enzyme.

2.7 Acknowledgement

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2.8 References


Chapter 3

Cysteine sulfinic acid decarboxylase activity of Aedes aegypti aspartate 1-decarboxylase: the structural basis of its substrate selectivity


Author contribution:
Pingyang Liu, design and perform the experiment; write and correct the manuscript; Patrick M. Torrens-Spence, help perform activity assay; Haizhen Ding, design the primer and help express the protein; Bruce M. Christensen, correct the manuscript; Jianyong Li, design the experiment; write and correct the manuscript
Abstract

Insect aspartate 1-decarboxylase (ADC) catalyzes the decarboxylation of aspartate to β-alanine. Insect ADC proteins share high sequence identity to mammalian cysteine sulfinic acid decarboxylase (CSADC), but there have been no reports indicating any CSADC activity in insect ADC or any ADC activity in mammalian CSADC. Substrate screening of Aedes aegypti ADC (AeADC), however, demonstrates that other than its activity to aspartate, the mosquito enzyme catalyzes the decarboxylation of cysteine sulfinic acid and cysteic acid as efficiently as those of mammalian CSADC under the same testing conditions. Further analysis of Drosophila melanogaster ADC also demonstrated its CSADC activity, suggesting that all insect ADC likely has CSADC activity. This represents the first identification of CSADC activity of insect ADC. On the other hand, HuCSADC displayed no detectable activity to aspartate. Homology modeling of AeADC and substrate docking suggest that residue Gln 377, localized at the active site of AeADC, could better interact with aspartate through hydrogen bonding, which may play a role in aspartate selectivity. A leucine residue in mammalian CSADC occupies the same position. A mutation at position 377 from glutamine to leucine in AeADC diminished its decarboxylation activity to aspartate with no major effect on its CSADC activity. Comparison of insect ADC sequences revealed that Gln 377 is stringently conserved among the available insect ADC sequences. Our data clearly established the CSADC activity of mosquito and Drosophila ADC and revealed the primary role Gln 377 plays in aspartate selectivity in insect ADC.

Keywords
aspartate 1-decarboxylase; insect; hypotaurine; taurine; β-alanine; cysteine sulfinic acid decarboxylase

3.1 Introduction

Aspartate 1-decarboxylase (ADC), also named aspartate-α-decarboxylase, catalyzes the production of β-alanine from aspartate (Fig. 1). There have been primarily two types of ADCs defined so far. The first type of ADC uses pyruvate as a cofactor and is present in bacteria (1,2). Bacterial ADC contains around 100 amino acids and is present as tetramer (1,2). The other type of ADC uses pyridoxal 5-phosphate (PLP) as a cofactor and to date has only been identified from insects (3). Accordingly, insect ADC belongs to the PLP-dependent decarboxylase group. No conserved sequence fragments were identified when insect ADC proteins were compared with bacterial ADC proteins, suggesting that these two types of ADC were evolved independently during evolution.

Insect ADC sequences share considerable sequence identity (~40%) with mammalian glutamate decarboxylase (GAD) sequences. Despite of their high sequence identity, GAD has no detectable activity to aspartate and ADC has no detectable activity to glutamate (4). Owing to their sequence similarity, however, some insect ADC enzymes have been annotated as GAD. For example, the recently verified *Aedes aegypti* ADC (*AeADC*) remains named as GAD in the NCBI Non-redundant protein database. Obviously, it is necessary to conduct more research and to better clarify the biochemical reactions and physiological functions
Critical sequence alignment of the previously verified mosquito ADC with database sequences, however, determined that mosquito ADC actually shares better (4-8% more) sequence identity with mammalian cysteine sulfinic acid decarboxylase (CSADC) (Fig. 2). The high sequence identity between insect ADC and mammalian CSADC questions if insect ADC has activity towards cysteine sulfinic acid (CSADC activity) or mammalian CSADC has activity to aspartate (ADC activity). To clarify these, we expressed and purified AeADC and human CSADC (HuCSADC), and then analyzed and compared their substrate specificity and biochemical properties side by side. This led to the discovery that AeADC is also an efficient CSADC enzyme (Fig. 1), but no ADC activity of the HuCSADC was observed.
Subsequent substrate screening of *Drosophila* ADC proved its CSADC activity as well. Homology modeling, substrate docking, and mutagenesis of *Ae ADC* provide some structural basis to better explain the shared substrate specificity between insect ADC and mammalian CSADC as well as the inability of the mammalian CSADC to use aspartate as a substrate.

Figure 3.2: Sequence alignment of *Ae ADC* and *Hu CSADC*. The amino acid sequences of *Ae ADC* (XP 001658435.1) and *Hu CSADC* (NP 001231634.1) were aligned using ClustalW program with default parameters. Residues in red and blue correspond to their respective conserved and similar resides between *Ae ADC* and *Hu CSADC*. 
3.2 Materials and Methods

3.2.1 Chemicals

All chemicals used in this report were purchased from SigmaAldrich unless specified otherwise.

3.2.2 Expression and purification of enzymes

Enzymes used in the manuscript were AeADC and HuCSADC. The expression and purification procedures for AeADC were as previously described (4). To express HuCSADC (NP 001231634), a forward primer (5’-AAAGAATGCTATGGCTGACTCAGAAGCACTCCCC TC-3’) containing a BsmI (underlined nucleotides) and a reverse primer (5’-AAAGAATTCT CACAGGTCCTGGCCTAGCCGCTC-3’) containing an EcoRI site (underlined nucleotides) were designed and used to amplify HuCSADC from human liver cDNA. The amplified cDNA product was cloned into an Impact-CN expression vector that allows the fusion of the cleavable intein tag to the N-terminus of a target protein (New England Biolabs) and frame of the HuCSADC insert was verified by DNA sequencing. Subsequent bacterial transformation of the recombinant plasmid, expression and purification of the recombinant HuCSADC were the same as previously described (4). The purity of the isolated AeADC and HuCSADC was assessed by SDS-PAGE and the protein concentrations were determined by a Bio-Rad protein assay kit with bovine serum albumin as a standard. The spectra of AeADC and
HuCSADC were recorded using a Hitachi U2800 UVvisible spectrophotometer.

### 3.2.3 Substrate screening

Purified recombinant AeADC and HuCSADC were used for substrate screening. Briefly, a reaction mixture of 200 µl containing 20 µg of purified recombinant protein and 5 mM aspartate, 5 mM cysteine sulfinic acid, or 5 mM cysteic acid was prepared in 150 mM phosphate buffer (pH, 7.0) containing 0.40 µM PLP. The reaction mixtures were incubated for 15 min at 25 °C and the reaction was stopped by adding two volumes of 100% ethanol. The mixture was derivatized by two volumes of o-phthaldialdehyde (OPT) agent as described in a previous method (4). Determination of the products was based on the detection of OPT derivatives by reverse-phase liquid chromatography with electrochemical detection. The mobile phase consisted of 50 mM phosphate buffer (pH, 3.5) containing 25% acetonitrile at a flow rate of 0.5 ml per min. The oxidation potential was set to +750 mV. The activities of AeADC and HuCSADC towards aspartate, cysteine sulfinic acid, or cysteic acid were calculated based on standard curves generated with authentic β-alanine, hypotaurine, or taurine standard at the identical conditions.

### 3.2.4 Computer modeling

Activity screening revealed that AeADC has both ADC and CSADC activities. In contrast, HuCSADC (with CSADC activity as its name implied) has no detectable activity to
aspartate. To elucidate the structural basis of \( \text{AeADC} \) substrate selectivity, its structural model was generated using the atomic coordinates of \( \text{HuCSADC} \) (PDB: 2JIS) as templates. \( \text{AeADC} \) shares 52% sequence identity with \( \text{HuCSADC} \) overall (Fig. 2) and 56% identity between their PLP-binding domains. The \( \text{AeADC} \) structural model was then used to assess the potential structural basis of its substrate binding through molecular docking (5). AutoDockTools and Autodock Vina were used to evaluate the interactions of the substrate and the enzyme. Autodock Vina uses a scoring function to calculate the global minimum chemical potential of the enzyme and its substrate, which determines the conformation preference and the free energy of binding (5). The substrates for docking were prepared using PRODRG (6).

### 3.2.5 Site-directed mutagenesis study of \( \text{AeADC} \)

The position of aspartate, docked in \( \text{AeADC} \) active site, provided some basis to predict that Gln 377 might play a role in aspartate binding through hydrogen bond interactions. The corresponding position in mammalian CSADC was occupied by a hydrophobic leucine residue. To evaluate Gln 377 in aspartate selectively, it was mutated to leucine in \( \text{AeADC} \). A forward primer (5'-\text{AAAGCTCTTCACGTCAATGCTCCACC}-3') and a reverse primer (5'-\text{AAAGCTCTTCACAGCGGAGCGGCGA}-3') with both containing a SapI restriction site (underlined nucleotides) and codon for the mutated residue (italic nucleotides) were designed and paired with the original 3- and 5-end primers, respectively, to amplify two cDNA fragments. Both fragments were digested with SapI (which generated cohesive site
for the two fragments for specific ligation) and then ligated together with the aid of T4 ligase. After amplification of the ligated product with original 5- and 3-end primers, the product was then ligated into an Impact-CN plasmid (New England Biolabs). The frame of the Q377L AeADC mutant was verified by DNA sequencing. The Q377L AeADC mutant was then expressed and purified in the same manners as those described for its native enzyme (4). The AeADC mutant was then screened for substrate preference as those described in substrate screening.

### 3.2.6 Kinetic analysis

The catalytic efficiency of AeADC and AeADC Q377L to cysteine sulfinic acid was determined by incubating the protein in the presence of varying concentrations of cysteine sulfinic acid at 25 °C. LineweaverBurk double reciprocal plots (1/V vs 1/(S)) were used to determine their MichaelisMenten constant \( K_m \), the maximal velocity \( V_{max} \), turnover number \( k_{cat} \), and catalytic efficiency \( k_{cat}/K_m \).

### 3.3 Results

#### 3.3.1 UV-visible spectra absorbance

Both ADC and CSADC are PLP-dependent decarboxylases and their proteins display a visible yellow color. Analysis of the purified AeADC and HuCSADC showed that their
spectral characteristics were quite similar except for minor differences in the ratio of UV-visible peak ratio (Fig. 3). It has generally been considered that the UV peak and the visible peak correspond to the enolimine form and zwitterion form of the internal aldimine, respectively (7). In PLP containing decarboxylases, PLP is invariably associated with the enzymes through a conserved active-site lysine residue; consequently, the ionization status of the internal aldimine is impacted by residues that are in close proximity and by residues that interact with the cofactor. The similarity of their spectral characteristics between HuCSADC and AeADC suggested that their PLP cofactor faces similar active site environments.

Figure 3.3: UV-visible spectra of the purified AeADC and HuCSADC. UV-visible spectra of the purified AeADC (left) and HuCSADC (right). Extensively purified AeADC and HuCSADC in 25 mM phosphate buffer (pH, 7.0) containing 120 mM NaCl were diluted with 100 mM phosphate buffer (pH, 7.5) and their UV-visible spectrum from 300 nm to 480 nm were determined using a Hitachi U-2800A spectrophotometer.

3.3.2 Substrate specificity

When AeADC or HuCSADC was mixed with cysteine sulfinic acid, the accumulation of hypotaurine was observed in both AeADC and cysteine sulfinic acid (Fig. 4A) and HuCSADC
and cysteine sulfinic acid reaction mixtures (Fig. 4B), respectively. The specific activities towards cysteine sulfinic acid for \textit{AeADC} and \textit{HuCSADC} were calculated to be $6.7 \pm 0.8 \, \mu\text{mol min}^{-1} \, \text{mg}^{-1}$ and $4.1 \pm 0.5 \, \mu\text{mol min}^{-1} \, \text{mg}^{-1}$, respectively in the presence of 5 mM cysteine sulfinic acid; accordingly, \textit{AeADC} has a greater specificity activity than \textit{HuCSADC} to cysteine sulfinic acid under comparable conditions. When the same amounts of human or mosquito enzyme were mixed with cysteic acid, the accumulation of taurine was observed in both reaction mixtures (Fig. 4C and 4D). The amount of taurine produced in the reaction mixture containing the mosquito enzyme was much greater than that in reaction mixture containing the human enzyme. These data indicate that \textit{AeADC} has the typical CSADC activity.

When the same amounts of either \textit{AeADC} or \textit{HuCSADC} were mixed with aspartate, the accumulation of $\beta$-alanine was observed in the \textit{AeADC} and aspartate reaction mixture (Fig. 4E), but essentially no $\beta$-alanine was produced in the \textit{HuCSADC} and aspartate reaction mixture (Fig. 4F). The chromatographic and detection conditions were identical for all three different assays and figure 3G illustrates the retention times of authentic hypotaurine, taurine and $\beta$-alanine standards under the same conditions of reverse-phase chromatography and detection. It is clear that \textit{HuCSADC}, as its name applied, can use cysteine sulfinic acid and cysteic acid as substrates, but it has no activity to aspartate.
3.3.3 **AeADC homology model**

The primary sequences of *AeADC* and *HuCSADC* share 52% identity and 74% similarity (Fig. 2). To understand the similarity and difference in substrate selectivity, a homology
model of AeADC was generated using HuCSADC structure as a template. The overall Anolea profile indicated that the quality of the model was sound, including loop regions (data not shown). Figure 5A illustrates the overall structure of the AeADC model superimposed with the HuCSADC structure with an root-mean-square deviation (RMSD) of 1.6 Å and Figure 5B shows the close-up view of their PLP-binding site, where residues Gly 218, Ser 219, Asp 339, Asn 368, Lys 371 from chain A and Gly 422 from chain B in AeADC are in close proximity of PLP and likely interact directly with PLP. These residues are conserved and similarly oriented in both enzymes (Fig. 5B).

3.3.4 Evaluation of substrate interacting residues through molecular docking

Aspartate and cysteine sulfinic acid were used as substrates to perform the docking experiment. The optimum docking position (predicted by Autodock Vina) was based on chemical potential, both the bound conformation preference and the free energy of binding (i.e., the ligand in the position has the lowest energy) (5). The orientation of the same substrate was somewhat different at the active site between the two enzymes, but residues that likely interacted with the substrate were mostly conserved (Fig. 5C-5F). Distances between the \( \alpha\)-N atom of the substrates and the Schiff base carbon of the lysine-PLP were also similar in both models irrespective to which substrate docked at the active site. Results showed that aspartate fits better at the active site of AeADC than that of HuCSADC with more hydro-
Figure 3.5: Computational analyses of AeADC and HuCSADC. A: A model of the overall structure of AeADC is shown as red and pink ribbon superimposed on the dimeric structure of the HuCSADC template (blue and cornflower blue ribbon). The PLP-binding sites are indicated with orange arrows. B: A model showing the PLP-binding site of AeADC (red and pink ribbons) superimposed on the structure of HuCSADC (blue and cornflower blue ribbons). Residues interacting with PLP are labeled. The loops from the other monomer of AeADC and HuCSADC are shown in pink and cornflower blue ribbon, respectively. C: Active site of modeled AeADC with aspartate. D: Active site of HuCSADC with aspartate. E: Active site of modeled AeADC with cysteine sulfinic acid. F: Active site of HuCSADC with cysteine sulfinic acid. Hydrogen bond interactions are marked with green lines.
gen bonds (Fig. 5C and 5D), which is likely due to the presence of an active site glutamine residue (Gln 377) in AeADC because Gln 377 can form two hydrogen bonds with aspartate (Fig. 5C). In contrast, the corresponding position in HuCSADC is occupied by a leucine residue (Leu 311) that cannot form hydrogen bond with aspartate (Fig. 5D). Although active site histidine residue (His 304) in HuCSADC could form a hydrogen bond with the α-carboxyl group of aspartate (so could His 370 in AeADC), overall aspartate seems to be better stabilized by the two additional hydrogen bonds contributed by Gln 377 (Fig. 5C). Similarly, Gln 377 in AeADC also forms hydrogen bond with cysteine sulfinic acid (Fig. 5E), but Leu 311 in HuCSADC does not (Fig. 5F).

3.3.5 Substrate specificity and spectral characteristics of AeADC Q377L mutant

Substrate docking provided some basis to predict that Gln 377 likely plays a role in aspartate selectivity. When Gln 377 in AeADC was mutated to Leu 377 and its mutant protein was tested for ADC and CSADC activities, it was found that the ADC activity of the AeADC Q377L mutant was diminished to less than 20% of the wild-type enzyme activity, but its CSADC activity was not significantly affected, maintaining about 90% of the wild-type CSADC activity (Fig. 6A-6D). In addition, there was noticeable difference in the UV-visible spectrum of AeADC Q377L mutant (Fig. 7). Specifically, the relative ratio of its UV peak (with a λ at 342 nm) to its visible peak (with a λ at 425 nm) was smaller than that of the
wild-type enzyme (Fig. 7). Based on $\lambda_{max}$ of the different internal aldimine tautomers, the Q377L mutation increased its zwitterion form (see scheme 1).

Figure 3.6: The UV-visible spectrum of AeADC Q377L. Purified AeADC Q377L in 25 mM phosphate buffer (pH, 7.0) containing 120 mM NaCl was diluted with 100 mM phosphate buffer (pH, 7.5) and its UV-visible spectrum from 300 nm to 480 nm was recorded using a Hitachi U-2800A spectrophotometer.

3.3.6 Kinetic properties of AeADC and AeADC Q377L to aspartate and cysteine sulfenic acid

To assess the effect of Gln 377 in AeADC substrate selectivity and catalysis, the kinetic properties of the mutant was critically compared with those of the wild-type enzyme (Table 1). Although the AeADC Q377L mutant still was able to use aspartate as a substrate, its affinity to the substrate was greatly decreased. In contrast, the affinity and catalytic efficiency of the mutant enzyme to cysteine sulfenic acid were essentially the same as those
Table 3.1: Kinetic parameters of \( AeADC \) and \( AeADC\) Q377L

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>( K_m ) (mM)</th>
<th>( V_{max} ) (( \mu )mol min(^{-1}) mg(^{-1}))</th>
<th>( k_{cat} ) (s(^{-1}))</th>
<th>( k_{cat}/K_m ) (mM(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( AeADC )</td>
<td>Aspartate</td>
<td>2.04±0.28</td>
<td>8.47±0.81</td>
<td>7.03</td>
<td>3.44</td>
</tr>
<tr>
<td>wild type</td>
<td>Cysteine sulfonic acid</td>
<td>1.16±0.1</td>
<td>7.03±0.6</td>
<td>5.86</td>
<td>5.05</td>
</tr>
<tr>
<td>( AeADC )</td>
<td>Aspartate</td>
<td>3.18±0.12</td>
<td>2.54±0.2</td>
<td>2.11</td>
<td>0.66</td>
</tr>
<tr>
<td>Q377L</td>
<td>Cysteine sulfonic acid</td>
<td>1.16±0.15</td>
<td>6.63±0.9</td>
<td>5.52</td>
<td>4.84</td>
</tr>
</tbody>
</table>

3.3.7 CSADC activity of \( Drosophila melanogaster \) ADC (\( DrADC \))

and conservation of Gln 377 in available insect ADC sequences

Substrate screening of recombinant \( DrADC \) established that, in addition to its ADC activity, the \( Drosophila \) enzyme clearly has CSADC activity as well as ADC activity (Fig. 8), suggesting that most insect ADC enzymes (if not all) likely have CSADC activity. Sequence
Comparison determined the presence of glutamine residue (equivalent to AeADC Gln 377) in DrADC. Blast search using the AeADC sequence against insect protein databases further confirmed that the Gln 377 equivalent was highly conserved in ADC sequences from individual insect species whose protein databases are currently available (Fig. 9). The presence of the conserved glutamine in all available insect ADC sequences indicates that this conserved residue may serve as one of the criteria towards ADC identification.
Figure 3.8: CSADC activity and ADC activity of DrADC. (A) CSADC activity and (B) ADC activity of DrADC. The expression, purification, and activity assay for DrADC were identical as those for AeADC. To express DrADC (NP 476788), a forward primer (5'-AAACATATGACGAGCACCAAGGATGATCTATC-3) containing a NdeI (underlined nucleotides) and a reverse primer (5'-AAAGAATTCAACAAGTTTTGGGCAGCGATTC-3) containing an EcoRI site (underlined nucleotides) were designed and used to amplify DrADC from Drosophila melanogaster total cDNA.

3.4 Discussion

Even though taurine is a major free amino acid in insect hemolymph (8), its biosynthesis has not been clearly described and discussed, which limits our understanding of the physiological roles of taurine in insects. In mammalian species, CSADC is the major enzyme responsible
Figure 3.9: Multiple sequence alignment of ADC illustrating the conserved Q residue from *Aedes aegypti* (XP 001658435.1), *Acyrthosiphon pisum* (XP 001950847.1), *Culex quinquefasciatus* (XP 001851393.1), *Anopheles darlingi* (EFR27272.1), *Tribolium castaneum* (ABX89951.1), *Pediculus humanus corporis* (XP 002424881.1), *Daphnia pulex* (EFX86096.1), *Acromyrmex echinatior* (EG166289.1), *Solenopsis invicta* (EFZ12137.1), *Camponotus floridanus* (EFN75005.1), *Harpegnathos saltator* (EFN89498.1), *Nasonia vitripennis* (XP 001606153.1), and *Apis mellifera* (XP 395238.2). ClustalW program was used for the alignment with default parameters. The red box indicated the conserved Q residue among the sequences. Residues in blue, dark blue, green, and black correspond to their respective conserved, highly similar, similar, and dissimilar residues.

for hypotaurine and taurine synthesis (9-13), but no CSADC has been identified in insects. In this study, we revealed that *AeADC* resembles mammalian CSADC. Moreover, *AeADC* has higher specific activity to cysteine sulfenic acid than that of human enzyme under comparable assay conditions. The affinity ($K_m$) of *AeADC* to cysteine sulfenic acid is actually higher than its affinity to aspartate. This is the first time that any enzyme with an efficient CSADC activity has been experimentally verified from species other than vertebrates. Although cysteine dioxygenase that generates cysteine sulfenic acid from cysteine is present in bacteria (14), no CSADC has been identified in any bacterial species. Analysis of substrate interacting residues in *AeADC* through structural modeling and substrate docking provided some sensible basis to predict active site Gln 377 in *AeADC* aspartate selectivity and the
significant decrease in affinity of AeADC Q377L mutant towards aspartate provided tangible evidence to conclude a primary role of Gln 377 in aspartate binding of the enzyme. Verification of CSADC activity for Drosophila ADC affords the extension of CSADC activity to other insect ADC. Indeed, insect ADC could have been named CSADC if cysteine sulfinic acid was the tested substrate in earlier insect ADC activity assays. Accordingly, it is appropriate to term the insect enzyme as ADC/CSADC.

AeADC and other available insect ADC sequences share 46-50% sequence identity with mammalian CSADC. Therefore, it is not completely surprising for insect ADC to have CSADC activity, but the ability and inability to use aspartate by insect ADC and mammalian CSADC, respectively, are intriguing. Structural modeling and substrate docking suggest that Gln 377 may contribute to aspartate binding in AeADC. However, the AeADC model was based on native structure of HuCSADC and in reality there are likely some conformational changes of the active site upon substrate binding. During substrate docking, the active site conformation was treated as invariable, so AeADC substrate interacting residues based on substrate docking have some apparent limitations and need to be interpreted conservatively (For example, aspartate may have difficulty to gain the initial entry of the enzyme, but could be docked within its active site. This explains our conservation in elaborating the substrate interacting residues in the results section). Nonetheless, the active site hydrophilic Gln 377 in AeADC apparently contrasts to the hydrophobic Leu 311 at similar position. Gln 377 in AeADC could form two hydrogen bonds with the α-carboxyl group of aspartate, whereas no such interaction between Leu 311 and aspartate could be predicted in HuCSADC.
Some better interactions of Q377 with aspartate through hydrogen bonding and inability to establish the interactions between Leu 311 and aspartate in HuCSADC may explain in part why the mosquito enzyme can use aspartate as a substrate while the human enzyme cannot. The substrate specificity of AeADC Q377L mutant better resembles its HuCSADC counterpart but it still has some ADC activity, which somewhat contrasts to the human enzyme that has no detectable activity to aspartate under similar conditions. This suggests that some subtle differences play some minor parts in aspartate binding/catalysis, but it is difficult to decipher through computer modeling and substrate docking in AeADC. More detailed crystal structure data of both wild type AeADC and AeADC Q377L mutant (including the structures of their complex with substrate analogs) might better explain their substrate preferences.

Another finding was that AeADC Q377L has a relatively higher absorbance peak at 425 nm compared to wild-type AeADC. In PLP containing proteins, the 342 nm peak and 425 nm peak were proposed to be the enolimine form and zwitterion form of the internal aldimine (please see Scheme 1), respectively and a more hydrophobic environment favored the presence of the enolimine form (7). The mutation of Gln to Leu was predicted to increase the hydrophobicity of the active site environment; such mutation should favor the formation of the enolimine form (corresponding to 342 nm peak), but in reality the zwitterion form (425 nm peak) was increased. Based on AeADC structural model, Gln 377 is beyond the direct interacting distance of any functional group of PLP. One may argue that Leu 311 in AeADC mutant, not in close proximity to PLP, has no apparent effect on increasing 342
nm peak, but it should have no major effect the 425 nm peak as well. Detailed active site structures between wild-type AeADC and AeADC Q377L may help to answer this question.

Studies have indicated hypotaurine as an antioxidant in vivo and a neurotransmitter influencing nociceptive transmission and development (15-17); hypotaurine is most well known as the intermediate in taurine biosynthesis (9-11). Taurine is a sulfur-containing β-type amino acid that is abundant in many tissues of animals, especially in muscles and brain (18). Taurine plays important roles in many biological processes, such as immunity, development of the nervous system, calcium regulation, antioxidation, membrane stabilization, osmoregulation, and cardiovascular function (19). Amino acid analysis revealed that taurine was a major amino acid found in insect hemolymph (8,20). Studies in cockroaches showed that taurine and β-alanine could open a chloride channel to cause central synaptic block and transient paralysis (21). However, there have been no reports that discuss the biosynthesis of taurine in insects. The ability of the mosquito ADC to catalyze the decarboxylation of cysteine sulfinic acid and cystic acid to hypotaurine and taurine, respectively and the ability of Drosophila ADC in catalyzing the same reaction provide some basis to suggest that insect ADC likely is involved in taurine biosynthesis.

3.5 Conclusion

In summary, we demonstrate that AeADC has both ADC and CSADC activity. Based on homology modeling and substrate docking, we propose that Gln 377 at the active site
of AeADC is important for its aspartate binding and a 3-fold decrease in affinity of the AeADC Q377L mutant to aspartate supports a key role Gln 377 plays in aspartate selectivity. Although the detailed mechanisms in aspartate binding and catalysis remain to be thoroughly elucidated, our data provide a useful reference towards differentiating ADC from typical CSADC. This work also provides a starting point for achieving a more comprehensive understanding of the biochemistry and physiology of insect ADC enzymes, particularly their potential role in taurine synthesis.

3.6 Acknowledgements

This work was supported by NIH grant AI 19769.

3.7 References

60, 1355-1363


Chapter 4

Mechanism of cysteine-dependent inactivation of aspartate/glutamate/cysteine sulfinic acid $\alpha$-decarboxylases


Author contribution:

Pingyang Liu, design and perform the experiment; write and correct the manuscript; Patrick M. Torrens-Spence, help perform activity assay; Haizhen Ding, design the primer and help express the protein; Bruce M. Christensen, correct the manuscript; Jianyong Li, design the experiment; write and correct the manuscript
Abstract Animal aspartate decarboxylase (ADC), glutamate decarboxylase (GAD) and cysteine sulfinic acid decarboxylase (CSADC) catalyze the decarboxylation of aspartate, glutamate and cysteine sulfinic acid to β-alanine, γ-aminobutyric acid (GABA) and hypotaurine, respectively. Each enzymatic product has been implicated in different physiological functions. These decarboxylases use pyridoxal 5'-phosphate (PLP) as cofactor and share high sequence homology. Analysis of ADC activity to aspartate in the presence of different amino acids determined that product formation was diminished in the presence of cysteine. Comparative analysis established that cysteine also inhibits GAD and CSADC in a concentration-dependent manner. Spectral comparisons of free PLP and cysteine, together with ADC and cysteine, result in comparable spectral shifts. Such spectral shifts indicate that cysteine is able to enter the active site of the enzyme, interact with the PLP-lysine internal aldimine, form a cysteine-PLP aldimine, and undergo intramolecular nucleophilic cyclization through its sulfhydryl group, leading to irreversible ADC inactivation. Cysteine is building block for protein synthesis and a precursor of cysteine sulfinic acid that is the substrate of CSADC and therefore is present in many cells, but the presence of cysteine (at comparable concentrations to their natural substrates) apparently could severely inhibit ADC, CSADC, and GAD activity. This raises an essential question as to how animal species prevent these enzymes from cysteine-mediated inactivation. Disorders of cysteine metabolism have been implicated in several neurodegenerative diseases. Results of our study should promote research in terms of mechanism by which animals maintain their cysteine homeostasis and possible relationship of cysteine-mediated GAD and CSADC inhibition in neurodegenerative disease development.
Keywords
cysteine; inactivation; PLP-containing decarboxylase; hypotaurine; β-alanine; γ-aminobutyric acid

4.1 Introduction

Animal glutamate decarboxylase (GAD), aspartate decarboxylase (ADC, also called aspartate α-decarboxylase or aspartate 1-decarboxylase), and cysteine sulfinic acid decarboxylase (CSADC) catalyze the decarboxylation of α-carboxyl group of glutamate, aspartate, and cysteine sulfinic acid to produce γ-aminobutyric acid (GABA), β-alanine, and hypotaurine, respectively; these amine products play important role in living organisms. For example, GABA is a chief inhibitory neurotransmitter and a regulator of neuronal excitability in animals. Hypotaurine is the direct precursor of taurine, a chemical that plays several physiological functions in mammals (1-3). β-alanine is one of the two precursors for the formation of carnosine (β-alanyl-L-histidine), a peptide that plays an important role as an intracellular buffer and antioxidant (4-6). Carnosine has also been demonstrated to enhance muscular performance (7-9). As a result, β-alanine has been recommended as beneficial dietary supplement (7,8).

Although β-alanine plays several physiological roles in mammals, ADC has not been identified in any animal species except insects. In insects, β-alanine is used to synthesize N-β-alanyldopamine (NBAD) and N-β-alanylhistamine. NBAD is oxidized easily by insect phe-
noloxidase and laccase, and its oxidized form can react with nucleophilic groups on proteins, leading to protein crosslinking. This has been considered one of the primary mechanisms involved in insect cuticle hardening or sclerotization (10-14). \( \beta \)-alanine has also been shown to inactivate the neurotransmitter histamine within the synaptic clefts of insect photoreceptor cells (15,16). Therefore, \( \beta \)-alanine plays unique physiological functions in insects.

Despite playing different physiological roles in animal and insects, ADC, CSADC, and GAD all use PLP as cofactor and share fairly high sequence identity (>40%). Amongst these enzymes, GAD is ubiquitous and therefore is considered the prototype. As a result, the ADC and CSADC sequences in some sequenced genomes often have been named GAD or GAD-like protein without further differentiation. Our previous study determined that insect ADC and GAD have no overlap substrate specificity (17). Insects do not have a specific CSADC and there has been no report regarding the ADC in mammals. In our recent study, however, we demonstrated that insect ADC could use cysteine sulfinic acid and cysteic acid as its substrates (Fig. 1). Accordingly, insect ADC has the typical CSADC activity (18). The function of insect ADC in taurine production may be as important (if not more) as its function in \( \beta \)-alanine production and the enzyme could have been named CSADC if cysteine sulfinic acid were the first tested substrate. Overlap in substrate specificity between insect ADC and mammalian CSADC indicates that it is necessary to critically compare the substrate specificity and biochemical properties of these GAD-like enzymes. It has also been reported that the activity of human GAD is negatively affected by the presence of aspartate (19). Aspartate-induced GAD inhibition suggests that non-substrate amino acids
might interfere with the catalytic efficiency of GAD-like. Therefore, to more practically comprehend the regulation of these enzymes, it is necessary to understand conditions that affect the functions of GAD-like protein family.

In this study, we expressed several GAD-like enzymes, including human CSADC, mosquito GAD, mosquito ADC and Drosophila ADC, and assessed the activities of these enzymes to their natural substrates in the presence of other proteogenic amino acids. We initially observed that in the presence of similar concentrations of aspartate and cysteine sulfinic acid, the $\beta$-alanine production (ADC activity) was diminished and the reaction was driven primarily towards hypotaurine production (CSADC activity). Both aspartate and cysteine sulfinic acid likely are present in many tissues and cells. Subsequent screening of insect ADC with either aspartate or cysteine sulfinic acid in the presence of each of the other

Figure 4.1: Reactions catalyzed by insect ADC. A. Aspartate 1-decarboxylation. B. Cysteine sulfinic acid decarboxylation and cysteic acid decarboxylation.
proteogenic amino acids revealed that cysteine effectively inactivated the activity of insect ADC. Further analysis determined that the activities of mosquito GAD and human CSADC were also inactivated by cysteine in a concentration-dependent manner.

In this communication, we provide data that describe the effect of cysteine on the activities of GAD-like proteins. Specifically, we use insect ADC as a model enzyme to discuss the mechanism of cysteine-dependent inactivation of GAD-like enzymes.

### 4.2 Materials and Methods

#### 4.2.1 Chemicals

All chemicals used in this report were from SigmaAldrich unless specified otherwise.

#### 4.2.2 *AeADC* expression and purification

*AeADC* was expressed in BL21 and purified to homogeneity as those described in a previous study (17). A Bio-Rad protein assay kit was used to determine protein concentration with bovine serum albumin as a standard. *AeADC* was concentrated to 20 μg μl⁻¹ in 20 mM phosphate buffer (pH, 7.0).
4.2.3 Assays of ADC and CSADC activities of AeADC in the presence of different amino acids

Prior to enzyme activity assays, concentrated enzyme preparations were diluted with phosphate buffer containing 1 mg of bovine serum albumin (for enzyme stabilization). Amino acid solutions in this manuscript were adjusted to pH=7.0 before use. The typical reaction mixtures of 50 µl, containing 2 µg of purified AeADC, and 5 mM of aspartate or 5 mM of cysteine sulfinic acid, were prepared in 200 mM phosphate buffer (pH, 7.0) in the absence or presence of 5 mM each of the proteogenic amino acids (the final concentration for tyrosine was 2 mM due to its low solubility). The reaction mixtures were incubated at 25 °C and the reaction was stopped by addition of two volumes of 100% ethanol 10 min after incubation. The mixture was then treated with an equal volume of o-phthaldialdehyde (OPT) reagent as described in a previous method (17). Product formation was based on the detection of β-alanine-OPT derivative by reverse-phase liquid chromatography with electrochemical detection (HPLC-ED). The mobile phase consisted of 50 mM phosphate buffer (pH, 3.5) containing 25% acetonitrile at a flow rate of 0.5 ml per min. The oxidation potential of the working was maintained at +750 mV with an Ag/AgCl reference electrode.

4.2.4 Time and dose dependence of cysteine inhibition on AeADC

ADC activity assays in the presence of different amino acids identified cysteine as an effective inactivator of AeADC activity. To assess the type of ADC inhibition by cysteine, a series
of solutions of 400 µl containing 6.25 mM aspartate and a varying concentration of cysteine were freshly prepared in 100 mM phosphate buffer (pH, 7.0) and mixed with 100 µl of AeADC solution to determine the effect of cysteine concentration on ADC activity. The final concentration of reaction contained 5 mM of aspartate and a series of cysteine concentrations (0.05 mM, 0.1 mM, 0.2 mM, 1 mM, 2 mM, and 5 mM). The enzyme preparation contained 0.2 mg of AeADC and 1 mg of bovine serum albumin (for enzyme stabilization) per ml and was prepared in the same buffer. The reaction was initiated by mixing 100 µl enzyme preparation into 400 µl aspartate and cysteine solution. At 2-, 4-, 6-, and 8-min intervals, 50 µl of the reaction mixture was withdrawn and mixed with 100 µl of 100% ethanol to stop reaction, followed by the described product derivatization and quantification procedures.

4.2.5 Spectral changes of AeADC or PLP in the presence of cysteine

Initial data indicated that inhibition of AeADC by cysteine seemed to be due to its interaction with PLP cofactor, which likely led to the noticeable spectral changes of the enzyme. To evaluate the possible interaction of aspartate, cysteine sulfinic acid, or cysteine and AeADC, 10 µl of 5 mM aspartate, cysteine sulfinic acid or cysteine was first mixed with 80 µl of 200 mM phosphate buffer (pH, 7.0), followed by rapid addition of 10 µl of 20 µg/µl of AeADC and spectral analysis. The spectral changes of the AeADC and amino acid mixtures from 300 nm to 480 nm were monitored at each 45 s interval for a 10-min period using an Ali-
gent 8453 UV-visible spectrophotometer. Data were processed using the Agilent UV-visible ChemStation software. To determine the possible interaction of free PLP with aspartate, cysteine sulfinic acid or cysteine, free PLP was prepared in phosphate buffer and mixed with each of the compounds (with final concentration of PLP and cysteine at 0.2 mM and 0.5 mM) and the possible spectral changes of the PLP and cysteine mixture was monitored from 300 nm to 480 nm as those described for AeADC and amino acid mixtures.

4.2.6 Effect of cysteine on Anopheles gambiae GAD (AeGAD), HuCSADC, and AeADC Q377L activities

Inhibition of AeADC by cysteine was verified. This questions if cysteine has similar effect on other GAD-like proteins. To assess the general effect of cysteine on GAD-like enzymes, AnGAD, HuCSADC, and AeADC Q377L (a mutation showing different substrate selectivity) (18) were expressed as AeADC and the effect of cysteine on their activity was analyzed similarly as those described for AeADC. Typically, reaction mixtures of 50 µl, containing 2 µg of purified AnGAD or HuCASDC and 5 mM of glutamate or 5 mM of cysteine sulfinic acid, were prepared in 200 mM phosphate buffer (pH, 7.0) in the absence or presence of 5 mM of cysteine. The other conditions, including incubation time, product derivatization, and product quantitation, were identical to those described for AeADC activity assays, except for the increase of acetonitrile (50%) in the mobile phase during detection GABA.
4.2.7 Computer modeling

Enzyme activity assays and spectral changes of \textit{AeADC} in the presence of cysteine indicated that cysteine could enter into the active site of \textit{AeADC} and interact with the internal aldimine, leading to the formation of PLP-cysteine complex. To elucidate the structural basis of cysteine inhibition, \textit{AeADC} structural model was generated using the atomic coordinates of \textit{HuCSADC} (PDB: 2JIS) as templates. \textit{AeADC} shares 52\% sequence identity with \textit{HuCSADC} overall and 56\% identity between their PLP-binding domains. The predicted \textit{AeADC} structural model was then used to assess the structural basis of cysteine binding through molecular docking (20). AutoDockTools and Autodock Vina were used to evaluate the interaction of the substrate and the enzyme (20). The structure of cysteine for docking was prepared using PRODRG (21). Cysteine is one of the preferred substrates for animal kynurenine aminotransferases and these enzymes are not inactivated by cysteine. To further understand and clarify the mechanism of cysteine inhibition to GAD-like proteins, the predicted \textit{AeADC} structural model was compared with an \textit{Aedes aegypti} kynurenine aminotransferase structural model with cysteinyl aldimine (PDB: 2R5C) (22). The active site for cysteine is found in one of the biological dimer (22).
4.3 Results

4.3.1 Presence of cysteine sulfinic acid on AeADC activity to aspartate

When AeADC was mixed with aspartate, accumulation of β-alanine was observed in the reaction mixture, but when the same concentration of cysteine sulfinic acid was also incorporated into the reaction mixture, production of β-alanine was diminished and instead a high level of hypotaurine was accumulated (Fig. 2A and 2B). When the concentration of aspartate was increased to 4 fold of cysteine sulfinic acid in the reaction mixture, the ADC activity was still inhibited (Fig. 2C), suggesting that AeADC favors cysteine sulfinic acid as its substrate or cysteine sulfinic acid could much better compete with aspartate for binding/catalysis. The same results were obtained when DmADC was analyzed in the same manners (not shown).

4.3.2 Effects of amino acids on AeADC activities

When AeADC was incubated with cysteine sulfinic acid in the presence of each of 20 proteogenic acids, its CSADC activity was not significantly affected by any of the tested amino acids (Fig. 3A). When AeADC was incubated with aspartate in the presence of each of 19 remaining proteogenic amino acids, its ADC activity was diminished in the presence of cysteine. No other amino acids showed no significant effect on either increase or decrease of
Figure 4.2: The ADC or CSADC activity of *Ae*ADC in the presence of aspartate or cysteine sulfinic acid. A. The ADC activity of *Ae*ADC with 5 mM of aspartate. B. The CSADC activity of *Ae*ADC with 5 mM of cysteine sulfinic acid. C. The ADC activity of *Ae*ADC with 5 mM of aspartate in the presence of 5 mM of cysteine sulfinic acid. D. The ADC activity of *Ae*ADC with 20 mM of aspartate in the presence of 5 mM of cysteine sulfinic acid.

its ADC activity (Fig. 3B). The selective cysteine-dependent inhibition of the ADC activity but not the CSADC activity of *Ae*ADC raises an essential question regarding the chemical mechanism of such selective inhibition.
Figure 4.3: The effect of amino acids on the activities of AeADC. A. The CSADC activity of AeADC with the addition of different amino acid. B. The ADC activity of AeADC with the addition of different amino acid. Each 50 µl reaction mixture contained 5 mM of substrate (either aspartate or cysteine sulfinic acid), 200 mM phosphate buffer (pH, 7.0), 0.4 µM of PLP, and 5 mM of amino acid addition. The reaction was triggered by adding 2 µg AeADC, incubated at 25 °C for ten minutes, and stopped by adding two volume of 100% ethanol.

4.3.3 Time and concentration dependence of cysteine inhibition

When AeADC was mixed with solutions containing 5 mM of aspartate and a varying concentration of cysteine, the level of ADC activity inhibition was approximately proportional
to the concentrations of cysteine in the reaction mixtures (Fig. 4A), suggesting somewhat a competitive nature of the inhibition. Based on the specific activity calculated during a 10-min incubation period, the presence of 2 mM of cysteine or above resulted in 80% inhibition of ADC activity (Fig. 4A). However, a progressive decrease in the specific activity was observed when reaction mixtures containing AeADC, 5 mM of aspartate and 1 mM of cysteine were stopped at 2, 4, 6 and 8 min after incubation (Fig. 4B). This suggests that the interaction of cysteine with AeADC actually led to progressive and irreversible inactivation of the enzyme. The kinetics of AeADC inactivation by cysteine was calculated to be $K_{obs} = -0.364$ min$^{-1}$. The inactivation efficiency can reach almost 100% beyond 10 min incubation (not shown). Prolonged pre-incubation of cysteine with AeADC will completely kill the ADC activity of the enzyme (Fig. 5).

The time and concentration dependency of cysteine inactivation of the AeADC activity differed from the cysteine inactivation of CSADC activity. When AeADC was first incubated in 5 mM of cysteine for 5 or 10 min and then mixed with cysteine sulfinic acid, the CSADC activity of the AeADC was greatly decreased compared to a cysteine-free control reaction. The level of cysteine-AeADC inactivation increased proportionally to cysteine pre-incubation time (Fig. 6). Under the applied conditions, the specific activity was 2.2 $\mu$mol min$^{-1}$ mg$^{-1}$ for the 10-min cysteine-pre-incubated ADC as compared to 5.5 $\mu$mol min$^{-1}$ mg$^{-1}$ for the enzyme without cysteine pre-incubation. These results indicate that cysteine sulfinic acid competes effectively with cysteine to bind to AeADC, which prevents or greatly slows down the interaction of cysteine with the enzyme so that the rate of product formation was not
Figure 4.4: The dose and time dependence of cysteine inhibition on ADC activity. A. Different concentrations of cysteine were tested on its inhibition of ADC activity. B. The velocity of ADC activity was measured at different time point when the reaction mixture contained 5 mM of aspartate and 1 mM of cysteine.

noticeably affected during a 10-min incubation period (see Fig. 3A). In contrast, cysteine competed more effectively than aspartate to react with AeADC when mixed with the enzyme at the same time, leading to a progressive inactivation of the enzyme and diminished product formation (see Fig. 3B).
Figure 4.5: Prolonged pre-incubation of cysteine with AeADC could completely inactivate the ADC activity of the enzyme. 2 µg AeADC and 1 mM of cysteine in 200 mM phosphate buffer pH=7.5 were incubated for 10 min at 25 °C prior to adding 5 mM of aspartate. The total reaction volume was 50 µl. The production of β-alanine was measured at 10 min after adding aspartate at 25 °C.

4.3.4 Interaction of aspartate, cysteine sulfinic acid or cysteine with AeADC

AeADC contains a PLP cofactor that is covalently linked with a conserved lysine residue as an internal aldimine. This internal aldimine produces absorbance peaks with λmax around 335 and 405 nm. The AeADC spectrum remains unchanged after two hours of incubation at 25 °C. This indicates that the AeADC is stable at 25 °C. When either aspartate or cysteine
Figure 4.6: The effect of cysteine pre-incubation on the CSADC activity of AeADC. 10 µl of 25 mM cysteine was added into each 50 µl reaction mixture containing 2 µg AeADC, 200 mM phosphate buffer (pH, 7.0), and 0.4 µM of PLP for 0 min (A), 5 min (B), or 10 min (C) at 25 °C. The reaction was triggered by adding 10 µl of 25 mM of cysteine sulfinic acid and incubated at 25 °C for ten minutes before it was stopped.

sulfinic acid (each at 0.5 mM final concentration) was mixed with AeADC in phosphate buffer at pH=7.0, there were no obvious changes of the spectrum of the enzyme (Fig. 7A and 7B). When 0.5 mM of cysteine was mixed with AeADC solution, progressive increase and decrease of the 335 nm peak and the 405 nm peak, respectively, were observed (Fig. 7C). Increasing the concentration of cysteine in the mixture increased the rate of spectral change (not shown). When aspartate and cysteine (each at 0.5 mM final concentration) was mixed with the enzyme, a similar increase of the 335 nm peak and decrease of the 405 nm
peak were observed (Fig. 7D). However, addition of 0.5 mM of cysteine and cysteine sulfinic acid did not result in significant change in the spectra of the enzyme within a 10 min period (Fig. 7E).

4.3.5 Effects of different amino acids on the UV-visible spectrum of free PLP

Free PLP has a peak absorbance around 390 nm and a small peak (or a shoulder) at 330 nm at pH=7.0. When cysteine was mixed with free PLP, a progressive decrease of the 390 nm peak and increase of the 335 nm peak were observed (Fig. 8A). These spectral changes are similar to those observed in *AeADC* and cysteine mixture except that the decrease was at 405 nm for the enzyme as apposed to the decrease at 390 nm for free PLP (Fig. 7C). In contrast, no apparent spectral changes of the PLP were observed when aspartate or cysteine sulfinic acid was mixed with free PLP (Fig. 8B). These results indicate that cysteine forms complex with PLP under physiological pH conditions.

4.3.6 Reaction of cysteine with the internal aldimine in *AeGAD*, *HuCSADC*, and *AeADC Q377L*

Cysteine is chemically similar to glutamate, aspartate, and cysteine sulfinic acid. It was predicted that cysteine is likely able to enter the active sites of GAD and CSADC, react with the internal aldimine, form cysteine-PLP complex, and inhibit their activities in the
Figure 4.7: The UV-visible spectra of AeADC under different conditions. A. The spectra of AeADC with the addition of aspartate (0.5 mM final concentration). B. The spectra of AeADC with the addition of cysteine sulfinic acid (0.5 mM final concentration). C. The spectra of AeADC with the addition of cysteine (0.5 mM final concentration). D. The spectra of AeADC with the addition of aspartate and cysteine (each 0.5 mM final concentration). E. The spectra of AeADC with the addition of cysteine sulfinic acid and cysteine (each 0.5 mM final concentration). The total reaction volume is 100 µl. 10 µl of 20 µg/µl AeADC was incubated with 0.5 mM of different compounds at 25 °C for ten minutes in 200 mM phosphate buffer (pH, 7.0). The change of spectra was monitored in a period of 10 min and each recording was taken at an interval of 45 s.
Figure 4.8: The UV-visible spectra of free PLP under different conditions. A. The spectra of free PLP with the addition of cysteine. The total reaction volume is 100 µl. 0.2 mM of PLP was incubated with 0.5 mM of cysteine at 25 °C for ten min in 200 mM phosphate buffer (pH, 7.0). Each spectrum was taken at an interval of 45 s. B. The spectra of free PLP under different conditions. The total reaction volume is 100 µl. Red line showed the spectrum of 0.2 mM of PLP in 200 mM phosphate buffer (pH, 7.0) at 10 min. Dark blue line showed the spectrum of 0.2 mM of free PLP and 0.5 mM of aspartate in 200 mM phosphate buffer (pH, 7.0) at 10 min. Light blue line showed the spectrum of 0.2 mM of free PLP and 0.5 mM of cysteine sulfenic acid in 200 mM phosphate buffer (pH, 7.0) at 10 min. Orange line showed the spectrum of 0.2 mM of free PLP and 0.5 mM of cysteine in 200 mM phosphate buffer (pH, 7.0) at 10 min.
same manners. When AeGAD was mixed with glutamate in the presence of cysteine, the inhibition of GAD was clearly observed (Tab. 1) and such inhibition was approximately proportional to the concentration of cysteine in the reaction mixtures (not shown). Both human CSADC and AeADC use cysteine sulfinic acid as substrate, but human CSADC has no activity to aspartate. When human CSADC was mixed with both cysteine sulfinic acid and cysteine at the same time, the activity of the enzyme was decreased, which is different from that observed from AeADC (Tab. 1).

We recently proposed that an active site glutamine residue (Gln 377) in AeADC plays a major role in its ability to use aspartate as substrate because mutation of the active site Gln 377 to leucine (Leu 377) diminished its ADC activity with no apparent effect on its CSADC activity. When the mutant AeADC was mixed with both cysteine and cysteine sulfinic acid at an equal molar concentration, the CSADC activity of the mutant AeADC was considerably reduced (Fig. 9 and Tab. 1).

Table 4.1: The activities of wild type AeADC, AeADC Q377L, AnGAD, and HuCSADC with or without cysteine addition

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Specific activity (µmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>control</td>
</tr>
<tr>
<td>AeADC</td>
<td>Aspartate</td>
<td>7.39±0.6</td>
</tr>
<tr>
<td>wild type</td>
<td>Cysteine sulfinic acid</td>
<td>6.70±0.8</td>
</tr>
<tr>
<td>AeADC</td>
<td>Aspartate</td>
<td>2.14±0.2</td>
</tr>
<tr>
<td>Q377L</td>
<td>Cysteine sulfinic acid</td>
<td>5.82±0.4</td>
</tr>
<tr>
<td>AnGAD</td>
<td>Glutamate</td>
<td>1.07±0.2</td>
</tr>
<tr>
<td>HuCSADC</td>
<td>Cysteine sulfinic acid</td>
<td>4.89±0.5</td>
</tr>
</tbody>
</table>
Figure 4.9: The effect of cysteine on the AeADC Q377L. 10 μl of 25 mM of cysteine was added into each 50 μl reaction mixture containing 2 μg AeADC Q377L, 5 mM of cysteine sulfinic acid, 200 mM phosphate buffer (pH, 7.0), and 0.4 μM of PLP. The reaction was stopped and derivatized after ten minutes incubation at 25 °C. The production of hypotaurine for control group (A) and cysteine-addition group (B) was tested and compared. The electrochemical detection was the same as previously.

4.3.7 Analysis of the interaction between cysteine and PLP-dependent enzymes through molecular docking

To assess the mechanism of cysteine-dependent inactivation against GAD-like proteins, cysteine was used as the ligand to perform the docking experiment in AeADC. The orientation
of cysteine within AeADC active site and its interaction with the active site residues were compared with those of cysteiny1-aldimine in a kynurenine aminotransferase that uses cysteine as a substrate (22,23). The optimum docking position (predicted by Autodock Vina) was based on chemical potential that takes into account both the bound conformation preference and the free energy of binding (i.e., the ligand in the position has the lowest energy) (20). The orientation of cysteine was slightly different at the active site between the two enzymes (Fig. 10A and 10B). Distances between the α-N atom or sulfhydryl group of the substrates and the C4’ atom of the lysine-PLP or PLP were labeled. Results showed that the sulfhydryl group was cycled back, making it within 5 Å to the C4’ atom of the PLP in AeADC (Fig. 10A). Notice that the cysteine does not form a Schiff base with PLP in the predicted model of AeADC, making the distance between the sulfhydryl group and the imine carbon slightly longer than it should have been. In contrast, the cysteine was stretched at the active site of the kynurenine aminotransferase; the sulfhydryl group and carboxyl group of cysteiny1 aldimine were interacting with the surrounding residues (Gln 44, Arg 405, and Asn 193), physically restraining the sulfhydryl group from interacting with the external aldimine (Fig. 10B).

4.4 Discussion

This is the first detailed report concerning the potential effect of cysteine on the regulation of animal ADC, CSADC, and GAD. Our study determined that 1) cysteine can enter into
Figure 4.10: Computational analysis of interactions between cysteine and enzymes. A. The relative position of cysteine and the PLP at the active site of predicted $Ae$ADC structure. B. A model showing the relative position of cysteinyl aldimine and the active-site residues of $Ae$KAT1. The homology model of $Ae$ADC was shown as red and pink ribbons. The structural model was generated with Swiss-model using the atomic coordinates of $Hu$CSADC (PDB: 2JIS) as templates. The crystal structural model of $Ae$KAT1 was shown as blue and cornflower blue ribbons. The distances were indicated with green dash lines.
the active site of the above enzymes and undergo transaldimination (i.e., the replacement of \(\epsilon\)-amino group of internal aldimine with the amino group of an incoming substrate to form external aldimine), resulting in the formation of cysteine-PLP aldimine and 2) the aldimine intermediate then undergoes intramolecular cyclization (nucleophilic addition) through the sulfhydryl group to form a stable covalently linked complex (Fig. 11), leading to the irreversible inactivation of the enzyme. In addition to the above findings, we demonstrate that the cysteine inactivation of GAD-like proteins is dependent on the relative affinities of cysteine and natural substrates of the GAD-like proteins. This is clearly illustrated by the varied efficiency of cysteine-dependent inactivation of ADC, CSADC, and ADC Q377L (due presumably to enhanced inactivator binding). Our results lead to an intriguing question as to how living species prevent the inactivation of GAD-like proteins by cysteine. This manuscript should serve as a useful reference towards comprehensive understanding of the regulation of GAD-like proteins in vivo. This work also highlights a possible research direction regarding the effect of the disorder of cysteine regulation on the physiological functions of GAD-like proteins. The toxicity of excess cysteine has been demonstrated in rabbits and rats (24-26).

Cysteine is indispensible in animals as a building block for protein synthesis and a precursor for the production of glutathione and taurine. This is particularly true for young individuals as growth is always accompanied with active protein synthesis, which requires adequate supplies of cysteine and which may explain in part the relatively high levels of cysteine in young individuals. The plasma cysteine concentration ranges from 80 to 200 \(\mu\)M in healthy
individuals (27) and cysteine tend to decrease along the aging process (28,29). Cysteine is reactive and potentially toxic because oxidation of cysteine in the presence of oxygen may produce reactive oxygen species, but under other conditions it may help scavenge radical species if the reactive compounds are already formed (30,31). Disorders of cysteine regulation often result in disease conditions (32). Relative to the control children, the children with autism had lower plasma cysteine concentration (33). In addition, cysteine, together with its metabolic derivative homocysteine, is defined as exotoxin (34). For example, malfunction of cysteine dioxygenase (the primary cysteine catabolism enzyme) can lead to elevated cysteine levels that have been associated with several major neurodegenerative diseases (34-38). The association of cysteine disorders to neurodegenerative diseases is more related to ageing.
process (39) and the precise role cysteine disorder in neurodegenerative diseases remains to be established.

In addition, the hepatic CDO activity increases while CSADC activity decreases as the intake of sulfur-containing amino acids elevates (40). Cysteine dioxygenase catalyzes the oxidation of cysteine to cysteine sulfinic acid that is then decarboxylated by CSADC in the taurine biosynthesis pathway (41). Cysteine dioxygenase has important medical implications and is generally considered as an important enzyme regulating cysteine (42). Accordingly, it seems reasonable to suggest that a deficiency of the enzyme could potentially lead to the cysteine accumulation that in turn may affect the biosynthesis of GABA and taurine due to the cysteine-dependent inactivation of GAD and CSADC, respectively. The inactivation of decarboxylase by cysteine may explain in part for the tight regulation of cysteine in mammals and other species.

Many decarboxylases contain PLP as a cofactor that forms an internal aldimine with a conserved lysine residue. The internal aldimine is essential to all PLP-dependent decarboxylation (43). Formation of internal aldimine between active site lysine and PLP involves the formation of a carbinolamine intermediate and subsequent dehydration to form imine (internal aldimine). Recent studies determined that a conserved active site cysteine residue (Cys 446 in \textit{HuGAD67}; Cys 455 in \textit{HuGAD66}) that promotes the dehydration of the carbinolamine intermediate is essential for the formation of the internal aldimine in human GAD (43). We initially speculated that free cysteine might form disulfide bond with this active site cysteine, resulting in a progressive inactivation of the enzyme. However, the relative
rapid rate of AeADC inactivation by cysteine excludes such possibility because formation of disulfide bond between free cysteine and residue cysteine requires time-consuming oxidation.

When AeADC was incubated with its substrate or non-substrate amino acids, there was no noticeable change of the spectrum of AeADC. However, the apparent spectral changes of AeADC in the presence of cysteine clearly indicate the interaction of cysteine with enzyme-bond PLP. Amino acids react with PLP and the interaction of cysteine and PLP results in the formation of imine and subsequent intramolecular cyclization of the site chain to form stable complex (Fig. 11) (44,45). The incorporation of cysteine to PLP led to progressive 390 nm peak decrease and 335 nm peak increase that corresponded to the changes observed from AeADC with cysteine, except that the peak for AeADC was at 405 nm instead of 390 nm. Formation of thiazolidinic compound between PLP and cysteine was demonstrated by nuclear magnetic resonance (46). The spectral characteristics of the cysteine-PLP derivative, isolated from cysteine-treated AeADC (Fig. 12), is also consistent with that of thiazolidinic species reported in literature.

It has generally been considered that PLP can react with the amino group of amino acids, leading to imine production, but there has been no detailed discussion about the pathway in vivo. Although the reaction likely proceeds under physiological conditions, the equilibrium unlikely favors the production of their imine complex between free PLP and any given amino acids. Nucleophilic addition to the carbonyl carbon is an acid-promoted reaction. To undergo nucleophilic addition to the carbonyl carbon of PLP, however, the weak nucleophilic amino group needs to be unprotonated. This may explain some increase in imine formation between
Figure 4.12: Spectrum of supernatant from heat-denatured AeADC sample. Purified AeADC was concentrated to 10 mg per ml and treated with 2 mM of cysteine for 30 min. The treated sample was then separated by gel filtration chromatography to separate the enzyme from free cysteine. The collected protein fraction was heat-denatured and the supernatant was collected by centrifugation. The spectral characteristics of the supernatant were then analyzed using Hitachi U-2008 spectrophotometer.

PLP and amino acids at relative basic conditions \textit{in vitro} (although the carbonyl carbon of PLP should be more reactive at relatively acidic condition) (47). Because the amino group of any given amino acids is in general positively charged, imine formation between PLP and amino acids should be a minor pathway \textit{in vivo} (no PLP would have been available for decarboxylases and aminotransferase otherwise). Indeed if aldehydes could react very easily with the amino group of amino acids and other biological amines, it would have been a disaster as numerous potentially toxic imine complexes could have been circulated in the bodies of living species (including humans). This leads to a question why cysteine readily
reacts with PLP.

Although the pKa of amino group is around 9 or above, there should be a very small fraction of any give amino acids with their amino group unprotonated at physiological pH. It is reasonable to predict that, when PLP is mixed with different amino acids, an insignificant level of Schiff base might be formed and the reaction is in dynamic process (association-dissociation) in the solution under physiological pH (this explains studies regarding the Schiff base formed between amino acids and PLP were done in either basic conditions or organic solutions (47). Like any other amino acids, the formation of imine between cysteine and PLP should be a minor pathway, but the subsequent intramolecular nucleophilic addition of the sulphydryl group towards the imine (which apparently proceeds easily) helps to eliminate the imine complex, which likely breaks the equilibrium and drives the reaction towards imine formation. This may explain the rather progressive accumulation of the cyclized cysteine-PLP complex because intramolecular cyclization to form the thiazolidine ring through the sulphydryl group should be fairly rapid if it were not due to the rate-limiting step of imine formation.

Insect ADC shares high sequence identity with mammalian CSADC and both can use cysteine sulfinic acid as a substrate. Aspartate could inactivate GAD by converting the holoenzyme to apoenzyme as a result of the transamination of aspartate (19), but glutamate could not affect insect ADC (Fig. 3). When AeGAD was mixed with an equal molar concentration of cysteine and glutamate, its GAD activity was diminished (Tab. 1). The CSADC activity of insect ADC was not significantly affected by cysteine unless cysteine was pre-incubation
with the enzyme. In contrast, when HuCSADC was added to a mixture of an equal molar concentration of cysteine and cysteine sulfenic acid, the production of hypotaurine was diminished (Tab. 1). These results indicate that cysteine is an inactivator of decarboxylases that use aspartate, glutamate and cysteine sulfenic acid as their substrates. There has been no report discussing cysteine as an inactivator of PLP-dependent decarboxylases in literature although cysteine was reported to inhibit the activity of crayfish GAD (48). Therefore, our data regarding the potential regulation of these enzymes by cysteine fill some knowledge gap in this area.

Our study provides solid evidence indicating that cysteine could inactivate GAD-like proteins through its reaction with the enzyme-bound PLP. PLP serves as the cofactor for many aminotransferases and decarboxylases; ~4 % of all classified enzymatic activities are PLP-dependent (49). Based on the reaction mechanisms, however, one may argue that if cysteine can inactivate GAD-like proteins, why many other PLP-containing enzymes do not seem to be affected by this amino acid. Moreover, some aminotransferases actually use cysteine as one of their preferred substrate (22,23). In reality, a number of factors likely limit the ability of cysteine to form cyclized complex with protein-linked PLP. First, cysteine may not be able to enter the active site of many PLP containing proteins. Second, cysteine may enter into the active site, but its amino group may not be able to be positioned at close proximity to the imine bond of the internal aldimine to initiate transaldimination. Third, even the amino group of cysteine can interact with the internal aldimine and replace the amino group of ε-amino group of active site lysine to form external aldimine, the active
site residues may restrict the flexibility of the sulphydryl group of cysteine from undergoing nucleophilic addition to form complex with PLP. For example, kynurenine aminotransferases can use cysteine as a substrate (22,23), but the interactions of several active site residues with the carboxyl group and sulphydryl group restrict the flexibility of the thiol group; thereby physically obstructing it from participating nucleophilic addition to PLP (Fig. 10).

In addition to those mentioned above, the affinity of GAD-like proteins to their nature substrate also seems to affect the efficiency on their inactivation by cysteine. Cysteine is not quite effective on inactivating the CSADC activity of \textit{Ae}ADC. The binding affinity of \textit{Ae}ADC to cysteine sulfenic acid is higher than that to aspartate; this could partly explain why cysteine sulfenic acid competes more effectively than aspartate to react with the internal aldimine when cysteine is present. We noticed that \textit{Ae}ADC Q377L showed diminished CSADC activity when it was mixed the substrate and cysteine at the same time, suggesting that the conformation and/or hydrophobicity of the active-site environment of \textit{Ae}ADC may also affect cysteine inactivation efficiency (Fig. 10 and Tab. 1). Analysis of cysteine on mutant \textit{Ae}ADC suggests that the active site glutamine in wild type \textit{Ae}ADC may impart negative effect on cysteine-mediated \textit{Ae}ADC inhibition. Glutamine is more hydrophilic than leucine and also can form hydrogen bonds with aspartate and cysteine sulfenic acid (Fig. 13A and 13B). Cysteine is much more hydrophobic than cysteine sulfenic acid and aspartate. The increase in hydrophobicity at position 377 may favor the binding of cysteine in \textit{Ae}ADC mutant. However, the exactly structural and chemical basis remains to be clarified.
4.5 Conclusion

In this study, cysteine was identified as an inactivator of aspartate/glutamate/cysteine sulfinic acid α-decarboxylases. The inactivation was demonstrated to be due to the interaction between cysteine and bound PLP from those enzymes. The possibility of such cysteine-dependent inactivation was dependent on the relative position of cysteine and the
bound PLP at the active site. The efficiency of such inactivation is likely affected by the hydrophobicity of certain active site residues. The discovery of cysteine-dependent inactivation of aspartate/glutamate/cysteine sulfenic acid $\alpha$-decarboxylases questions if pressure from the cysteine inhibition of GAD-like enzymes could have been one of the driving forces for a tight regulation of cysteine \textit{in vivo} and if inactivation of GAD-like proteins due to disorders of cysteine metabolism could eventually lead to neurological symptoms. These apparently deserve further investigations.

### 4.6 Acknowledgements

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1652S-1659S


Chapter 5

Role of Glutamate Decarboxylase-like Protein 1 (GADL1) in Taurine Biosynthesis

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Author contribution:

Pingyang Liu, design and perform the experiment; write and correct the manuscript; Xiaomei Ge, help perform the tissue distribution of mRNA and protein; Haizhen Ding, design the primer and help express the protein; Bruce M. Christensen, correct the manuscript; Honglin Jiang, correct the manuscript; Jianyong Li, design the experiment; write and correct the manuscript
Abstract This manuscript concerns the tissue specific transcription of mouse and cattle glutamate decarboxylase-like protein 1 (GADL1) and the biochemical activities of human GADL1 recombinant protein. Bioinformatic analysis suggested that GADL1 appears late in evolution, only being found in reptiles, birds, and mammals. RT-PCR determined that GADL1 mRNA is transcribed at high levels in mouse and cattle skeletal muscles and also in mouse kidneys. Substrate screening determined that GADL1, unlike its name implies, has no detectable GAD activity, but it is able to efficiently catalyze decarboxylation of aspartate, cysteine sulfinic acid, and cysteic acid to β-alanine, hypotaurine, and taurine, respectively. Western blot analysis verified the presence of GADL1 in mouse muscles, kidneys, C2C12 myoblasts, and C2C12 myotubes. Incubation of the supernatant of fresh muscle or kidney extracts with cysteine sulfinic acid resulted in the detection of hypotaurine or taurine in the reaction mixtures, suggesting the possible involvement of GADL1 in taurine biosynthesis. However, when the tissue samples were incubated with aspartate, no β-alanine production was observed. We proposed several possibilities that might explain the inactivation of ADC activity of GADL1 in tissue protein extracts. Although β-alanine-producing activity was not detected in the supernatant of tissue protein extracts, its potential role in β-alanine synthesis cannot be excluded. There are several inhibitors of the ADC activity of GADL1 identified. The discovery of GADL1 biochemical activities, in conjunction with its expression and activities in muscles and kidneys, provide some tangible insight towards establishing its physiological function(s).

Keywords
β-alanine; hypotaurine; taurine; muscle; decarboxylase; mammal; amino acid; aspartate; GADL1

5.1 Introduction

γ-aminobutyric acid (GABA) is found in every class of living organisms (1). In higher organisms, GABA functions as an inhibitory neurotransmitter; for those without nervous system, such as plants and bacteria, GABA is considered an important signaling molecule (2, 3). GAD catalyzes the synthesis of GABA from glutamate. Even though GAD is a pyridoxal 5-phosphate (PLP)-containing enzyme, bacterial/plant and animal GADs are quite different (4, 5). Animal GAD is present in Parazoa and all later groups of animals with ~40% sequence similarity throughout evolution (Fig. 1). In most animal species, there are two isoforms of GAD, which could be required for tissue-specific or developmental regulation and in turn reflect the important roles of GABA in living species (6-8). Other similar PLP-containing acidic amino acid decarboxylases, such as aspartate 1-decarboxylase (ADC), cysteine sulfinic acid decarboxylase (CSADC), and GADL1, seem to have evolved later than GAD in species evolution (Fig. 1).

Animal ADC is only found in insects (Fig. 1). Sequence analysis indicates that insect ADC shares high sequence homology with mammalian CSADC. Recently, we determined that insect ADC also is able to catalyze the decarboxylation of cysteine sulfinic acid to hypotaurine, the typical CSADC activity, but human CSADC has no activity to aspartate
HuGADL1 equivalent sequences are present in other available mammalian genomes, but there have been no publications addressing its activity or function. Despite its name, HuGADL1 actually shares higher sequence identity to human CSADC (59% identity) than to human GADs (50% and 51% identity). HuGADL1 also shares sequence similarity to insect ADC (51% identity). Therefore, it is possible that HuGADL1 could have activity for aspartate and/or cysteine sulfinic acid. Compared to the three-step reductive uracil degradation processes for β-alanine synthesis in mammals and some other species (10), insects seem to use a much more straightforward enzymatic pathway to produce β-alanine through aspar-
Mammals need β-alanine for the synthesis of a number of β-alanine containing dipeptides (particularly carnosine), leading to the question of why a seemingly direct and simple aspartate to β-alanine pathway has not evolved in mammals. It has been established that skeletal muscles contain high concentration of carnosine whose synthesis occurs primarily in muscles (based on the presence and activity of muscle carnosine synthase) (11,12). β-alanine is indispensible for carnosine synthesis and oral consumption of β-alanine could significantly improve the muscular carnosine concentrations, though a considerable portion of the β-alanine would be digested before reaching to muscles (13,14). This suggests that in situ synthesis of β-alanine would be advantageous for carnosine synthesis. Muscles have high levels of taurine concentration while the muscular taurine is mostly synthesized in livers and transported to muscles via the taurine transporter (15). It has generally been considered that taurine and β-alanine are not synthesized in muscles, but the similarity of GADL1 to mammalian CSADC and insect ADC, together with its high expression levels in muscles, provides a basis to speculate that GADL1 could use cysteine sulfinic acid or/and aspartate as a substrate, therefore likely involving in taurine or/and β-alanine biosynthesis.

In this study, we expressed recombinant human GADL1 and examined its activity to different amino acids, which resulted in the detection of decarboxylation activity of both aspartate and cysteine sulfinic acid. GADL1 does not work on glutamate as its name suggests. Subsequently, we analyzed the transcript and protein levels of GADL1 in mice and cattle, determining that its mRNA and protein were present primarily in skeletal muscles of both species. The transcription and expression of GADL1 in muscles and the ability of its recom-
binant protein to produce β-alanine and hypotaurine through decarboxylation of aspartate and cysteine sulfinic acid, respectively, suggesting that the decarboxylation of aspartate and cysteine sulfinic acid could be a route of β-alanine and hypotaurine synthesis in skeletal muscles. Then, we were able to detect the hypotaurine-producing activities in the supernatant of protein extracts from muscle and kidney tissues. A number of endogenous compounds were shown to inhibit the ADC activity of GADL1.

5.2 Materials and Methods

5.2.1 Chemicals

All chemicals used in this report were from SigmaAldrich unless specified otherwise.

5.2.2 Tissue collection

Mouse tissues were collected from 2 male mice around 8 weeks old. Bovine tissues were collected from 2 Holstein bulls around 5 years old at slaughter. The tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until RNA isolation.
5.2.3 Cell culture

C2C12 myoblasts were cultured in growth medium (DMEM with 10% FBS and 1% ABAM). C2C12 myotubes were cultured in differentiation medium (DMEM with 2% HS and 1% ABAM) for 72 h before experiments. DMEM; FBS; ABAM: antibiotic antmyotic.

5.2.4 Expression and purification of GADL1

To express HuGADL1, a forward primer (5-AAA\textbf{CATATGATTCCAAGTAAGAAGAATGCT-}
3) containing a NdeI site (underlined nucleotides) and a reverse primer (5-\textbf{AAAGAATTCCACA}
TGTCTTTACCCAGTAAGTCTA-3) containing an EcoRI site (underlined nucleotides) were
designed and used to amplify HuGADL1 from human liver cDNA. The amplified HuGADL1
cDNA was cloned into an Impact-CN plasmid (New England Biolabs) for expression of its
recombinant protein. The frame of the HuGADL1 was verified by DNA sequencing. Esch-
erichia coli BL 21 cells with the expression vector were induced at 0.15 mM of isopropyl
\(\beta\)-D-1-thiogalactopyranoside when optical density OD reached 1.0 and grew for 24 h at 15 °C
before breaking the cells in a suggested lysis buffer. The recombinant enzyme was obtained
from Escherichia coli BL 21 cells. The concentrated protein sample was further purified by
ion exchange and gel filtration chromatographies (Mono-Q column and Sepharose 12, GE
Health) with 20 mM phosphate buffer (pH, 7.0). Protein concentrations were determined
by a Bio-Rad protein assay using bovine serum albumin as a standard. The spectrum of
HuGADL1 was recorded using a Hitachi U2800 UVvisible spectrophotometer.
5.2.5 Substrate screening

The purified recombinant *HuGADL1* was used for substrate screening. Each reaction mixture of 200 µl containing 10 µg of purified recombinant protein and 40 µl of 50 mM of a substrate (aspartate, cysteine sulfinic acid, cysteic acid, or glutamate) was prepared in 150 mM phosphate buffer (pH, 7.0) with 0.40 µM of PLP. The reaction mixtures were incubated for 10 min at 25 °C and stopped by adding two volumes of 100% ethanol. The specific activities and standard deviations were calculated based on the averages of triplicates. The mixtures were derivatized by two volumes of o-phthaldialdehyde (OPT) agent as described in a previous method (9). Determination of the products was based on the detection of OPT derivatives by reverse-phase liquid chromatography with electrochemical detection. The mobile phase consisted of 50 mM of phosphate buffer (pH, 3.5) containing 25% acetonitrile at a flow rate of 0.5 ml per min. The oxidation potential of the working electrode was set to +0.75 V. The activities of *HuGADL1* towards the substrates were calculated based on standard curves generated with authentic standards at the identical conditions.

After the substrate specificity of GADL1 was determined, protein extracts from either mouse muscles or mouse kidneys were obtained and assayed for decarboxylation of aspartate and cysteine sulfinic acid. The extracts were obtained from raw tissues by homogenization in 100 mM HEPES buffer (pH, 7.5). 1 mM of MgCl₂, 10 µM of PLP, 2 mM of β-mercaptoethanol, and 20 µM of phenylmethanesulfonylfluoride (protease inhibitor) were added into the homogenization buffer. The components of homogenization was the same to the previous lysis buffer except that the buffer compound is HEPES instead of Tris as the amino group of
tris(hydroxymethyl)aminomethane will interfere with the following OPT assay. The protein extract was directly used for activity assay.

### 5.2.6 Kinetic assays

The catalytic efficiencies of *HuGADL1* towards aspartate and cysteine sulfinic acid were determined by incubating 20 µg of enzyme in the presence of varying concentrations (0.1 mM, 0.5 mM, 1 mM, 2 mM, 5 mM, 10 mM, and 20 mM) of aspartate and cysteine sulfinic acid in 500 µl of 150 mM of phosphate buffer (pH, 7.0) containing 0.40 µM of PLP. Fifty millimolars of aspartate and cysteine sulfinic acid were prepared as stock and adjusted to pH=7.0 using 1 M of phosphate buffer (pH, 7.0). Each reaction was incubated for 10 min at 25 °C before analysis. The kinetic parameters and standard deviations were calculated based on the averages of triplicates. Lineweaver-Burk double reciprocal plots (1/V vs 1/S) were used to determine the Michaelis-Menten constant $K_m$ and the maximum velocity $V_{max}$.

### 5.2.7 RNA isolation

Total RNA was isolated using TRI Reagent according to the manufacturers instruction (MRC, Cincinnati, OH). The extracted RNA was dissolved in diethylpyrocarbonate-treated water. Concentrations of total RNA were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).
5.2.8 RT-PCR

Total RNA (0.1µg) was reverse-transcribed into cDNA in a total volume of 20 µl using the ImProm-II reverse transcriptase (Promega) according to the manufacturers instruction. Ribosomal 18S RNA was used as internal standard. To amplify the target gene, 5 ng of cDNA was mixed with 12.5 µl of 2 x PCR Master Mix (Promega) and 10 pmol of each corresponding primer in a total volume of 25 µl. The conditions for PCR were 32 cycles at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s. The primers used for RT-PCR are shown in Tab. 1.

<table>
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<th>Mouse primers</th>
<th>mGADL1-F</th>
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<td>mGADL1-R</td>
<td>5’-CGGCGGTGCTTTCTTGACAG-3’</td>
</tr>
<tr>
<td>m18s-F</td>
<td>5’-TTAAGAGGGACGGGCCGCGGGG-3’</td>
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</tr>
<tr>
<td>m18s-R</td>
<td>5’-CTCTGGCTCCGTCTTGCGCCCG-3’</td>
<td></td>
</tr>
<tr>
<td>Bovine primers</td>
<td>bGADL1-F</td>
<td>5’-CTGGGGGGCCTCGATCTT-3’</td>
</tr>
<tr>
<td></td>
<td>bGADL1-R</td>
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<tr>
<td>b18s-R</td>
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5.2.9 Western blot analysis

Frozen tissues (~0.5 g) and C2C12 cells were lysed ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH=8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitors (Roche Diagnostics Corporation, Indianapolis, IN) and phosphatase inhibitors (0.5 mM Na$_3$VO$_4$, 5 mM Na pyrophosphate, 50 mM NaF, 10 mM Na-glycerophosphate). The lysates were centrifuged at 12,000 x g
for 15 min at 4 °C. The final supernatant were collected and stored at 80 °C. Protein concentrations of the supernatants were determined with a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). For western blot analyses, 40 µg of isolated total protein or 10 µg purified CSADC protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% nonfat dried milk in Tris-Buffered Saline and Tween 20 (TBST) buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween-20) and incubated with GADL1 antibody (Thermo Scientific, Rockford, IL) at 1:100 dilution in TBST with 5% BSA overnight at 4 °C. This antibody was detected using a horseradish peroxidase-conjugated goat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific, Rockford, IL). Following detection of GADL1 protein, the membrane was stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and re-probed with the β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

5.3 Results

5.3.1 Presence of GADL1 gene in species

Although not specified in the databases, human GADL1 likely is the first one named as GADL1 and the other GADL1 sequences likely have been based on the human sequence.
To date, there have been several GADL1 genes in the databases. The deduced sequences from these genes are highly conserved. For example, GADL1 sequences from human, cattle, and mice share more than 90% sequence identity (Fig. 2). In human, GADL1 also shares considerable similarity with human GAD65 and GAD67, which likely explains why it has been named GAD-like protein. However, GADL1 shares noticeable better similarity (59% identity) with CSADC than with mammalian GAD sequences (50% and 51% identity) (Fig. 3).

5.3.2 UV-visible spectrum of recombinant HuGADL1

The concentrated protein solution showed a visible light yellow color. Spectral analysis of purified HuGADL1 revealed the presence of typical visible absorbance peaks with their $\lambda_{\text{max}}$ at 340 nm and 430 nm, respectively (Fig. 4). These visible absorption peaks were due to the presence of PLP cofactor. The ratio of the 340 nm peak versus 430 nm peak reflected the relative contents of PLP tautomers. Overall, the visible spectrum of HuGADL1 was quite similar to that of HuCSADC (9), except that HuGADL1 had a higher 340 nm/430 nm peak ratio (Fig. 4).

5.3.3 Substrate specificity of HuGADL1

HuGADL1 was screened against all 20 proteogenic amino acids and also cysteine sulfinic acid and cysteic acid for decarboxylation activity. Among them, HuGADL1 showed decarboxyla-
Figure 5.2: Multiple sequence alignment of GADL1 from *Homo sapiens* (NP 997242), *Bos taurus* (NP 001095751), and *Mus musculus* (NP 082914) using ClustalW program with default parameters. Residues in red and blue correspond to their respective conserved and similar residues between *Ae*ADC and *Hu*CSADC. Conserved (*), highly similar (:), and similar residues (.) are shown below the alignment.

Figure 5.2: Multiple sequence alignment of GADL1 from *Homo sapiens* (NP 997242), *Bos taurus* (NP 001095751), and *Mus musculus* (NP 082914) using ClustalW program with default parameters. Residues in red and blue correspond to their respective conserved and similar residues between *Ae*ADC and *Hu*CSADC. Conserved (*), highly similar (:), and similar residues (.) are shown below the alignment.

tion activities to aspartate, cysteine sulfinic acid, and cysteic acid (Fig. 5A-C), but displayed no activity to glutamate (Fig. 5D) and other amino acids. Figure 5A-C demonstrates that *Hu*GADL1 has both ADC activity and CSADC activity. Under the applied assay conditions, the specific activity of *Hu*GADL1 was 1.3 ± 0.2 μmol min⁻¹ mg⁻¹ to aspartate, 2.08 ± 0.3 μmol min⁻¹ mg⁻¹ to cysteine sulfinic acid, and 0.46 μmol min⁻¹ mg⁻¹ to cysteic acid, respec-
Figure 5.3: Sequence alignment of HuGAD65, HuGAD67, HuCSADC, and HuGADL1. HuGAD65 (NP 000809.1), HuGAD67 (NP 000808.2), HuCSADC (NP 057073.4), and HuGADL1 (NP 997242). Conserved (*), strongly similar (:), and weakly similar residues (.) are shown below the alignment.
Extensively purified *Hu*GADL1 was prepared in 20 mM phosphate buffer (pH, 7.0) and its absorbance from 300 nm to 480 nm was determined using a Hitachi U-2800A spectrophotometer. The insert illustrated purified protein and reference molecular weight marker.

Based on its substrate specificity, *Hu*GADL1 could be called a *Hu*CSADC isozyme or named *Hu*ADC. When the enzyme was incubated with aspartate and cysteine sulfinic acid at the same time, its CSADC activity was not affected to any significant degree, but its ADC activity was considerably reduced (Fig. 5E), indicating that cysteine sulfinic acid competes much more effectively than aspartate in *Hu*GADL1 binding. For example, in the presence of 10 mM of cysteine sulfinic acid and 5 mM of aspartate, the calculated CSADC
activity and ADC activity were approximately 1.67 $\mu$mol min$^{-1}$ mg$^{-1}$ and 0.06 $\mu$mol min$^{-1}$ mg$^{-1}$ (Fig. 5E left), respectively. When GADL1 was incubated with cysteine sulfinic acid and aspartate with each at 10 mM final concentration, its CSADC activity and ADC activity were approximately 1.41 $\mu$mol min$^{-1}$ mg$^{-1}$ and 0.12 $\mu$mol min$^{-1}$ mg$^{-1}$ (Fig. 5E middle). When the enzyme was incubated with 5 mM of cysteine sulfinic acid and 10 mM of aspartate, the CSADC activity and ADC activity were approximately 1.29 $\mu$mol min$^{-1}$ mg$^{-1}$ and 0.22 $\mu$mol min$^{-1}$ mg$^{-1}$ (Fig. 5E right). Figure 5F shows the elution profile of hypotaurine, taurine, $\beta$-alanine, and GABA in our assay conditions.

### 5.3.4 Kinetic parameters of HuGADL1 to aspartate and cysteine sulfinic acid

HuGADL1 showed moderate substrate affinity to aspartate and cysteine sulfinic acid. Although the enzyme showed overall better affinity and catalytic efficiency to cysteine sulfinic acid than to aspartate (Tab. 2), the concentration of aspartate is generally much higher than that of cysteine sulfinic acid in many tissues. Therefore, the enzyme could be functional primarily as ADC or CSADC depending on its locations.

#### Table 5.2: Kinetic parameters of HuGADL1

<table>
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<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ ($\mu$mol min$^{-1}$ mg$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
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<td>Cysteine sulfinic acid</td>
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Figure 5.5: Substrate specificities of recombinant \( H_u \)GADL1. Chromatograms (A), (B), and (C) show the accumulation of hypotaurine in a \( H_u \)GADL1 and cysteine sulfinic acid reaction mixture, the accumulation of taurine in a \( H_u \)GADL1 and cysteic acid reaction mixture, and the accumulation of \( \beta \)-alanine in a \( H_u \)GADL1 and aspartate reaction mixture, respectively. Chromatogram (D) shows the absence of GABA in the \( H_u \)GADL1 and glutamate reaction mixture. Chromatogram (E) shows a comparative study of the substrate preference of \( H_u \)GADL1 under different concentrations of substrates. Chromatogram (F) illustrates the retention time of hypotaurine, taurine, \( \beta \)-alanine, and GABA standards under the same assay conditions.
5.3.5 Tissue distribution of GADL1 mRNA

Tissue distributions of GADL1 mRNA of mice and cattle were determined by RT-PCR using ribosomal 18S RNA as an internal standard for cDNA normalization. In mice, GADL1 mRNA expression was detected at high levels only in skeletal muscles and kidneys (Fig. 6A). In cattle, GADL1 mRNA was detected in skeletal muscles and hearts, but transcript abundance was much greater in muscles than in hearts (Fig. 6B). GADL1 mRNA was not found in mouse brains (Fig. 7). The GADL1 mRNA was detected from C2C12 myoblasts but not from C2C12 myotubes.

Figure 5.6: RT-PCR analysis of GADL1 in mice and cattle tissues. The tissue distribution of GADL1 mRNA was studied in mice (A) and cattle (B). Ribosomal 18S RNA was used as internal standard for both species.
5.3.6 Western blot analysis

The presence of GADL1 was investigated in mouse muscles, kidneys, C2C12 myoblasts, and C2C12 myotubes. Antibody against β-actin was used as an internal control. Purified recombinant mouse CSADC was used to test the specificity of the GADL1 antibody. The result showed that GADL1 is present in muscles, kidneys, C2C12 myoblasts, and C2C12 myotubes (Fig. 8A and 8B). These results are comparable with the RT-PCR data, except that GADL1 transcript was not detected from C2C12 myotubes (Fig. 7). It is possible that GADL1 in C2C12 myotubes might be carried over from the myoblast stage of the muscle cells. It was also noted that the GADL1 antibody cross-reacted with mouse CSADC.
(Fig. 8C). Trace amount of CSADC was detected from kidney samples but not from muscle samples (Fig. 8A).

![Western blot analysis of GADL1 in tissues and cell culture.](image)

Figure 5.8: Western blot analysis of GADL1 in tissues and cell culture. (A) The presence of GADL1 in mouse kidneys and muscles. Noticed that a lower band was detected in kidneys; this band corresponded to CSADC. (B) The presence of GADL1 in mouse C2C12 myoblasts and myotubes. (C) Ten micrograms of purified mouse CSADC was used to detect whether the GADL1 antibody had cross-reactivity. Antibody against β-actin was used as a loading control.

5.3.7 Activity assay using protein extracts

The ADC and CSADC activities were investigated with fresh protein extracts from kidneys and muscles, respectively. When the supernatant of freshly prepared muscle protein extracts was mixed with cysteine sulfinic acid and incubated for 10 min, no apparent accumulation of hypotaurine was observed, but the relative amount of taurine (Fig. 9A) was much greater than that of the endogenous taurine present in the supernatant of muscle protein extracts (see Fig. 9B as a reference). When the freshly prepared muscle sample was mixed with aspartate
for 10 min, no apparent accumulation of β-alanine was observed in the reaction mixture (Fig. 9B). As only aspartate was incubated with the supernatant, the taurine peak corresponded to the relative base level of taurine in the supernatant of muscle protein extracts (Fig. 9A and 9B). Based on the apparent increase of taurine concentration in the reaction mixture with cysteine sulfinic acid as a substrate, it seemed that no hypotaurine was produced in the reaction mixture, but hypotaurine, once formed, was converted to taurine. When hypotaurine was directly mixed with muscle extract sample, hypotaurine was also converted to taurine (Fig. 10), suggesting that crude muscle extract contains factor(s) capable of oxidation of hypotaurine to taurine. Compared to the high concentration of endogenous taurine, no endogenous β-alanine peak was observed in the supernatant of muscle protein extracts. Also, no apparent decrease of β-alanine was observed when the compound was incubated with muscle extract sample (Fig. 11).

When the supernatant of freshly prepared kidney protein extracts was incubated with cysteine sulfinic acid as a substrate, accumulation of hypotaurine was observed in the reaction mixture (Fig. 9C). When the kidney sample was incubated with aspartate, no apparent accumulation of β-alanine was observed in the reaction mixture (Fig. 9D). It is worth noting that, when substrate was incubated with the supernatant of muscle protein extracts, the majority of product was found in the form of taurine, not hypotaurine (Fig. 9A). The CSADC activity in muscle and kidney protein extracts was calculated around 11 nmol min⁻¹ mg⁻¹ and 13 nmol min⁻¹ mg⁻¹, respectively. GADL1 was not stable in protein extracts because attempts to get rid of the endogenous taurine in protein extracts, such as dialysis and
ammonia sulfate precipitation/resuspension, led to the disappearance of CSADC activity in both muscle and kidney tissue samples (data not shown).

5.4 Discussion

In this manuscript, we reported for the first time the biochemical activities of recombinant *Hu*GADL1 and the distribution of GADL1 mRNA in mice and cattle. The recombinant
Figure 5.10: The conversion of hypotaurine to taurine when hypotaurine was incubated with the supernatant of freshly prepared muscle protein extracts. The 50-µl reaction mixture had 0.2 mM of hypotaurine and contained 40 µl of supernatant of freshly prepared muscle protein extracts. The reaction was stopped at 0 min (A) and 10 min (B), and subjected to OPT-derivatization analysis. The hypotaurine concentration in (B) was around 0.06 mM. The supernatant contained around 90 mg/ml total protein.

*HuGADL1*, expressed in *Escherichia coli*, has a similar UV-visible spectrum as other identified glutamate/cysteine sulfinic acid/aspartate 1-decarboxylases. *HuGADL1* has different
Figure 5.11: The stability of $\beta$-alanine in supernatant of the freshly prepared muscle protein extracts. (A) Two millimolars of $\beta$-alanine solution prepared in the supernatant of freshly prepared muscle protein extracts. The solution was incubated at 25 °C for 20 min before analysis. (B) Two millimolars of $\beta$-alanine solution prepared purified water.

substrate usage than the name would imply. The enzyme catalyzes decarboxylation of $\alpha$-carboxyl group of cysteine sulfinic acid and aspartate but not glutamate. GADL1 mRNA showed tissue-specific expression in cattle and mice, with relatively high levels in the muscle of both species. High levels of GADL1 transcript were also detected in mouse kidneys. The presence of GADL1 in muscles and kidneys has been confirmed by western blot analysis and activity assays with tissue samples. It is likely that GADL1 may contribute to in
vivo taurine or/and β-alanine biosynthesis (Fig. 12). Consistent with our results in terms of GADL1 expression, online human microarray data indicated that GADL1 expression went up significantly under conditions of neuromuscular pain (ArrayExpress: E-GEOD-7307, Gene Expression Altas http://www.ebi.ac.uk/gxa/). These online data have been derived from a number of independent DNA array studies. The linking of GADL1 with muscular pain conditions provides some basis to suggest that GADL1 plays a physiological role in muscles.

There are only a limited number of GADL1 genes in the Gene database with most of them from mammals. It was interesting to observe that although GADL1 from cattle and mice is highly similar in primary sequence and both are transcribed primarily in skeletal muscles, the mouse gene is also transcribed a lot in kidney and the cattle gene is transcribed a little in heart. In addition, GADL1 mRNA was detected in C2C12 myoblasts, a cell line corresponding to earlier stages of muscle ontogeny, but it disappeared in the next C2C12 myotube stage, indicating that GADL1 might be tightly regulated during myogenesis. The western blot data indicated that the presence of GADL1 in mouse muscles, kidneys, C2C12 myoblasts, and C2C12 myotubes, which is comparable with our RT-PCR data except for the C2C12 myotube result. Hypotaurine is the indispensable intermediate along the cysteine sulfinic acid to taurine pathway. Accumulation of taurine in the supernatant of muscle extracts and cysteine sulfinic acid reaction mixture determined the CSADC activity in muscles. Based on the western blot analysis, however, typical CSADC was absent in muscles. Accordingly, the CSADC activity in muscles likely is primarily due to the presence of GADL1, while in kidney tissues activity might well be a contribution by both typical CSADC and
GADL1 as seen in Figure 8A. It has been suggested that the hypotaurine-to-taurine reaction was enzyme-mediated, but there have been no reports or evidence showing the presence of such an enzyme. Conversion of hypotaurine to taurine in muscle samples suggested that a
molecule/enzyme, capable of catalyzing the production of hypotaurine to taurine, is present in muscles (Fig. 10). No such activity was detected from brains and kidneys (data not shown). It is therefore worthwhile to work on the exact enzyme that was able to catalyze the conversion of hypotaurine to taurine.

*Hu*GADL1 is active to cysteine sulfinic acid. Accordingly, it is reasonable to name it CSADC or CSADC isozyme. Although CSADC has generally been considered the primary enzyme for taurine synthesis, only a few mammalian CSADC enzymes have been experimentally characterized (16-18). Typical CSADC catalyzes cysteine sulfinic acid to hypotaurine that is the precursor of taurine and can be oxidized *in vivo* to taurine, but the enzyme has no activity to aspartate (9). Although past studies are not very consistent regarding the tissue distribution of mammalian CSADC, it is generally agreed it is expressed in both liver and brain (16,19,20). Although hearts and skeletal muscles have high concentrations of taurine, CSADC was not considered to be present in skeletal muscles and kidneys (16) and it was suggested that taurine was produced elsewhere and transported to these tissues (21,22). In this manuscript, the expression and biochemical activity of GADL1 in muscles suggest that muscle taurine might be produced *in situ*.

The other product of recombinant GADL1-catalyzed reactions is β-alanine, a naturally occurring -amino acid that is commonly found in many living species. β-alanine is a central component of pantothenate (vitamin B5), the essential precursor of coenzyme A (23). β-alanine is also an important component of several dipeptides in animals, such as carnosine, carnine, and *N*-β-alanyldopamine (NBAD). In humans (perhaps all other mammalian
species as well), β-alanine is necessary for carnosine synthesis. Carnosine, the dipeptide between L-histidine and β-alanine, has long been considered the dominant buffering component in muscles (24). Increasing the intracellular carnosine levels has been exploited for improving the buffering capacity of skeletal muscles, thus enhancing exercise performance (14,25). Carnosine is present at high concentrations in vertebrate skeletal muscles (24). In addition to muscles, carnosine is also found in brains and hearts and is therefore suspected to be more than a buffering reagent in physiology (26-30). Studies of carnosine have suggested that it is of neuroprotective (31,32), antiglycative (33), antioxidative (34), and anti-aging activities (35,36).

Living species seem to have evolved different biochemical processes to generate β-alanine and some have more than one β-alanine producing pathway. It has been a general consensus that β-alanine is mainly derived from the degradation of uracil in plants, fungi, and vertebrates (37). In contrast, β-alanine is produced primarily through one-step decarboxylation of α-carboxyl group of aspartate by ADC in bacteria and insects, although the insect ADC and bacterial ADC share no sequence homology and are recruited independently by convergent evolution (38,39). The bacterial ADC is a homotetramer with a covalently bound pyruvoyl cofactor (40), while the insect ADC is a dimer with PLP as a cofactor (9).

The discovery of ADC activity of recombinant HuGADL1 is interesting and potentially quite important. Currently, except for insect ADC, no animal enzyme is known that is capable of catalyzing the decarboxylation of the α-carboxyl group of aspartate to β-alanine. Endogenous carnosine is synthesized by carnosine synthetase using histidine and β-alanine
as substrates in muscles (41,42). β-alanine is the limiting compound in carnosine synthesis because its plasma concentration is much lower than that of histidine (24) and the enzyme has a lower affinity to β-alanine than to histidine (43,44). All the currently known β-alanine-synthesizing enzymes have low expression levels in muscles, where the demand for β-alanine is great; accordingly, it is generally considered that the intramuscular β-alanine is transported from elsewhere to make carnosine (13). A study with chicken embryonic muscle cell culture indicated that a β-amino-acid transporter is present in the cells (45); hence the sarcoplasmic β-alanine delivery is the rate-limiting factor for muscle carnosine synthesis. β-alanine supplementation has been exploited to boost the blood β-alanine concentration, drive the carnosine synthesis in muscles, and improve muscle performance (46). Long-term β-alanine intake has been shown to augment muscle carnosine concentrations (25,47,48), but such delivery is inefficient and somewhat problematic. Even with multiple high doses of 800 mg β-alanine per day over 4 weeks, the mean increase of carnosine was only about 60% (24). Moreover, acute oral β-alanine intake (>800 mg) can cause paraesthia, generally known as the feelings of pins and needles (49). Compared to the enzymatic β-alanine production and transportation pathways mentioned above, GADL1-mediated β-alanine production could be the simple and energy-efficient pathway.

ADC activity in muscle extracts remains to be established. Our recent data showed that cysteine could inactivate the insect ADC and the two activities of the enzyme responded differently to cysteine inactivation, with its ADC activity being more potently inactivated (50). Cysteine sulfinic acid or/and hypotaurine can inhibit the ADC activity of GADL1
Further analysis indicated that cysteine and taurine could severely inhibit the ADC activity of GADL1 as well (Fig. 13). It is possible that homogenization disrupted most organelles, which might expose GADL1 to endogenous inhibitors, which may explain in part our inability to detect ADC activity in the extracted protein samples.

Figure 5.13: Taurine and cysteine could inactivate the ADC activity of GADL1. (A) Two micrograms of purified recombinant human GADL1 was incubated with 10 mM of aspartate. The specific ADC activity was 1.42 µmol min⁻¹ mg⁻¹. (B) Two micrograms of purified recombinant human GADL1 was incubated with 10 mM of taurine for 10 min before adding 10 mM of aspartate to the solution. The specific ADC activity was 0.66 µmol min⁻¹ mg⁻¹. (C) Two micrograms of purified recombinant human GADL1 was incubated with 1 mM of cysteine for 10 min before adding 10 mM of aspartate to the solution. The specific ADC activity was 0.38 µmol min⁻¹ mg⁻¹. The reaction was prepared in 150 mM phosphate buffer (pH, 7.0) with 0.40 µM of PLP. The reaction mixtures were incubated for 10 min at 25 °C and stopped by adding two volumes of 100% ethanol. The aspartate and taurine stocks used in the reaction were adjusted to pH=7.0.
From an evolutionary perspective, GAD and CSADC appeared early in evolution, but so far GADL1 has only been found only in birds, reptiles, and mammals (Fig. 1). Although insect ADC possesses a similar substrate usage to GADL1 (9,50), insect ADC-type enzymes have not been found in non-insect species (Fig. 1). GADL1 appeared late in species evolution. One might argue that GADL1 is still an evolving protein and its appearance may help those animals better adapt the much more complex terrestrial environment and whose better survival may somewhat depend on their muscle performance.

5.5 Conclusion

In summary, although the \textit{in vivo} ADC activity of GADL1 is still in question, the distribution and biochemical activities of the recombinant \textit{Hu}GADL1 provide a basis to more intelligently explore its physiological function(s). The hypotaurine-generating activity of GADL1 was shown in muscles and kidneys; the up-regulation of GADL1 in muscles under disease conditions (reported by independent mRNA array analyses) indicates that the protein plays a physiological function(s) in muscles. Consequently, results of this study provide 1) useful information towards a more appropriate classification/annotation of GADL1 proteins, 2) a tangible basis in terms of directions for revealing its true physiological function(s), and 3) a stimulating momentum for studying these intriguing proteins.
5.6 Acknowledgements

This work was supported by NIH grant AI 19769.

5.7 References

Chapter 6

Biochemical evaluation of insect L-3, 4-dihydroxyphenylalanine decarboxylase (DDC) and 3, 4-dihydroxylphenylacetaldehyde (DHPAA) synthase reveals their catalytic mechanisms

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L-3, 4-dihydroxyphenylalanine decarboxylase (DDC) catalyzes decarboxylation of L-3, 4-
dihydroxyphenylalanine (L-DOPA) to produce dopamine; 3, 4-dihydroxylphenylacetal-
dehyde (DHPAA) synthase catalyzes decarboxylation-deamination of L-DOPA to produce DH-
PAA. DHPAA synthase has been only identified from insects and plants. Both enzymes bind
pyridoxal 5-phosphate (PLP) as the cofactor and share around 50% protein sequence simi-
larity. Insect DDC and DHPAA synthase are involved in different steps of catecholamines
biosynthesis during insect cuticle hardening. However, the structural elements that result
in the subtle differences in the reactions of both enzymes are unclear. In this manuscript,
site-directed mutagenesis studies of insect DDC have revealed a few conserved residues that
can partially convert the decarboxylation activity of typical DDC to the decarboxylation-
deamination activity of DHPAA synthase. The manuscript has shown that a catalytic loop
tyrosine residue and an active site histidine residue are important for the DDC activity whereas a phenylalanine residue and an asparagine residue at the corresponding positions within insect DHPAA are important for decarboxylation-deamination activity. The results of this manuscript could help to understand the catalytic similarity and difference of DDC and DHPAA synthase.

Keywords
3, 4-dihydroxylphenylacetaldehyde, L-DOPA, dopamine, α-methyl dopa-resistant, decarboxylation-deamination, PLP, insect cuticle, decarboxylation, and decarboxylation-deamination

6.1 Introduction

3, 4-dihydroxylphenylacetaldehyde (DHPAA) synthase have been characterized in flies and mosquitoes (1,2). DHPAA synthase is a pyridoxal 5-phosphate (PLP)-containing enzyme that shares ~48% sequence similarity with L-3, 4-dihydroxyphenylalanine decarboxylase (DDC) (Fig. 1) (3); due to its phenotype with α-methyl dopa resistance, DHPAA synthase was previously known named as α-methyl dopa-resistant protein before its actual substrate was revealed (4).

The physiological role of DHPAA synthase has been extensively studied before its biochemical activity was determined. Mutations of Drosophila melanogaster DHPAA synthase gene have been shown to exhibit a dual phenotype of recessive lethality and dominant conditional lethality (5). The recessive lethal phenotype is during embryonic hatching, when the dying
embryos have abnormal cuticles and necrotic anal organs (6); the dominant phenotype is manifested by the lower LD_{50} to α-methyl dopa (6). It was speculated that, though its reaction was not known at that time, the enzyme was involved in the biosynthesis of catecholamines involved in insect cuticle hardening. Particularly, the tissue specific distribution of this enzyme has a strong correlation with the regions of the soft and flexible cuticle formation (7,8). Moreover, the clear flexible pupal cuticle produced during pupariation has been reported to be affected when the activity of the enzyme is compromised (9).
Though the gene coding for DHPAA synthase has been known for a long time, until very recently its biochemical activity had not been experimentally identified. Compared to DDC, this enzyme catalyzes the decarboxylation-deamination of L-DOPA to produce DHPAA (2). While insect DDC and DHPAA synthase catalyze the decarboxylation and decarboxylation-deamination of the same substrate, L-DOPA, there has been no study about the structural basis for their different catalytic mechanisms. Plant aromatic amino acid decarboxylases (AAADs) can catalyze either decarboxylation or decarboxylation-deamination reaction (10-13). Recently, a study of plant AAADs revealed that a conserved catalytic loop tyrosine residue is primarily responsible for dictating typical decarboxylase activity whereas a phenylalanine at the corresponding position is associated with aldehyde synthase activity (14).

In this manuscript, several conserved residues between DDC and DHPAA synthase were chosen as candidates for site-directed mutagenesis. AeDDC mutants were designed, cloned, expressed, and purified for biochemical assays, particularly whether there is any hint of DHPAA synthase activity of those mutants. Our current results have illustrated that, in addition to the catalytic loop tyrosine/phenylalanine residue that has been illustrated in plant and mammalian AAADs/aromatic acetaldehyde synthases (AASs) (14,15), there is at least one more site that is partially responsible for dictating decarboxylation and decarboxylation-deamination activity. We are actively conducting more experiments to reveal the governing residues in insect DDC/DHPAA synthase and the mechanisms behind those residues.
6.2 Materials and Methods

6.2.1 Chemicals

All chemicals used in this report were from SigmaAldrich unless specified otherwise. 3, 4-dihydroxyphenyl ethanol was purchased from Cayman chemical company.

6.2.2 Site-directed mutagenesis study of AeDDC

The conserved residues of AeDDC for mutagenesis were His 82, His 202, and Tyr 342; each was mutated to its corresponding residue at Aedes aegypti DHPAA synthase. A forward primer (with the codon of the mutated residue) and a reverse primer (with the anticodon of that mutated residue), both containing a SapI restriction site, were designed and paired with the original 3- and 5-end primers, respectively, to amplify two cDNA fragments. Both fragments were digested with SapI (which generated a cohesive site for the two fragments for specific ligation) and then ligated together with the aid of T4 ligase. Primers for mutagenesis were shown in Tab. 1. After amplification of the ligated product with original 5- and 3-end primers, the product was then ligated into an Impact-CN plasmid (New England Biolabs). The frame of the mutant was verified by DNA sequencing.
Table 6.1: Primers used for the cloning of wild type AeDDC and AeDDC mutants

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</tbody>
</table>

6.2.3 Protein expression and purification

Recombinant IMPACT-CN plasmid with the amplified sequence was transformed into *Escherichia coli* BL21 and purified to homogeneity. AeDDC mutants were expressed and purified as those described in a previous study (3). A Bio-Rad protein assay kit was used to determine protein concentration with bovine serum albumin as a standard. Both enzymes were concentrated to around 25 µg µl⁻¹ in 20 mM phosphate buffer (pH, 7.0).

6.2.4 Biochemical activity assays of AeDDC mutants

Purified recombinant AeDDC mutants were used for DHPAA synthase activity assays. Briefly, a reaction mixture of 100 µl containing 25 µg of purified recombinant protein and 8 mM of L-DOPA was prepared in 100 mM of phosphate buffer (pH, 6.8) containing 20 µM of PLP. At 10, 15, 20, 30, or 45 min after incubation, 16 µl of the reaction mixture was mixed with 4 µl ethanol-saturated NaBH₄ (to reduce any aldehyde formed in the reaction for easy detection). The mixtures (now 20 µl) were incubated at 25 °C for 5 min and then 20 µl of 0.8 M of formic acid was incorporated into the mixtures (to decompose remaining NaBH₄).
After centrifugation, the acidified reaction mixtures were analyzed by reverse-phase high performance chromatography (HPLC) followed by electrochemical detection. The mobile phase consisted of 50 mM of phosphate buffer (pH, 3.5) containing 10% acetonitrile and 0.5 mM octyl sulfate at a flow rate of 0.5 ml per min. The oxidation potential was set to +750 mV. The decarboxylation-deamination of wild type DHPAA synthase is concurrent, so the enzyme could not catalyze dopamine to produce DHPAA through deamination (2). To test whether the reaction mechanism changed in \( AeDDC \) mutants, 5 mM of dopamine was used as the substrate to replace L-DOPA in reaction mixture. The reaction was triggered by the addition of 25 \( \mu \)g of each of the purified recombinant \( AeDDC \) mutant and measured 20 min after incubation.

### 6.3 Results

#### 6.3.1 The evolutionary relationship of insect DDC and DHPAA synthase

Blast search of DHPAA synthase sequence in animal protein databases indicated that DH-PAA synthase is exclusively found in insects, though most of the putative DHPAA synthases (based on higher sequence similarity to identified DHPAA synthase than to other enzymes) have not been experimentally verified. Insect DDC and DHPAA synthase share around 50% sequence similarity; further phylogenetic analysis of insect DDC and DHPAA synthase has
shown that insect DHPAA synthase could be grouped into two clusters (Fig. 2). One cluster is composed of DHPAA synthases mostly from flies and mosquitoes; the other cluster contains the putative DHPAA synthases that lack either one or two residues at the catalytic loop region. The putative *Tribolium castaneum* DHPAA synthase was cloned; the recombinant protein has been expressed and purified in the laboratory. The biochemical assays of the putative *Tribolium castaneum* DHPAA synthase, have shown that the decarboxylation-deamination activity could not be detected from the wild type enzyme; however, when inserting the missing residues at the catalytic loop region, a minor presence of DHPAA could be observed when incubating with L-DOPA (data not shown). It is suspected that the length or residues at the catalytic loop region of DHPAA synthase may at least be one contributing factor to its activity. Scientists in our laboratory are working on discovering differences between the two clusters of DHPAA synthase for a thorough and systematic explanation to the disappearance of the decarboxylation-deamination activity observed in those insect species.

By comparing DDCs and DHPAA synthases from *Aedes aegypti* and *Drosophila melanogaster*, two conserved residues (His 82 and His 202) were picked as candidates for mutagenesis in DDC (Fig. 3). One additional residue (Tyr 342) was picked for mutagenesis mainly because of the study done in plant and mammalian AAADs (14,15), even though all currently identified mosquito DHPAA synthases contain a Tyr at the corresponding position (Fig. 3).
Figure 6.2: A phylogenetic tree of identified and putative DDCs and DHPAA synthases from insects. DHPAA synthase was named as AMD protein before its real substrate is identified. Sequences were aligned using ClustalW, and the dendrogram was generated using the neighbor-joining method. Numbers at nodes show bootstrap values based on 1,000 replicates. The sequences of AeADC, DrADC, AeGAD, DrGAD were used as outliers when the phylogenetic tree was made.

6.3.2 UV-visible spectrum of recombinant wild type AeDDC and AeDDC mutants

The concentrated protein solution showed a visible light yellow color. Spectral analysis of purified wild type AeDDC revealed the presence of typical visible absorbance of PLP-dependent decarboxylases with a major peak $\lambda_{max}$ at 330 nm and a broad shoulder $\lambda_{max}$ at 430 nm, respectively (Fig. 4A). On the other hand, for wild type Aedes aegypti DHPAA synthase, the ratio between the two major peaks with $\lambda_{max}$ at 340 nm and 430 nm is smaller than that of wild type AeDDC (Fig. 4B) (2). These visible absorption peaks were due to the
Figure 6.3: Sequence alignment of DDC and DHPAA synthase of *Aedes aegypti* and *Drosophila melanogaster*. The amino acid sequences of *Ae*DDC (XP 001648263.1), *Dr*DDC (NP 724163.1), *Aedes aegypti* DHPAA synthase (XP 001661056.1), and *Drosophila melanogaster* DHPAA synthase (NP 476592.1) were aligned using ClustalW program with default parameters. Conserved (*), strongly similar (:), and weakly similar residues (.) are shown below the alignment.
presence of PLP cofactor. The ratio of the 340 nm peak versus 430 nm peak reflected the relative contents of PLP tautomers (16). As for AeDDC mutants, the spectrum of AeDDC H82Q is similar to that of wild type Aedes aegypti DHPAA synthase while the spectra of AeDDC H202N and AeDDC Y342F are similar to that of wild type AeDDC (Fig. 4C, D, &E).

Figure 6.4: UV-visible spectra of the purified recombinant wild type AeDDC, AeDDC mutants, and wild type Aedes aegypti DHPAA synthase. The UV-visible spectrum of wild type AeDDC (A), wild type Aedes aegypti DHPAA synthase (B), AeDDC H82Q (C), AeDDC H202N (D), and AeDDC Y342F (E). Their UV-visible spectra from 300 nm to 480 nm were determined using a Hitachi U-2800A spectrophotometer.
6.3.3 Biochemical activities of AeDDC mutants

The results showed that while AeDDC H82Q had no aldehyde synthase activity at all, both AeDDC H202N, and AeDDC Y342F had at least detectable levels of aldehyde synthase activity (Fig. 5). Neither AeDDC H202N nor AeDDC Y342F could catalyze the deamination of dopamine to produce DHPAA (Fig. 6). The specific activities of decarboxylation and decarboxylation-deamination of AeDDC mutants have been calculated at 25 °C with 8 mM of L-DOPA incubating for 15 min (Tab. 2).

Table 6.2: Specific activities of wild type textitAeDDC, textitAeDHPAA synthase, and AeDDC mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
<th>Specific activity (µmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µmol min⁻¹ mg⁻¹)</td>
</tr>
<tr>
<td>DDC</td>
<td>DDC activity</td>
<td>1.62±0.18</td>
</tr>
<tr>
<td></td>
<td>DHPAA activity</td>
<td>Not detectable</td>
</tr>
<tr>
<td>DHPAA synthase</td>
<td>DDC activity</td>
<td>Not detectable</td>
</tr>
<tr>
<td></td>
<td>DHPAA activity</td>
<td>2.30±0.15</td>
</tr>
<tr>
<td>H82Q</td>
<td>DDC activity</td>
<td>1.58±0.18</td>
</tr>
<tr>
<td></td>
<td>DHPAA activity</td>
<td>Not detectable</td>
</tr>
<tr>
<td>H202N</td>
<td>DDC activity</td>
<td>0.72±0.05</td>
</tr>
<tr>
<td></td>
<td>DHPAA activity</td>
<td>0.056</td>
</tr>
<tr>
<td>Y342F</td>
<td>DDC activity</td>
<td>1.10±0.15</td>
</tr>
<tr>
<td></td>
<td>DHPAA activity</td>
<td>0.051</td>
</tr>
</tbody>
</table>

6.4 Discussion

In nature, there are only a few enzymes that can catalyze decarboxylation-deamination reaction. The structural basis that governs their reaction mechanism and substrate selectivity is of both scientific interest and industrial application. In this manuscript, taking the site-
directed mutagenesis approach, the authors have studied the residues that can differentiate the DHPAA synthase from DDC in insects. So far, two positions have been detected to convert decarboxylation to decarboxylation-deamination between insect DDC and DHPAA
Figure 6.5: The activity of \textit{Ae}DDC H82Q, \textit{Ae}DDC H202N, and \textit{Ae}DDC Y342F in the presence of 8 mM of L-DOPA dopamine as the substrate at 10 min, 15 min, 20 min, 30 min, and 45 min after the start of reaction. (A) \textit{Ae}DDC H82Q, (B) \textit{Ae}DDC H202N, and (C) \textit{Ae}DDC Y342F.

Studies of plant phenylacetaldehyde synthase (PAAS) have shown that the enzyme can catalyze both a decarboxylase reaction and an oxidase reaction at the same active site with PLP as the cofactor (11). Plant PAAS and insect DHPAA synthase likely share the same reaction mechanism though one uses phenylalanine and the other works on L-DOPA. An earlier study of a parsley enzyme has shown that it can catalyze decarboxylation-deamination of tyrosine and L-DOPA (13). Although PLP-dependent enzymes capable of both decarboxylation and decarboxylation-deamination have been reported in a few species, the oxidative decarboxylation reactions are quite slow compared to the non-oxidative decarboxylation (17-19). So
Figure 6.6: The activity of AeDDC H202N and AeDDC Y342F in the presence of dopamine as the substrate. (A) AeDDC H202N and (B) AeDDC Y342F.

far, enzymes that can efficiently couple a decarboxylase reaction and an oxidative deaminase reaction have only been experimentally identified in plants and insects. This coupling has been suggested to be the result of easy access of O₂ to the active site, stabilization of the peroxide anion through protonation (17), and enhanced radical character of the reaction intermediates (20).
Previous studies have shown that the above conversion from exclusive decarboxylation to exclusive decarboxylation-deamination is achievable in plant and mammalian AAADs by changing the catalytic loop tyrosine to phenylalanine alone and the mechanism has been proposed (14,15,21). Protonation at the Cα of external aldimine has been proposed to be crucial for the non-oxidative decarboxylation (Scheme 1) (15,22); otherwise, O2 would attack the C4 of external aldimine, resulting in the release of aldehydes product and ammonia (Scheme 1). Initially, when only the catalytic loop tyrosine was found important for the non-decarboxylation activity, the tyrosine has been proposed to promote the protonation of the Cα of external aldimine (15). Recently, careful analysis of the active site conformation revealed that the side chain hydroxyl group of tyrosine is closer to the imidazole amine of a conserved histidine than to the Cα of external aldimine and the imidazole amine of histidine is in close proximity to the Cα of external aldimine (14). Hereby, a functional role for tyrosine and histidine has been proposed respectively: histidine contributes to the protonation at the Cα of external aldimine and tyrosine helps to stabilize the deprotonated imidazole amine of histidine (14). It would be interesting to test whether a phenylalanine residue at the catalytic loop is required for aldehydes synthase activity in plant and mammalian enzymes. Currently, it seems that tyrosine is required for decarboxylation activity. It could be that tyrosine controls the reaction selectivity of decarboxylation and mutation of tyrosine in decarboxylase would break the exclusive reaction restriction of this group of enzyme. How about mutating tyrosine to other amino acid residues other than phenylalanine?

Although the above hypothetical reaction mechanism of DDC and aldehyde synthase cor-
Scheme 6.1: Proposed pathways for decarboxylation-deamination in the presence of O$_2$ and decarboxylation. Left, decarboxylation-deamination in the presence of O$_2$. Right, decarboxylation.
responded to the observation in this manuscript, in that both AeDDC H202N and AeDDC Y342F have the appearance of DHPAA synthase activity. Still, the mechanistic differences between insect DDC and DHPAA synthase have not been fully explained from the proposed reaction mechanism based on studies of plant and mammalian PLP-dependent decarboxylases. First, there is just minor conversion of non-oxidative decarboxylation to oxidative decarboxylation in AeDDC Y342F, while the contribution of tyrosine to the Cα protonation in plant and mammalian PLP-containing decarboxylase is dominant. In addition, mosquito DDC and DHPAA synthase both have Tyr at the catalytic loop. To summarize, the actual contribution of Tyr to ensure the exclusive decarboxylation activity of insect DDC is weakened. Second, the conservation of active site Asn residue has only been observed from insect aldehyde synthases and its contribution to the decarboxylation-deamination activity has only been tested in insect enzymes. AeDDC H202N has some DHPAA synthase activity while the majority of its activity is still L-DOPA decarboxylase activity. It would be interesting to test if the mutation of His to Asn in plant or mammalian AAADs would lead to the appearance of aldehydes synthase activity in those enzymes. The next step would be to design plant or mammalian AAADs with His-to-Asn mutation and test the activities of these mutants. In addition, in insect DHPAA synthases, how important is Asn to the decarboxylation-deamination activity? Will the Asn-to-His mutant of DHPAA synthase retain most or just a little of the decarboxylation-deamination activity?

Finally, the kinetic parameters of wild type DDC, wild type DHPAA synthase, and DDC mutants need to be calculated and compared to quantify the influence of each of the mutation
at the active site of the enzymes.

The importance of this work serves to better understand the mechanism of PLP-dependent aldehyde synthase, a group whose members and reactions are far less documented among PLP-containing enzymes. The result of this manuscript would help to understand the reaction mechanism of decarboxylation-deamination and the competence between decarboxylation and decarboxylation-deamination.

As a large portion of insect DHPAA synthases are currently speculated to be non-functional (though more candidates from this group need to be tested to confirm the hypothesis) and could be referred as still evolving, the enzyme offers an exciting chance to observe the evolution of a protein within species. By knowing the residues that can convert the activity and comparing the appearance of those residues in different insect species, people could figure out the detailed evolutionary blueprint for DHPAA synthase and help to understand the role of DHPAA synthase to insect cuticle formation.

6.5 Conclusion

In this manuscript, biochemical evaluation of insect DDC and DHPAA synthase has been performed. Mutagenesis studies in DDC have shown that its decarboxylation activity could be converted to the decarboxylation-deamination by changing a few residues at the active site. So far, this conversion has not been as complete as it was in plant AAAD mutants. More detailed studies are needed to find out more residues involved and to confirm the exact
role of each residue. The reaction mechanism of insect DHPAA synthase, together with that of plant AAS, would shed light on the structural elements that result in subtle differences in the reactions catalyzed by those closely related PLP-dependent decarboxylases.

6.6 Acknowledgements

This work was supported by NIH grant AI 19769.

6.7 References


Chapter 7

Conclusion

Insect cuticle hardening is a complicated process that involves crosslinks between cuticular proteins and sclerotizing reagents (Chapter 2). Several enzymes involved in the biosynthesis of sclerotizing reagents are unique to insects and thus have been considered ideal targets for insecticide design (Chapter 2). This dissertation collectively presents our biochemical studies of aspartate 1-decarboxylase (ADC) and 3, 4-dihydroxylphenylacetaldehyde (DHPAA) synthase, both of which are important enzymes in the insect cuticle hardening process.

ADC is responsible for the biosynthesis of a well-studied and major sclerotizing reagent, N-β-alanyldopamine (NBAD). The activity of insect ADC also regulates the level of cuticle melanization by affecting the concentration of free dopamine in insect cuticle. Our studies reported the usage of cysteine sulfinic acid and cysteic acid as substrates in addition to aspartate by insect ADC (Chapter 3). Typical cysteine sulfinic acid decarboxylase (CSADC)
has been only experimentally confirmed in mammalian species; while both enzymes could work on cysteine sulfinic acid and cysteic acid, only insect ADC could work on aspartate (Chapter 3). The presence of a conserved glutamine residue at the active site of insect ADC has contributed to the substrate usage of aspartate by insect ADC (Chapter 3).

Further biochemical characterization of insect ADC revealed that cysteine is a potent inactivator, though cysteine inactivates the two reactions of insect ADC to different extents (Chapter 4). The mechanism of cysteine inactivation has been proposed and confirmed to be the formation of a 4-thiazolidinecarboxylic acid derivative between the incoming cysteine and the active site cofactor, pyridoxal 5-phosphate (PLP) (Chapter 4). Such inactivation has been observed in glutamate decarboxylase (GAD) and CSADC as well, making cysteine an inactivator to this specific group of PLP-dependent decarboxylases (Chapter 4).

Mammalian glutamate decarboxylase like-1 (GADL1) shares around 40% protein sequence identity with insect ADC, mammalian CSADC, and GAD, but the biochemical activity of GADL1 has not been confirmed. Our biochemical studies show that this enzyme does not work on glutamate as its name suggests. Instead, it shares the same substrate usage as that of insect ADC (Chapter 5). It is suspected that the enzyme is found in mammalian species, reptiles, and birds (Chapter 5). Since no study has really shown the presence and distribution of the mRNA and protein of GADL1, our subsequent experiments have indicated that the mRNA and protein of this enzyme both are found in mammalian skeletal muscles and kidneys (Chapter 5). The CSADC activity of the enzyme has been shown in protein extracts of mouse skeletal muscles and kidneys (Chapter 5). This work has identified an
enzyme that could participate in the metabolism of taurine and/or β-alanine in mammalian species.

Finally, some metabolic engineering work has been performed on L-3, 4-dihydroxyphenylalanine decarboxylase (DDC) and DHPAA synthase. Both enzymes are PLP-containing enzymes and participate in the biosynthesis of sclerotizing reagents in insect cuticle hardening; they use the same substrate and carry different reactions. The difference of such catalytic mechanism is unknown yet but is important for us to understand the regulation of insect cuticle formation. DDC catalyzes the decarboxylation of DOPA to produce dopamine and DHPAA synthase catalyzes the decarboxylation-deamination of DOPA to produce DHPAA, an unstable and reactive compound that is likely involved in the soft and flexible cuticle formation. We have shown that the different reactions of DDC and DHPAA synthase could be converted by mutating a few conserved amino acid residues. The design of one mutation is based on the study discussed in Appendix A while others are still being researched.

The major studies of this dissertation lie in insect cuticle hardening but some interesting results have been detected along the way and serve to promote interesting research in other fields. The identification of substrate usage of insect ADC provides a starting point for achieving a more comprehensive understanding of the biochemistry and physiology of insect ADCs, particularly their potential role in taurine biosynthesis (Chapter 3). High concentrations of taurine are detected in insect hemolymph but there has been no report before our study showing any enzyme of taurine- or hypotaurine-producing ability. Insect ADC could be the target to be further exploited to study the physiological roles of taurine in
insect species (Chapter 3). The importance of the inactivating feature of cysteine detected in insect ADC and other PLP-dependent decarboxylases goes beyond entomology (Chapter 4). It raises an essential question as to how animal species prevent these PLP-dependent decarboxylases from cysteine-mediated inactivation (Chapter 4). Cysteine is an important building block in living species; however, the concentrations of cysteine need to be tightly controlled as disorders of cysteine metabolism have been implicated in several neurodegenerative diseases. Results of our study might establish a linkage between cysteine-mediated inactivation of GAD and CSADC to symptoms of neurodegenerative disease development (Chapter 4). The cysteine inactivation work serves to promote research in terms of mechanism by which animals maintain their cysteine homeostasis (Chapter 4). The discovery of GADL1 biochemical activities, in conjunction with its expression and activities in muscles and kidneys, provides some tangible insight towards establishing its physiological function(s) (Chapter 5). Although currently unfinished, the studies of DHPAA synthase could update the knowledge of insect cuticle hardening, especially the knowledge of soft and flexible cuticle formation (Chapter 6). In addition, the metabolic engineering of insect DHPAA and DDC could promote the understanding of reaction mechanisms of these PLP-dependent enzymes (Chapter 6).
Chapter 8

Appendix A. Biochemical Evaluation of The Decarboxylation and Decarboxylation-Deamination Activities of Plant Aromatic Amino Acid Decarboxylases

This chapter was adapted from the article below: This research was originally published in Journal of Biological Chemistry. Torrens-Spence, M. P., Liu, P., Ding, H., Harich, K., Gillaspy, G., and Li, J. Biochemical evaluation of the decarboxylation and decarboxylation-deamination activities of plant aromatic amino acid decarboxylases. J Biol Chem. 2012; (ID M112.401752) in publish. the American Society for Biochemistry and Molecular Biology.

Author contribution:
Patrick M. Torrens-Spence, design and perform the experiment; write and correct the manuscript; Pingyang Liu, help perform activity assay; Haizhen Ding, design the primer and help express the protein; Kim Harich, perform the mass spectrometry; Glenda Gillaspy, correct the manuscript; Jianyong Li, design the experiment; write and correct the manuscript

**Abstract** Plant aromatic amino acid decarboxylase (AAAD) enzymes are capable of catalyzing either decarboxylation or decarboxylation-deamination on varied but stringent combinations of aromatic amino acid substrates. These two different activities result in the production of arylalkylamines and the formation of aromatic acetaldehydes respectively. Such variations in product formation enable individual enzymes to play different physiological functions. Despite these catalytic variations, arylalkylamine and aldehyde synthesizing AAADs are indistinguishable without protein expression and characterization. In this study, extensive biochemical characterization of plant AAADs was performed to identify residues responsible for differentiating decarboxylation AAADs from aldehyde synthase AAADs. Results demonstrated that a tyrosine residue located on a catalytic loop proximal to the active site of plant AAADs is primarily responsible for dictating typical decarboxylase activity whereas a phenylalanine at the same position is primarily liable for aldehyde synthase activity. Mutagenesis of the active site phenylalanine to tyrosine in *Arabidopsis thaliana* and *Petroselinum crispum* aromatic acetaldehyde synthases converts the enzymes activity from decarboxylation-deamination to decarboxylation. The mutation of the active site tyrosine to phenylalanine in the *Catharanthus roseus* and *Papaver somniferum* aromatic amino acid decarboxylases changes the enzymes decarboxylation activity to a decarboxylation-deamination activity. Generation of these mutant enzymes enables the production of unusual AAAD enzyme products including indole-3-acetaldehyde, 4-hydroxyphenylacetaldehyde, and
phenylethylamine. Our data indicated that the tyrosine and phenylalanine in the catalytic loop region could serve as a signature residue to reliably distinguish plant arylalkylamine and aldehyde synthesizing AAADs. Additionally, the resulting data enables further insights into the mechanistic roles of active site residues.

**Keywords**

aromatic acetaldehyde synthase; phenylacetaldehyde synthase; aromatic amino acid decarboxylase; tryptophan decarboxylase; indole-3-acetaldehyde; phenylethylamine; auxin

### 8.1 Introduction

In many species, including humans, there is only one decarboxylase that selectively catalyzes the decarboxylation of aromatic amino acids. This enzyme is commonly named aromatic amino acid decarboxylase (AAAD). Within plants and insects, however, the similar enzyme has undergone extensive evolutionary divergence, resulting in multiple paralogs with divergent functions. The divergent functions of plant and insect AAADs are closely related to their corresponding enzymes substrate selectivity and catalytic reactions. For example, tyramine derived from tyrosine is the essential precursor for the biosynthesis of many plant secondary metabolites (including \( N \)-hydroxycinnamic acid amides and benzylisoquinoline alkaloids) \( (1-5) \). Tryptamine and 5-hydroxytryptamine (5-HT), produced by specific plant AAADs, are precursors for the synthesis of thousands of indole alkaloid compounds \( (1,6-7) \). Because their substrate specificity provides functional relevant information, some AAADs
have been further annotated on their principle substrate as tyrosine decarboxylases (TyDCs) and tryptophan decarboxylases (TDCs) (8,9).

Recent studies of AAAD proteins demonstrated that in addition to the typical decarboxylation activity established in TyDCs and TDCs, some annotated plant and insect AAAD proteins are actually aromatic acetaldehyde synthases (AASs) (10-12). These AASs catalyze a rather complicated decarboxylation-oxidative deamination process of aromatic amino acids, leading to the production of aromatic acetaldehydes, CO$_2$, ammonia, and hydrogen peroxide rather than the AAAD derived arylalkylamines and CO$_2$ (Fig. 1). Research has implicated plant AAS enzymes in the production of volatile flower scents, floral attractants, and defensive phenolic acetaldehyde secondary metabolites (10-12) and insect AAS in soft cuticle hardening (13). While the physiological functions of true AAADs vary with their substrate selection, AAS proteins perform additional distinct physiological roles as compared with true AAADs. Consequently, it is essential to be able to distinguish AAS proteins from true AAAD enzymes. Despite variations in substrate specificity and catalytic reactions, plant AAS proteins share great sequence similarity to true AAADs. Such high homology is specifically emphasized through the comparison of a characterized *Arabidopsis thaliana* AAS and a *Capsicum annuum* TDC (11,14). Although the *C. annuum* TDC only catalyzes decarboxylation on indolic substrates and the *A. thaliana* AAS only catalyzes aldehyde synthase on phenolic substrates, these two enzymes retain 74% identity and 84% homology. This extensive homology has lead to a major problem in distinguishing AAS enzymes from typical AAAD proteins. For example, all currently verified plant AAS proteins were ini-
tially annotated as TyDCs. Additionally, our recent study showed that even experimentally investigated AAAD enzymes are capable of inaccurate characterization. In the aforementioned study we demonstrate that the previously characterized *Petroselinum crispum* TyDC (Q06086) (15) is actually an AAS that principally catalyzes the conversion of tyrosine to p-hydroxyphenylacetaldehyde (12).

![Figure 8.1: Relative activities of AAAD and AAS.](image)

To narrow the location of activity dictating AAAD and AAS residues we initiated this study by generating a chimeric enzyme, containing the N-terminal half of the *T. flavum* TyDC and the C-terminal half of the *P. crispum* AAS. The 70% identity between the *P. crispum* AAS and *Thalictrum flavum* TyDC (AAG60665) (12) suggests that their respective AAS and TyDC activities are likely dictated by very limited number of active site residues. Activity analysis of the chimeric enzyme determined that the *T. flavum-P. crispum* hybrid has typical AAS activity. The resulting aldehyde synthase activity indicates that residues located in the mutants C-terminal half are predominantly responsible for activity differentiation. Next, analyses of published crystal structures were preformed to identify putative activity differentiating residues with in plant AAAD and AAS enzymes. Several structures were analyzed, but due to the strong plant AAAD homology, the conservation of active site residues, and sub-
stantial electron density of an active site proximal catalytic loop (absent from other AAAD structures), the human histidine decarboxylase (HuHDC) structure was chosen as the primary model (16). Investigation of the human HDC (PDB: 4E1O) crystal structure identified residues located with in 6 Å of the pyridoxal 5-phosphate (PLP) inhibitor adduct. Comparison of these active site residues to their homologous residues in characterized plant AAAD and AAS sequences enabled the identification of active site residues potentially responsible for activity differentiation. Analysis of residues conserved in arylalkylamines synthesizing AAADs and absent in aromatic acetaldehyde synthesizing AASs enabled the exclusion of all but one putative activity dictating residue. The resulting analyses enabled the identification of a putative residue located in a catalytic loop proximal to the active site. The candidate residue is represented by a tyrosine 347 in the *T. flavum* enzyme and as a phenylalanine 346 in the *P. crispum* enzyme. Mutation of this candidate residue from phenylalanine to tyrosine within the *Arabidopsis thaliana* (NP 849999) and the *P. crispum* (Q06086) aldehyde synthases converts the enzymes activities from decarboxylation-oxidative deamination to decarboxylation. Mutation of the homologous tyrosine residue in *Catharanthus roseus* TDC (P17770) and *Papaver Somniferum* TYDC 9 (AAC61842) converted the enzymes activities from decarboxylation to decarboxylation-oxidative deamination. Our data demonstrated that the active site tyrosine and phenylalanine in the flexible loop of plant AAADs plays a dictating role for true AAAD activity and AAS activity respectively. Our progress represents a step forward towards achieving a comprehensive understanding of substrate specificity and catalytic reactions in plant AAAD proteins.
8.2 Materials and Methods

8.2.1 Chemicals

Acetonitrile, 2-mercaptoethanol, formic acid, tryptophan, tryptamine, indole-3-acetaldehyde-
sodium bisulfite, indole-3-ethanol, phenylalanine, phenylethylamine, phthaldialdehyde, PLP, 
sodium bisulfite, and sodium borohydride were purchased from Sigma (St. Louis, MO). The 
IMPACT-CN protein expression system was obtained from New England Biolabs (Ipswich, 
MA).

8.2.2 Plant material and growth conditions

*P. crispum* seeds, *T. flavum* seeds, *P. Somniferum* seeds, and *C. roseus* seeds were ob-
www.horizonherbs.com, respectively. *A. thaliana* seeds were from The Arabidopsis Biological 
Resource Center (Columbus, Ohio). Seeds from these plants were germinated in Sunshine 
Pro Premium potting soil and grown under a 16 h photoperiod at 23 °C at 100 microeinsteins.

8.2.3 RNA isolation, cDNA amplification, and mutagenesis

Total RNA samples were isolated from 12 week old *A. thaliana, C. roseus, P. Somniferum,* 
*P. crispum* and *T. flavum* plants using an Ambion mirVana™ miRNA Isolation Kit. These 
RNA samples were treated with DNase (Ambion TURBO DNA-free™ Kit). First strand cD-
NAs were synthesized by RT-PCR of total RNA samples using Invitrogen™ SuperScript™ III and a poly T17- primer. Specific primer pairs (Tab. 1), designed based on coding sequences (CDS) of A. thaliana AAS (NP 849999), P. crispum AAS (Q06086), T. flavum (AAG60665), P. Somniferum TyDC (AAC61842), and C. Roseus TDC (P17770) were synthesized and used for the amplification of the respective coding sequences. Amplified full-length CDS cDNA were subsequently ligated into IMPACT-CN expression vector for protein expression. Completed expression vectors were used as templates to produce the AAS to AAAD and AAAD to AAS mutants.

To generate T. flavum-P. crispum chimeric enzyme, a specific reverse primer based on codons for T. flavum TyDC (AAG60665) residues 294-300 and a forward primer based on P. crispum AAS (Q06086) residues 300-306 (with both primers containing a SapI restriction site) were synthesized (Tab. 1). The new T. flavum reverse primer was paired with the original T. flavum forward primer and the new P. crispum forward primer was pared with original P. crispum AAS reverse primer to amplify (using the respective full-length T. flavum TyDC and full-length P. crispum AAS CDS sequences as template) a DNA fragment encoding for the first 300 amino acid of T. flavum TyDC and a DNA fragment encoding for the P. crispum residues 300-514, respectively. The two cDNA fragments were digested with SpaI to generate cohesive 3-end for the T. flavum cDNA fragment and cohesive 5-end for the P. crispum cDNA fragment. The two digested fragments were ligated together with the aid of T4 DNA ligase to produced a chimeric CDS coding for the 300 N-terminal side residues of the T. flavum TyDC and 214 C-terminal site of the P. crispum AAS (17).
Table 8.1: Primer sequences for the amplification and metagenesis of AAAD and AAS proteins

<table>
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<th>Primer Name</th>
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<td>AAS-R</td>
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<td>AAS F338Y-F</td>
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<tr>
<td>AAS F338Y-R</td>
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<td><strong>C. roseus</strong></td>
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<td><strong>P. crispum</strong></td>
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Full-length CDS cDNA containing the F338Y mutation in *A. thaliana* AAS, the F346Y mutation in *P. crispum* AAS, the Y348F mutation in *C. roseus* TDC, and the Y350F mutation in *P. somniferum* TyDC were generated using a SapI mutagenesis procedure similar to the procedure described for the production of the chimeric *T. flavum-P. crispum* enzyme. The variable region of the SapI restriction site was used to generate codon mutagenesis (Tab. 1).
8.2.4 Protein expression and purification

All cDNA products encoding for wild type TyDCs, AASs, TDCs and their mutants were individually ligated into IMPACT-CN bacterial expression plasmids. DNA sequencing was utilized to verify the sequence and frame of each cDNA insert. Recombinant IMPACT-CN plasmids were used to transform *E. coli* DE3. Transformed bacterial colonies expressing the target proteins were selected and used for large-scale expression (16-20 liters of *E. coli* cells) of individual recombinant proteins. The detailed conditions for recombinant expression and purification were essentially the same as previously described (18).

8.2.5 Activity assays

Typical reaction mixtures of 100 or 200 µl, containing 20-30 µg of recombinant TyDC, TDC, AAS, hybrid enzyme or mutant protein and 5 mM of substrate (tyrosine, dopa, tryptophan or phenylalanine) were prepared in 50 mM potassium phosphate buffer (pH, 7.5) and incubated at 25 °C in a water bath. The reactions were stopped (at a time point between 5 and 60 min) through the addition of an equal volume of 0.8 M formic acid. Supernatants of the reaction mixtures, obtained by centrifugation, were analyzed with (Aqueous) Pierce Quantitative Peroxide Assay Kit to determine AAS activity. The supernatants were also analyzed by High-performance liquid chromatography (HPLC) with electrochemical detection (HPLC-EC) or HPLC with ultraviolet detection (HPLC-UV). Details of HPLC-EC or HPLC-UV conditions were described in figure captions. Product production was verified though the
comparison of various aromatic amine standards under identical chromatography conditions.

### 8.2.6 AAAD mutant product verification by High-performance liquid chromatography/Tandem mass spectrometry (HPLC/MS/MS)

To further verify the identity of the mutant enzymatic products, physiological substrates and mutant reaction mixtures were analyzed by LC/MS/MS. Reaction mixtures of 100 µl, containing 30 µg of *C. roseus* TDC Y348F and 10 mM of tryptophan, or 30 µg of *A. thaliana* AAS F338Y and 10 mM of phenylalanine were prepared in 50 mM of phosphate buffer (pH, 7.5) and incubated at 25 °C in a water bath for 30 minutes. Reactions were stopped by mixing an equal volume of 0.8 M of formic acid. Prior to injection, indole-3-acetaldehyde, generated in the *C. roseus* TDC Y348F mutant and 10 mM tryptophan reaction mixture, was derived to indole-3-acetaldehyde-sodium bisulfite addition compound using sodium bisulfite. Samples were analyzed by a LC-3200 Q Trap MS/MS system (AB Sciex) in either positive and negative ion mode, as appropriate. Identification of phenylethylamine from the *A. thaliana* AAS F338Y mutant reaction mixture and indole-3-acetaldehyde-sodium bisulfite addition compound from the derived *C. Roseus* TDC Y348F mutant reaction mixture was based on their retention time and MS/MS spectra in comparison with those produced from authentic phenylethylamine and indole-3-acetaldehyde-sodium bisulfite addition compound standards at identical analytic conditions.
8.2.7 Kinetic analysis

After the substrate specificity and catalytic reaction of enzymes were verified, the kinetic parameters were evaluated. The *A. thaliana* AAS (NP 849999) wild type and F338Y mutant enzymes were evaluated using phenylalanine as a substrate. The *C. Roseus* TDC (P17770) wild type and Y348Y mutant enzymes were evaluated using tryptophan as a substrate. Kinetic data points were performed in triplicate and kinetic values were evaluated by hyperbolic regression. Reaction mixtures of 100 µl containing 5 µg of enzyme recombinant protein and varying concentrations of substrate (0.1 to 40 mM of phenylalanine, 0.005 to 16 mM of tryptophan) were prepared in 50 mM phosphate buffer (pH, 7.5) and incubated at 25 °C. To detect phenylethylamine generated by *A. thaliana* F338Y, two volumes of 100% ethanol were added to the reaction mixtures (to stop the reaction) at 5 min after incubation and supernatants, obtained by centrifugation (14,000g for 5 min at 4 °C) were derived using OPT reagent and then analyzed by HPLC-EC and quantitated based on standard curve generated using authentic OPT derived phenylethylamine (19). Tryptamine generated by wild type *C. Roseus* TDC was measured by stopping the reaction mixture using an equal volume of 0.8 M formic acid, obtaining the supernatant via centrifugation, and analyzing by HPLC-EC against a standard curve of tryptamine. To determine the AAS activity for the *C. Roseus* TDC mutant and the wild type *A. thaliana* enzyme, an equal volume of 0.8 M formic acid was added to the reaction mixtures and supernatants (obtained by centrifugation) were analyzed with (Aqueous) Pierce Quantitative Peroxide Assay Kit to measure peroxide production. The amounts of H₂O₂ product in each reaction mixture was quantitated based on
a standard curve generated using authentic peroxide.

8.3 Results

8.3.1 AAAD/AAS chimeric mutant analysis

To narrow the location of the AAAD vs AAS activity differentiating residue/residues, a chimeric (hybrid) protein composed of *T. flavum* TyDC (AAG60665) residues 1-300 and *P. crispum* AAS (Q06086) residues 300-514 was generated and its catalytic reaction was assessed with tyrosine as a substrate. Incubation of the *T. flavum*-*P. crispum* chimeric enzyme in the presence of tyrosine resulted in the detection of a major broad peak overlapping with a minor sharp peak during HPLC-EC analysis (Fig. 2A). The broad peak was considered to be p-hydroxyphenylacetaldehyde (HPAA) because it displayed the same chromatographic behavior as the HPAA formed in the wild type *P. crispum* AAS and tyrosine reaction mixture (Fig. 2C). When both the hybrid enzyme and the wild type *P. crispum* enzyme reaction mixtures were treated with borohydride prior to HPLC-EC analysis, the broad product peaks (Fig. 2A and Fig. 2C respectively) were converted to sharp product peaks (Fig. 2B and Fig. 2D respectively) that coeluted with authentic 4-hydroxyphenylethanol (4-HPEA) (tyrosol) at different chromatographic conditions. Unlike the wild type AAS and tyrosine reaction mixture, a small amount of tyramine was formed in the mutant enzyme and tyrosine reaction mixture (Fig. 2A and 2B). Despite this minor activity, the hybrid enzyme
behaved primarily as a wild type *P. crispum* AAS. These results suggested that residues in the C-terminal half of plant AAADs are likely responsible for constraining decarboxylation and aldehyde synthase reactions.

Figure 8.2: HPLC-EC analysis of *T. flavum*-*P. crispum* chimeric enzyme and *P. crispum* AAS activity with tyrosine as a substrate. Y-axis represents the output in microamps, the x-axis represents retention time. Reaction mixtures of 100 µl containing either 30 µg of *T. flavum*-*P. crispum* chimeric enzyme or 20 µg of wild type AAS and 5 mM of tyrosine were incubated at 25 °C and their reaction was stop at 40 min after incubation by adding an equal volume of 0.8 M of formic acid into the reaction mixture. The mixtures were centrifuged for 5 min at 14,000g and supernatants were injected for HPLC-EC analysis. To reduce HPAA to HPEA (tyrosol), some reaction mixtures were treated with an equal volume of 100% ethanol saturated with borohydride after 40 min incubation. The borohydride treated mixtures were incubated for 10 min on ice, treated with 0.8 M of formic acid to decompose remaining borohydride and centrifuged to obtain supernatants. Supernatants were injected for HPLC-EC analysis. Chromatograms (A and B) illustrate the accumulation of HPAA (the major broad peak) and tyramine (the minor sharp peak) in a *T. flavum*-*P. crispum* chimeric enzyme and tyrosine reaction mixture at 40 min after incubation and the reduction of 4-HPAA to 4-HPEA (tyrosol) in a borohydride treated reaction mixture. Chromatograms C and D show the production of 4-HPAA in a wild type *P. crispum* AAS and tyrosine reaction mixture at 40 min after incubation and the reduction of HPAA to HPEA (tyrosol) in a borohydride treated reaction mixture.
8.3.2 C-terminal active site structural analysis and sequence comparison

To impact the catalytic reaction, the activity dictating C-terminal residues should be in close proximity to the active site internal aldimine bond (that subsequent interacts with incoming substrate, leading to the formation of the external aldimine intermediate during catalysis). To elucidate active site proximal residues, the recently released mammalian histidine decarboxylase (HuHDC) (NP 002103) (16) was investigated in Pymol (20) to identify C-terminal residues located within 6 Å of the external aldimine inhibitor complex. The catalytic domain of HDC is homologous to that of plant AAADs. Additionally, most of the substrate interacting residues in AAADs are conserved in HDCs. Moreover, the active site loop of a ligand bound HDC complex displayed substantial electron density (absent from other AAAD structures). Results identified 13 active site proximal residues from the C-terminal portion of the HuHDC structure. A multiple sequence alignment of the HuHDC sequence, 4 characterized plant AAAD sequences, and 3 characterized plant AAS sequences was performed to identify homologous active site proximal residues from plant AAAD and AAS sequences (Fig. 3). The majority of these putative activity-dictating residues were subsequently dismissed as activity dictating residues due to inter activity conservation or lack of intra activity conservation. Ultimately, it became apparent that T. flavum TyDC Tyr347 was stringently conserved in all identified TyDC and TDC (as well as many other predicted AAADs), while the similar position was occupied by phenylalanine in identified AAS proteins (one verified Rosa AAS contained a valine at the corresponding position) (10).
The correlation between these hypothetical activity differentiating residues and their corresponding catalytic functions can be illustrated by viewing the multiple alignments of five characterized AAADs and 3 characterized AASs (Fig. 4). These analyses suggested that this tyrosine and/or phenylalanine might have an impact on AAAD and AAS catalytic reactions.

Figure 8.3: Sequence alignment of a P. crispum AAS, Rosa hybrid cultivar AAS, A. thaliana AAS, C. roseus TDC, O. sativa TDC, P. Somniferum TYDC 9, T. flavum TyDC, A. thaliana TYDC, and Human HDC. P. crispum AAS (Q06086), Rosa hybrid cultivar AAS (ABB04522), A. thaliana AAS (NP 849999), C. roseus TDC (P17770), O. sativa TDC (AK069031), P. Somniferum TYDC 9 (AAC61842), T. flavum TyDC (AAG60665), A. thaliana TYDC (NP 001078461), and Human HDC (NP 002103.2). The aforementioned plant sequences maintain an average 56% identity. The decarboxylation dictating tyrosine and the aldehyde synthase dictating phenylalanine are highlighted in yellow. The C-terminal residues with in 6 Å of the pyridoxal 5-phosphate inhibitor adduct from the Human HDC (4E1O A) crystal structure and their homologous residues with in plant AAAD and AAS sequences are highlighted in green. The Thalictrum-parsley hybrid protein is composed of the underlined sequence.

8.3.3 Conversion of A. thaliana and P. crispum AASs into AAADs through mutation

To compare with P. crispum AAS, we previously expressed A. thaliana AAS (NP 849999) and verified its activity (12) (the activity of the A. thaliana AAS enzyme has previously been investigated) (11). The A. thaliana AAS has a Phe338 occupying the same position as the
conserved tyrosine in the TyDC and TDC enzymes flexible loop region. To test the role this positional residue plays in catalysis, the *A. thaliana* AAS Phe338 was mutated to Tyr338 and its mutant protein was expressed, purified, and compared with the wild type AAS in catalytic reaction with phenylalanine as a substrate. After the F338Y mutant was incubated for different time periods with phenylalanine and then assessed for the production of $\text{H}_2\text{O}_2$ in
Figure 8.4: Sequence alignment of plant enzymes. *A. thaliana* AAS (NP 849999), *Rosa hybrid cultivar* AAS (ABB04522), *P. crispum* AAS (Q06086), *P. Somniferum* TYDC 9 (AAC61842), *T. flavum* TyDC (AAG60665), *A. thaliana* TYDC (NP 001078461), *C. roseus* TDC (P17770), and *O. sativa* TDC (AK069031). The aforementioned plant sequences maintain an average 56% identity. The decarboxylation dictating tyrosine and the aldehyde synthase dictating phenylalanine are highlighted in yellow.
the reaction mixtures, no apparent H$_2$O$_2$ accumulation was observed in the reaction mixtures (Fig. 5, mutant curve). In contrast, production of H$_2$O$_2$ was observed in the wide-type *A. thaliana* AAS and phenylalanine reaction mixtures and the relative amounts of H$_2$O$_2$ were approximately proportional to the incubation periods (Fig. 5, wild-type curve). Inability to produce H$_2$O$_2$ in the *A. thaliana* AAS F338Y mutant phenylalanine reaction mixtures suggests that this particularly phenylalanine residue is important for the AAS activity of the *A. thaliana* enzyme.

![Figure 8.5: Analysis of hydrogen peroxide production in reaction mixtures containing phenylalanine and wild type *A. thaliana* AAS or its F338Y mutant. Reaction mixtures of 0.2 ml containing 5 mM phenylalanine and 20 µg of the wild type *A. thaliana* AAS or 20 µg of its F338Y mutant were prepared in 50 mM phosphate buffer, pH=7.5. The reaction mixtures were incubated at 25 °C. At each 1 min interval, 20 µl reaction mixture was withdrawn and mixed into 150 µl Pierce peroxide assay reagents solution. The Wild Type curve and mutant curve illustrate the amount of H$_2$O$_2$ accumulated in 20 µl of reaction mixture at 1–8 min incubation periods.](image)

Based on high conservation of the active loop tyrosine in TyDC and TDC at the corresponding position, the generated *A. thaliana* F338Y mutation might result in mutant decarboxylase activity. When the phenylalanine and *A. thaliana* AAS F338Y mutant reaction mixtures were analyzed by HPLC with UV detection at 265 nm at the described HPLC-EC sepa-
ration conditions, a product with a retention time of 4.41 min was observed. The relative amounts of the product formed were approximately proportional to the incubation periods of the reaction mixtures (Fig. 6A-6C). The product has an identical retention time to the authentic phenylethylamine at the same HPLC separation conditions. HPLC/MS/MS analysis in positive ion mode of an A. thaliana AAS F338Y mutant and phenylalanine reaction mixture revealed that the products of the MH\(^+\) ion m/z 122.1 and the relative intensities of its tandem spectrum (MS/MS spectrum) were identical to those generated using authentic phenylethylamine at identical conditions (Fig. 6D-6E). In addition, the product reacted with OPA-thio reagent producing an electrochemically active compound as other primary amines, further verifying the presence of free primary amine of the product. To further verify the decarboxylation activity-dictating role of this active site tyrosine, we performed an additional phenylalanine to tyrosine mutation on the 346-phenylalanine residue of P. crispum AAS (Q06086). HPLC-EC comparison of the wild type enzyme versus the F346Y mutant confirms an alteration in the primary activity from decarboxylation-deamination to decarboxylation (Fig. 7). In addition to the primary decarboxylation activity, the P. crispum F346Y mutant retained a small percentage of decarboxylation-deamination activity. The change from AAS activity to true AAAD activity in the A. thaliana AAS F338Y and the P. crispum F350Y mutants, in conjunction with the strict conservation of Tyr338 equivalent with in identified TyDCs and TDCs (Fig. 2), indicates that this loop region tyrosine residue plays a primarily role in the typical decarboxylation reaction in plant AAADs.
Figure 8.6: Detection/identification of phenylethylamine produced in A. thaliana AAS F338Y mutant and phenylalanine reaction mixtures by HPLC-UV and LC/MS/MS. Chromatograms (A-C) illustrate the relative amount of phenylethylamine (4.41-min peak) formed in the reaction mixtures at 5, 10 and 15 min after incubation, respectively. Chromatogram and MS/MS spectrum (D) illustrate the MH$^+$ ion (122) and daughter ions of the product formed in A. thaliana AAS F338Y mutant and phenylalanine reaction mixture in comparison with those (E) produced by phenylethylamine standard under identical conditions of LC/MS/MS analysis.

8.3.4 Mutagenic conversion of C. roseus TDC and P. somniferum TyDC into an indole-3-acetaldehyde synthase and a 4-hydroxyphenylacetaldehyde synthase respectively

We have previously expressed a C. roseus TDC (P17770) and verified its substrate specificity to primarily tryptophan and also 5-hydroxytryptophan (5-HTP) at a reduced rate (12) (the
activity of the *C. roseus* TDC enzyme has previously been investigated) (9). This TDC, like other verified TDC and TyDC, contains the conserved Tyr348 at the position equivalent to Phe338 of the *A. thaliana* AAS. To determine its role in catalytic reaction, a Y348F mutant of the *C. roseus* TDC was produced and its catalytic reaction was assessed with tryptophan as a substrate. Similar to those obtained during analysis of the *P. crispum* AAS and tyrosine reaction mixtures (Fig. 2C), HPLC-EC analysis of the *C. roseus* TDC Y348F mutant and tryptophan reaction mixtures resulted in the detection of a very broad peak (Fig. 8A) and treatment of the reaction mixture with borohydride converted the broad peak to a sharp peak (Fig. 8B), which contrasts to the typical tryptophan decarboxylation reaction by the
wild type *C. roseus* TDC (Fig. 8C). The sharp peak, detected in the borohydride-treated reaction mixture, had identical retention time as authentic indole-3-ethanol under the same conditions of HPLC-EC analysis and coeluted with the standard at different mobile phase conditions during HPLC-EC analysis (not shown). Indole-3-ethanol is not charged easily by electrospray ionization, but the indole-3-acetaldehyde-bisulfite adduct (formed easily by reacting indole-3-acetaldehyde with bisulfite in aqueous solution) is negatively charged in aqueous solution even at relatively acidic pH. Analysis of a sodium bisulfite-treated *C. roseus* TDC Y348F mutant and tryptophan reaction mixture by HPLC/MS/MS in negative mode verified that the MS-MS spectrum of \([\text{M-H}]^-\) precursor ion of the adduct m/z 240.1 was identical to those of authentic indole-3-acetaldehyde-bisulfite under identical analysis conditions (Fig. 8D and 8E). The detection of a broad peak during HPLC-EC analysis of the TDC Y348F mutant and tryptophan reaction mixture, the conversion of the broad peak to a sharp peak by borohydride reduction, the coelution of the sharp peak with indole-3-ethanol, the reaction of the enzymatic product with bisulfite (indicative for the presence of aldehyde group) and the identical MS and MS/MS spectra of the its bisulfite adduct with those of indole-3-acetaldehyde-disulfite standards provide convincing evidences for the TDC Y348F enzymatic production of indole-3-acetaldehyde. Accordingly, mutation of Tyr348 to Phe348 changed *C. roseus* TDC into a tryptophan to indole-3-acetaldehyde catalyzing AAS.

In addition to indole-3-acetaldehyde, a small amount of tryptamine was also detected in TDC Y348F mutant and tryptophan reaction mixture. In order to substantiate the claim that this active site phenylalanine is responsible for decarboxylation-deamination activity,
an additional tyrosine to phenylalanine mutation was performed upon the *P. Somniferum* tyrosine-350 residue. Evaluation of the *P. Somniferum* wild type and Y350F mutant enzyme activities demonstrates a conversion of activity from decarboxylation to decarboxylation-deamination (Fig. 9). In addition to the primary decarboxylation-deamination activity, the *P. Somniferum* Y350F mutant retained a small percentage of its original decarboxylation activity. These *C. roseus* and *P. Somniferum* tyrosine to phenylalanine mutations in conjunction with the *P. crispum* and *A. thaliana* phenylalanine to tyrosine mutations further support the role the active site tyrosine and phenylalanine play in decarboxylation and decarboxylation-deamination activity, respectively.

### 8.3.5 Substrate specificity and kinetic analysis

The typical substrate of wild-type enzymes was used for activity assays of the mutant enzymes. After the change of catalytic reaction for the mutant enzymes was established, they were screened for activity against other aromatic amino acids. Similar to wild type *A. thaliana* AAS, its F338Y mutant displayed a very low activity to tyrosine (~5% of its activity to phenylalanine) and strong activity to dopa (Fig. 10), but showed no detectable activity to tryptophan and 5-HTP; while *C. roseus* TDC Y348F mutant showed activity to tryptophan and 5-HTP with no detectable activity to phenylalanine, tyrosine and dopa (not shown). These analyses established that although the catalytic reaction of these mutants was changed, their substrate specificity were the same as their wild type enzymes (9,11).
Figure 8.8: HPLC-EC and LC/MS/MS detection of indole-3-acetaldehyde generated from *C. roseus* TDC Y348F mutant and tryptophan reaction mixtures. (Chromatograms A-C) Y-axis represents the output in µA, the x-axis represents retention time. Chromatograms (A and B) illustrate the indole-3-acetaldehyde (the major broad peak) formed in a TDC Y348F mutant and tryptophan reaction mixtures after 15 min incubation and the detection of indole-3-ethanol (tryptophanol) in another 15 min incubated reaction mixture that was treated borohydride prior to HPLC-EC analysis. Chromatogram (C) shows the detection of tryptamine in a wild type TDC and tryptophan reaction mixture 15 min after incubation. Chromatogram and MS/MS spectrum (D) illustrate the [M-H]⁻ ion (240) and daughter ions of the bisulfite derivatized product formed in bisulfite-treated *A. thaliana* AAS F338Y mutant and phenylalanine reaction mixture in comparison with those (E) produced by indole-3-acetaldehyde-bisulfite standard under identical conditions of HPLC/MS/MS analysis.

Kinetic evaluations were conducted for both the wild type and mutant *A. thaliana* and *C. roseus* proteins using their respective physiological substrate. Under the applied conditions,
Figure 8.9: HPLC-EC analysis of *P. somniferum* wild type and Y346F enzymes with tyrosine as a substrate. Y-axis represents the output in µA, the x-axis represents retention time. Chromatogram A shows the production of tyramine in the wild type *P. somniferum* mutant and tyrosine reaction mixture at 40 min after incubation. Chromatograms B and C illustrate the accumulation of HPAA (the major broad peak) and tyramine (the minor sharp peak) in the *P. somniferum* Y346F enzyme and tyrosine reaction mixture at 40 min after incubation and the reduction of 4-HPAA to 4-HPEA (tyrosol) in a borohydride treated reaction mixture.

*A. thaliana* AAS F338Y mutant and *C. roseus* TDC Y348F mutant displayed a similar affinity to phenylalanine and tryptophan, respectively, as compared to their respective wild type enzymes (Tab. 2). Similar michaelis constants between wild type and mutant enzymes indicates that the mutation of the active site tyrosine to phenylalanine or vise versa does not apparently alter the substrate binding affinity of the enzymes. In contrast, the specific activities of the mutant enzymes were quite different to those determined for the wild-type enzymes (Tab. 2). The decarboxylase activity of *A. thaliana* AAS F338Y mutant was approximately 3 fold faster than the aldehyde synthase activity of its wild type enzyme to
Figure 8.10: HPLC-EC detection of dopamine and 3,4-dihydrophenylethanol produced in *A. thaliana* AAS F338Y mutant and wild type reaction mixtures respectively. Y-axis represents the output in microamps, the x-axis represents retention time. Chromatograms (A-C) illustrate the relative amount of dopamine formed in the reaction mixtures at 4, 12 and 24 min after incubation, respectively. Chromatograms D illustrates wild type enzyme and dopa reaction mixture at 24 min after incubation and the reduction of 3,4-dihydroxyphenylacetaldehyde to 3,4-dihydrophenylethanol in a borohydride treated reaction mixture. In the mutant enzyme reaction mixtures, the rate of product formation was decreased as dopamine accumulated in the reaction mixtures, the reaction could proceed basically to completion (CO$_2$ is released, so no equilibrium is reached during reaction). At the applied conditions, essentially all dopa was converted to dopamine, but no indication for further oxidative deamination of the dopamine product was observed.

The same substrate, while the specific aldehyde synthase activity of *C. roseus* Y348F mutant was approximately 17 fold slower than the decarboxylase activity of the wild type TDC to tryptophan (Tab. 2). Such alterations in activity likely are reflected by the complexity of
the reactions. The added chemistry necessary for decarboxylation-deamination as opposed to a simpler decarboxylation may result in the observed catalysis rate change.

Table 8.2: Kinetic parameters of *A. thaliana* and *C. roseus* wild type and mutant enzymes

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<th>Enzyme</th>
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<th>Activity</th>
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<td><em>C. roseus</em></td>
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<td>Decarboxylation</td>
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### 8.4 Discussion

Based on reactions they catalyze, plant AAADs can be classified as true AAADs (enzymes that catalyze the production of arylalkylamines from their respective aromatic amino acid substrates) or AASs (enzymes that catalyze the production of aromatic acetaldehydes from their respective aromatic amino acid substrates). Within each subclass, these enzymes can be further classified based on their substrate specificity, such as TyDCs and TDCs. Association of the substrate selectivity and catalytic reaction with a given AAAD protein provides relevant functional linkages. Unfortunately, high sequence identity across plant AAADs makes it difficult to accurately assign substrate specificity and catalytic reaction based on a primary sequence. Our conversion of two AASs into AAADs through active site phenylalanine to tyrosine mutation and our conversion of two AAADs into AASs through active site
tyrosine to phenylalanine mutation demonstrates that the conserved active site tyrosine and phenylalanine are predominantly responsible for their respective AAAD and AAS catalytic reactions. Although more experimental verification is necessary, our data, in conjunction with the strict conservation of the active site tyrosine in all verified TyDC and TDC, indicate the presence of active site tyrosine is a genuine criterion to predict the decarboxylation activity of a given member of the AAAD family. We submit that the identification of a homologous active site tyrosine or phenylalanine will enable accurate differentiation of plant AAAD and AAS enzymes from a primary sequence basis. In addition, the identification of an activity-dictating residue in AAADs and AASs provides some basis to expand on each activities respective reaction mechanisms.

PLP-dependent decarboxylases are believed to proceed by a single reaction mechanism (21). In this mechanism the external aldimine loses the $\alpha$-carboxyl group to form the carbanion quinonoid intermediate (due to its interaction with catalytic residues). Subsequent $\alpha$-carbon protonation by an active site residue generates the product monoamine. Previous investigations of AAAD enzymes suggest that the quinonoid intermediate may become protonated by the catalytic loop tyrosine to form the arylalkylamine product (22). The same report indicated that in the absence of $\alpha$-carbon protonation, molecular oxygen is capable of attacking the carbanion of the quinonoid intermediate to generate a peroxide. The peroxy-aldimine transition form will undergo heterolysis of the O-O bond allowing for the regeneration of PLP and formation of an imine complex that spontaneously decomposes to yield an aromatic aldehyde and ammonia. Based on the effective conversion of two AASs into AAADs
upon mutating the active site phenylalanine to tyrosine the proposed tyrosine α-carbon protonation mechanism seem plausible. However, the α-carbon protonation of the intermediate requires the ionization of the tyrosine phenolic hydroxyl group proton. With a pKa around 10, the phenolic hydroxyl group of tyrosine may not ionize easily under physiological conditions. Additionally, a lack of electron density for the loop region in the three available AAAD structures makes it difficult to accurately estimate the probable distance between the side chain hydroxyl group of tyrosine and the α-carbon of the external aldimine. Therefore, it is unknown whether the α-carbon and the catalytic loop tyrosine are within interacting distances.

Analysis of a recently released mammalian HDC (the only type II PLP decarboxylase with electron density for the catalytic loop) enables speculation on the interactions between the substrate α-carbon and the catalytic loop tyrosine (16). Careful examination of the HDC active site confirmation revealed that although its Tyr 344 from monomer B (the equivalent active site tyrosine in plant AAAD and mammalian DDC) is quite close to the α-carbon of the external aldimine complex (7.50 Å), its side chain hydroxyl group is actually much closer to the imidazole amine (ε-amine group) (4.42 Å) of monomer A His 194. Accordingly, within the HDC structure, the hydroxyl group of monomer B Tyr 344 should interact more easily with the imidazole of monomer A His 194 than it would with the α-carbon of the external aldimine complex. Such structural observations in conjunction with our experimental verifications of catalytic loop tyrosine mediated decarboxylation enable us to propose a new mechanism of α-carbon protonation. We believe that plant AAADs active site tyrosine does
not directly protonate the intermediate \( \alpha \)-carbon, but rather play a crucial role in stabilizing the proton transfer from His194 to the \( \alpha \)-carbon (in the HDC structure, Tyr 344 hydroxyl group is 4.39 Å from the histidine 194 \( N_2 \)). We propose that the phenolic hydroxyl group from the catalytic loop tyrosine forms a stabilizing hydrogen bond with the \( N_2 \) histidine to enable the proton transfer from the histidine imidazole to the \( \alpha \)-carbanion. The modeled active site conformation of *C. roseus* TDC and *A. thaliana* AAS (using 4E1O as template (16); generated through Modeler (23); visualized through Pymol (20)) help illustrating the indirect role the active site tyrosine may play in stabilizing protonation of the \( \alpha \)-carbon of the substrate-PLP complex through His 194 (Fig. 8). This proposed mechanism is further supported by the loss of decarboxylation activity in the tyrosine to phenylalanine AAAD mutants. Upon replacing the catalytic tyrosine with phenylalanine (Y348F mutation in *C. roseus* TDC, Y346F mutation in *P. somniferum* TyDC), there is no electronegative \( R \) group atom to stabilize histidine proton transfer. Without such a stabilizing interaction, the substrate \( \alpha \)-carbon is not protonated. This leaves the substrate carbanion susceptible to peroxidation and subsequent aromatic acetaldehyde formation.

The above hypothesis regarding the active site tyrosines role in \( \alpha \)-carbon protonation (though reasonable) remains speculative in nature. However, the predicted active site conformation of insect AAS proteins provides a more compelling argument for our mechanistic proposal. Unlike plant AAS proteins that contain an active site phenylalanine, the equivalent position in insect AASs remains as a tyrosine (moreover, this tyrosine residue is stringently conserved in all available insect AAADs, regardless of their substrate specificity and catalytic reactions).
This contrasts what is observed in plant AAS proteins. However, analysis of the His194 equivalent in insect AAAD and AAS determined that all typical insect AAADs contain the histidine in the position, but the active site histidine was replaced by an asparagine in all verified insect AAS proteins. Asparagine has a greatly reduced likelihood of proton donation (non ionizable nature of its R group) as compared to histidine. Consequently, in the presence of an active site asparagine in insect AAS, the active site tyrosine does not play a stabilizing role in α-carbon proton transfer (Fig. 11). The presence of tyrosine and absence of histidine with in insect AAS enzymes supports the role of tyrosine in promoting typical decarboxylation in plant AAADs through its interaction with the active site histidine. Through these analyses, it has become apparent that the active site tyrosine from insect AAAD and AAS proteins plays a different catalytic role from the tyrosine from plant AAADs. However, this by no mean indicated that this active site tyrosine is not critical for insect AAAD and AAS substrate binding and catalysis. Proximity to the active site and the stringent conservation in all insect AAAD and AAS proteins indicates a probable role in substrate binding or catalysis.

Although our data suggested some apparent similarities in catalytic reactions of plant and non-plant AAADs, there are also considerable differences regarding the structural basis of substrate binding and catalysis. Mammalian DDC has been the most extensively studied AAAD in terms of substrate specificity and catalytic reactions. Based on literature, mammalian DDC can use both dopa and 5-HTP as substrates (24,25), but the plant AAADs or AASs use aromatic amino acids with either indole ring or benzene ring, but never the
Figure 8.11: Models of active site residues and external aldimine interactions. The model on the left shows the relative locations of histidine 203, tyrosine 348, and the external aldimine from the *C. roseus* TDC model (generated using 4E1O). The model in the middle shows the relative locations of histidine 193, phenylalanine 338, and the external aldimine from the *A. thaliana* AAS model (generated using 4E1O). The model on the right shows the relative locations of asparagine 192, tyrosine 332, and the external aldimine from the *D. melanogaster* AAS model (generated using 4E1O). Residues shown in yellow correspond to the $\alpha$ chain whereas residues in green correspond to the $\beta$ chain.

Both (1,8,9). Other than its typical decarboxylation activity to dopa and 5-HTP, mammalian DDC can also catalyze oxidative deamination of dopamine and 5-HT (26,27). However, plant AAADs cannot catalyze any oxidative deamination of tyramine, dopamine or tryptamine (analyzed with two available plant TyDC, one plant TDC and three plant AASs in our laboratory). The same applies to the *A. thaliana* F338Y mutant as well. For example, at the applied assay conditions, essentially all dopa substrate has been converted to dopamine after 24 min incubation. HPLC-EC analyses showed no indication for any further oxidative deamination of dopamine product to its aldehyde derivative in the reaction mixture (Fig. 10C). This suggests that arylalkylamines, once released from the catalytic center of plant AAADs or AAS, cannot easily enter into their active site again, or that the external aldimine between the PLP cofactor and arylalkylamines cannot be easily formed at the active site of
plant AAADs. Addition of tyramine at mM concentrations into the wild type *P. crispum* AAS and tyrosine reaction mixtures also did not noticeably slow down the AAS catalyzed PHAA production from tyrosine. Additionally the tyramine levels remained at same level during incubation (not shown), indicating the absence of tyramine oxidative-deamination. This reinforces the notion that the α-carboxyl group is essential for plant AAS substrate binding. Apparently, extensive studies about AAADs are necessary before being able to achieve a better overall understanding of the large AAAD family proteins across species.

The interconversion of AAAD and AAS activities enabled the generation of several unusual plant AAAD and AAS enzyme products. These products include 4-hydroxyphenylacetaldehyde, phenylethylamine, indole-3-acetaldehyde, and 5-hydroxy indol-3-acetaldehyde. 4-hydroxyphenylacetaldehyde has only been documented as a product from a single AAS enzyme (12). Thus far the physiological function of this AAS product remains unknown. Phenylethylamine has only been observed as plant type II PLP decarboxylase product from a selection of *Solanum lycopersicum* enzymes (28). Plant AAAD enzymes typically retain approximately 40% identity to one another where as these *S. lycopersicum* enzymes only demonstrate approximately 11% identity to canonical AAADs. Further *S. lycopersicum* AAAD sequence analysis demonstrates strong homology (approximately 55% identity) to the characterized *Arabidopsis thaliana* (AF389349) and *Brassica napus* (BAA78331) serine decarboxylases (29). Therefore, these *S. lycopersicum* enzymes cannot be viewed as canonical plant AAAD enzymes making phenylethylamine a truly unique plant AAAD product. Finally, the enzymatic conversion of tryptophan to indol-3-acetaldehyde and 5-hydroxytryptophan to 5-
hydroxy indol-3-acetaldehyde by the C. roseus TDC mutant truly represents novel AAAD enzyme products. Amongst these unusual products, the C. roseus TDC mutant generated indol-3-acetaldehyde is likely the most interesting. Indol-3-acetaldehyde is a proposed intermediate in the tryptophan dependent indole-3-pyruvic acid (IPA) auxin biosynthetic pathway (30,31). Despite many references regarding this auxin (indole-3-acetic acid) intermediate, the biosynthetic route has not been verified. Although there have been verified tryptophan aminotransferases (32) capable of catalyzing the production of tryptophan to indole-3-pyruvic acid, there have been no experimental data showing the presence of an IPA decarboxylase in plants. Interestingly, our mutant is capable of performing the function of a tryptophan aminotransferase and the putative IPA decarboxylase (converting tryptophan to indol-3-acetaldehyde) in one step (Fig. 12). The final auxin biosynthetic step in this proposed pathway involves the conversion of indol-3-acetaldehyde to indole-3-acetic acid (IAA). This reaction has been demonstrated by aldehyde oxidases (33,34). Therefore, our TDC Y348F mutation could conceivably convert the standard TDC reaction into one capable of producing a key intermediate for auxin biosynthesis. The introduction of a stable and active TDC Y348F mutant into wild type and auxin deficient Arabidopsis plants should demonstrate the presence/absence of an IPA decarboxylase, demonstrate/disprove in vivo aldehyde oxidases activity, and produce insights into indol-3-acetaldehydes role as an auxin intermediate. Moreover, this proposed experiment might reflect on the recently proposed role of the YUCCA enzymes in the IPA pathway. Initial characterizations of the YUCCA enzymes indicated that these flavin monooxygenase-like proteins function in an alternate
biosynthetic route from the aforementioned IPA pathway (35). Activity assays revealed that YUCCA enzymes catalyze the hydroxylation of tryptamine to generate a proposed IAA intermediate \( N \)-hydroxytryptamine (36,37). However, new genetic and biochemical investigations of YUCCA enzymes have cast doubt on this tryptamine \( N \)-hydroxylation activity in favor of YUCCAs role in IPA to IAA catalysis (38,39). Failure to rescue the phenotypes from TDC Y348F auxin deficient mutants would lend further support to YUCCAs probable role in IAA biosynthesis. Alternatively, phenotype rescue of the auxin deficient mutants through the introduction of a TDC Y348F gene would cast doubt on YUCCAs role in the IPA pathway. Additionally, conversion of a regular TDC enzyme to a tryptophan aldehyde synthase only requires a single nucleotide substitution. One might think that somewhere and some point throughout plant evolution this might have occurred and granted some level of reproductive fitness. Such an enzyme might have evolved in plants and may be producing an alternate auxin biosynthetic route.

8.5 Conclusion

In summary, the AAAD family is a great example of protein functional expansion. Based on its ubiquitous distribution, it seems reasonable to consider that the typical DDC or TyDC, present from bacteria to humans, is the prototype of other more specific AAADs. Such divergent AAADs have likely evolved unique substrate specificities and activities to provide unique physiological requirements. The functions of AAADs vary considerably, depending
Figure 8.12: Intersection of the *C. roseus* TDC Y348F mutant and the proposed tryptophan dependent indole-3-pyruvic acid auxin biosynthetic pathway.

upon their substrate specificity and catalytic reaction, but their high sequence similarity makes it extremely difficult to tell them apart based on primary sequences. Our data indicated that the presence of the conserved tyrosine or phenylalanine in the active site loop region could serve as signature residues for reliably predicting typical plant AAAD and AAS, respectively. This should help to more precisely predict between true AAAD and AAS sequences available in the database and to aid the proper annotation of numerous incoming plant AAAD sequences currently undergoing genome-sequencing projects. Our comparative analyses of active site conformations between different AAAD and AAS proteins lead to a new hypothesis concerning the roles the active site tyrosine and phenylalanine play in their
typical decarboxylation reaction and decarboxylation-oxidative deamination, respectively. Although the hypothesis remains to be further substantiated, the proposed mechanism gives some reasonable explanation and insight for AAAD- and AAS-catalyzed reactions, respectively, which should stimulate research in this area. In addition, the ability to catalyze tryptophan to indole-3-acetaldehyde suggests a useful research tool in the investigation of auxin biosynthesis. Overall, our progress in differentiating plant AAAD and AAS proteins is major step forward towards proper classification/annotation of hundreds of individual plant AAADs in the database. However, much still remains to be established regarding the structural components and chemical mechanisms dictating the substrate specificity and variable activities of AAAD- and AAS-mediated reactions.

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8.7 References

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Appendix A. Biochemical Evaluation of Plant AAADs